Phospholipid Metabolism in the Lung of Fat-Fed Rats

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Phospholipid metabolism in the lung was studied because the lung was the first organ at which the ingested lipids arrived. It was revealed in this study that the lung contained phosphatidyl choline, ethanolamine, phosphoinositol, sphingomyelin and lyssolecithin, that the amount of phosphatidyl serine were very small in the lung, and that a phosphatidic acid was discovered in the lung after the administration of fats. Furthermore, an unknown phospholipid subfraction was revealed in the lung.

Fatty acid composition of phospholipid subfractions was investigated one and six hours after the administration of trilinolein.

The elongation of fatty acids occurred also in the lung. Phosphatidyl ethanolamine and an unknown subfraction were active in the elongation but phosphatidyl choline was stable.

It was resulted by ligation of the thoracic duct that appearance of a phosphatidic acid was delayed, and that saturated fatty acid increased, unsaturated decreased.

These findings suggest that the lung has an ability of lipid synthesis, and that phosphatidyl ethanolamine and an unknown subfraction were active in lipid metabolism although phosphatidyl choline was stable.

INTRODUCTION

It is well established that the liver plays an important role in lipid metabolism. On the other hand, little attention has been given to a possible role of the lung in the storage or excretion of lipids.

In considering the mechanism involved in the absorption of lipids, most of the ingested lipids pass through the lung before the liver by way of the thoracic duct. Therefore, it is naturally considered that the lung may have some roles in lipid metabolism because the lung is the first organ at which the ingested lipids (chylomicrons) arrive.

In the literature, there were scattered reports indicating the lung had also an important role in lipid metabolism.
administered cholesterol into a peripheral artery or into the portal vein, and found that even if cholesterol had to pass through
the liver before the lung its primary site of deposit was still the lung.

Cionti \(^2\) found that cholesterol in the lung was located in the reticu-
loendothelial cells of the alveolar wall.

Schrade \(^3\) administered olive oil to rats and found that the amount
of total lipids increased markedly in the lung.

OsaJima et al \(^4\) observed that the amount of total lipids, cholesterol
and phospholipids increased in the lung when triolein or trilinolein was
administered but did not increase when tripalmitin or tristearin was
administered.

inderbitzen \(^5\) observed that the disappearance of chylomicrons was
delayed when one lung was removed, and arrived at the conclusion
that the lung removed chylomicrons from the circulation.

In view of the fact obtained by the investigation Lipoprotein-Lipase
Activity, Heinemann \(^6\) and Yamada \(^7\) concluded that the lung also play-
ed an important role in lipid metabolism.

On the other hand, fatty liver was observed at a great rate in
patients with pulmonary tuberculosis \(^8\). This clinical observation may
suggest the role of the lung in lipid metabolism.

The present study was undertaken in an attempt to investigate a
possible role of the lung in lipid metabolism, especially from the stand-
point of phospholipid metabolism.

MATERIALS AND METHODS

Male albino rats of Wistar strain, weighing 160g to 260g, were
selected for the present investigation and were fed ad libitum a com-
mercial laboratory chow (Oriental chow).

At this investigation, rats were divided into three groups. Group
I was left overnight with water but without food, and then killed by exsanguination. This group was used for the control. Group II and
group III also were left overnight with water but without food, and
then either 0.5g of tripalmitin in 4ml of dehycol solution or 0.5g of
trilinolein in 4ml of dehycol solution was administered through a rub-
ber inserted into the stomach.

The animals were lightly anesthetized with ether so that they
were fully recovered by the time a cathether was removed.

Rats of group II and III were killed by exsanguination six hours
after tripalmitin or trilinolein was administered. It was on the basis
of the preliminary observation that rats of group II and III were killed
six hours after the administration of fats.

In order to investigate the effect of the thoracic duct on lipid meta-
bolism in the lung, ligation of the thoracic duct was accomplished by
the procedure of Bollman. After the operation, the rats were maintained on the same chow and water for a week, and then trilinolein was administered after fasting for twelve hours. At time intervals of one, three and six hours after the administration of trilinolein the rats were killed.

At the time of killing, lungs were removed and total lipids were extracted from the lungs by a modification of the method of Folch-Lees. The amounts of total lipids were calculated by a balance and phosphorus was measured by the method of Allen. The extracted lipids were dissolved in 10 ml of chloroform and this solution were used for the samples.

Chromatographic technique:

Glass plates (20 cm x 20 cm and 5 cm x 20 cm) were thoroughly cleaned with a detergent (H2SO4-potassium dichromate solution). The "neutral" plates were prepared by making a slurry 30 g of Kiesel Gel G with 65 ml distilled water and applying it according to the method of Stahl. After a layer of Kiesel Gel G had been applied the plates were left on the tray at room temperature for 30 minutes and then inserted on the light-alloy drying rack. The rack with the plates was inserted into the drying cabinet, which had been heated to 110°C. The door of the cabinet should be opened for the first ten minutes of drying to let the stream escape, and furthermore the plates were activated for 30 minutes. The hot rack was next removed, cooled for 10 minutes and stored in a cabinet with silica gel and phosphorus pentoxide. When it took longer than three days before the samples were applied on the plates, the plates had to be again activated at 80°C for 40 minutes just prior to the application of the samples. Depending on the amount of minor constituent to be detected, various volumes (10 μl–200 μl) of the samples were applied 2 cm from the bottom of the plates with a calibrated syringe for gas-liquid chromatography.

If it was necessary to detect a minor constituent on thin-layer chromatogram, the samples were applied in greater quantities although a major constituent could not be clearly separated.

Chromatographic chambers were prepared 60 minutes prior to the insertion of the plates and furthermore were lined on three sides with a filter paper in order to prevent drying of the solvent at the front. The edge of a thin-layer should be stripped off using the thumb as a guide in order to prevent the edge effect occured in the developing.

The plates were developed at room temperature until the solvent front reached 12 cm from the origin of spots.

At this investigation, a solvent mixture of chloroform-methanol-water (65 : 25 : 4) was used for the separation of phospholipid.

Detection of spots: In this investigation six different detection
methods were employed.

(1) Ninhydrin to detect amino-phospholipids. Phospholipids with free amino-group were revealed by ninhydrin spray (0.2% ninhydrin in butanol). (After spraying, the plates were heated for 5 minutes at 100°C).

Red-violet spots appeared on a white background.

(2) Choline-containing phospholipids were detected by dragendorff reagent (Bi). The plates were sprayed with a mixture of 4 ml of solution I, 1 ml of solution II and 20 ml distilled water. Solution I contained 1.7 g of Bi (NO₃)₃·5H₂O diluted to 100 ml with 20% v/v acetic acid. Solution II contained 40 g of KI in 100 ml water. As the plates were dried at room temperature, choline-containing phospholipids produced orange spots.

(3) Phosphoinositol was detected by ammonium silver nitrate (Ag). The plates were sprayed with a mixture equal volumes of 0.1 N AgNO₃ and 7 N ammonium hydroxide. The plates were than heated at 110°C until dark brown spot appeared on a white background.

(4) Lipids containing phosphorus (phospholipids) were detected by molybdic acid reagent (Mo). The plates were sprayed with a solution of 5 ml of 60% w/v perchloric acid, 10 ml of 1 N HCl, and 25 ml of 4% w/v ammonium molybdate. Blue spots appeared on a white background as the plates were dried at room temperature.

(5) Lipids were detected nonspecifically by iodine solution (I₂). The plates were sprayed with a solution of 1.0 g diluted 100 ml methanol. Brown spots appeared on a white background as the plates were dried at room temperature and the excess of iodine was evaporated.

(6) Lipids were detected semiquantitatively by sulfonic acid. The plates were sprayed with 50% sulfonic acid and then heated for 20 minutes at 100°C. During the first few minutes of the charring operation, free sterols and sterol esters gave a typical Lieberman-Burchard color reaction, and at a later stage glycolipids turned deep purple and phospholipids, pale brown.

Two-dimensional chromatography: As all of phospholipids could not be separated by one dimensional chromatography. Since phosphatidyl ethanolamine, serine and inositol could not be separated, two-dimensional thin-layer chromatography had to be used at the same time. The plates were developed at first with chloroform-methanol-7 N ammonium hydroxide (60:35:5) and then dried at room temperature. For two-dimensional chromatography, the plates were rotated 90 clockwise and placed in another developing chamber containing a solvent mixture of chloroform-methanol-7 N ammonium hydroxide (35:60:5).

Another solvent mixtures were used for two-dimensional thin-layer
chromatography. The solvent mixture contained chloroform-methanol-glacial acetic acid-water (250 : 74 : 19 : 3) or chloroform-methanol-7 N ammonium hydroxide (230 : 90 : 15). In this type of two-dimensional chromatography, the "basic" plates were used. The "basic" plates were prepared by making a slurry of 30g of Kiesel Gel G with 65 ml of 0.01 M Na$_2$CO$_3$.

Fatty acid composition: Fatty acid composition of phospholipid subfractions separated by thin-layer chromatography were investigated by gas-liquid chromatography.

The samples were applied as many small spots to 20cm x 20cm plates and then developed with chloroform-methanol-water (65 : 25 : 4). The plates were dried and partially masked by covering aluminium foil over the surface of silicic acid so that only one spot remained uncovered. Exposure to iodine solution showed the position of the lipids, and on this basis the cover area of silicic acid containing the unchanged lipids were scrapped off by a spatel. It could be checked by spraying the remainder of the plate with 50% sulfonic acid that the scrapped silicic acid contained only a phospholipid subfraction. This silicic acid containing a separated phospholipid subfraction was transferred to a small glass column 1 cm in diameter and was eluted with chloroform-methanol-water (65 : 25 : 4) and then with methanol.

The eluted solvents were evaporated in vacuum and used for the investigation of fatty acids.

RESULTS

By thin-layer chromatography lung lipids was separated and its results were shown in Fig (1) and Fig (2).

Five spots appeared on the chromatogram in group I (Fig 1). A spot which did not appeared in group I was revealed in group II and group III (Fig 2). That is to say, a new spot appeared only when fat was administered to rats. The applied samples on the chromatogram contained the same volumes in three groups. Spots on the chromatogram were investigated and its results were listed in Table (1). The spot at the solvent front was neutral lipids and cholesterol because the spot gave a typical Liebermann-Burchard color reaction by 50% sulfonic acid, and the other spots gave pale brown. Furthermore, each spot was scrapped off and it was investigated by the method of Wagner$^{16}$ whether it contained phosphorus or not. All spots except the spot at the solvent front contained phosphorus. On the other hand, only the solvent eluted from the spot at the solvent front contained cholesterol and did not contain phosphorus.

By the above examination, it was revealed that five spots and a
new spot were phospholipids.

Phospholipids were identified by observing the color reaction and its results were listed in Table (1).

No.1, 2 and 3 spot gave an orange color by spraying Dragendorff reagent. No.4 spot became red-violet by spraying ninhydrin reagent.

As compared with a relative pure phosphatidyl choline and ethanolamine obtained from soya-beaın lecithin, Rf value of No.3 spot was identical with that of soya-beaın phosphatidyl choline, and Rf value of No.4 spot was identical with that of soya-beaın phosphatidyl ethanolamine.

Two-dimensional thin-layer chromatogram of the lipids extracted

Table. 1

<table>
<thead>
<tr>
<th>Neutral lipid</th>
<th>I₂</th>
<th>H₂SO₄</th>
<th>Mo</th>
<th>N₅n</th>
<th>Bi</th>
<th>Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>+</td>
<td>brown</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phosphatic acid</td>
<td>+</td>
<td>brown</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Unknown</td>
<td>+</td>
<td>&quot;</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cephalin</td>
<td>+</td>
<td>&quot;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Choline</td>
<td>+</td>
<td>&quot;</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>+</td>
<td>&quot;</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lysophosphatidyl Choline</td>
<td>+</td>
<td>&quot;</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
from lungs of the rat given trilinolein was shown in Fig (3). As compared with the two-dimensional thin-layer chromatogram of the rat liver by Skidmore and Entenmann,\textsuperscript{13} it was revealed that a new spot appeared in the rat given fat was a phosphatidic acid, that the amount of phosphatidyl serine and phosphoinositol was small in the lung, and that No. 5 spot could not identified.

This identification of the spots was in accordance with the results obtained by the method of Abramson\textsuperscript{14} (Fig 4).

The effect of ligation of the thoracic duct was investigated. In the rats whose duct was ligated, a phosphatidic acid was not discovered one and three hours after the administration of trilinolein, but was discovered after six hours. On the other hand, a phosphatidic acid was discovered after three hours in the untreated rats.

Fig. (5) Fatty Acid Composition of Phospholipid.

- Control
- Tripalmitin
- Trilinolein
Fatty acid composition of phospholipid subfractions was investigated. The results obtained from the rat given tripalmitin were shown in Fig (5).

In phosphatidyl choline, the concentration of 20:4 decreased and 20:3 increased. In phosphatidyl ethanolamine, the decrease of 20:4 and the increase of 20:3 became more obvious than in phosphatidyl choline. In an unknown spot, the concentration of 16:0 and 20:3 increased and the concentration of 18:0, 18:1 and 18:2 decreased.

The result obtained from the rats given trilinolein were shown in Fig (5).

**Fig. (5) Fatty Acid Composition of Phospholipid**

![Graph showing fatty acid composition of phospholipid subfractions](image-url)
In phosphatidyl choline, the concentration of 16:0 slightly increased but the concentration of 18:2 did not increase. In phosphatidyl ethanolamine, the concentration of 16:0 and 18:2 decreased and the concentration of 20:4 increased.

In an unknown spot, the concentration of 16:0 and 18:1 increased and the concentration of 18:2 and 20:4 did not change.

Fatty acid composition was investigated one and six hours after the administration of trilinolein, and its results were shown in Fig(6). Fatty acid composition of the lung lipid one hour after the admi-
mistration of trilinolein was compared with that of the lung lipid six hours.

In phosphatidyl choline, concentration of 16:0 decreased and the concentration of 20:4 increased. In phosphatidyl ethanolamine, the concentration of 20:4 increased markedly, the concentration of 16:0 decreased at the same degree as the increase of 20:4, but the concentration of 18:2 did not change. In an unknown spot, the concentration of 18:1 and 18:2 increased and the concentration of 16:0 decreased.

Ligation of the thoracic duct was accomplished in order to investi
igate the effect of the thoracic duct on lipid metabolism in the lung, and fatty acid composition of the lung lipid in the rats given the surgical operation was compared with that of the lung lipid in the rats whose thoracic duct remained intact. Its results were shown in Fig(7).

In phosphatidyl choline, the concentration of 16:0 and 16:1 increased slightly, the concentration of 18:1 increased, and the concentration of 18:2 and 20:4 were unchanged. In phosphatidyl ethanolamine, the concentration of 16:0 and 20:4 decreased and the decrease of 20:4 was marked. In an unknown spot, the concentration of 16:0 and 18:0 increased, the concentration of 18:1, 18:2 and 20:4 decreased, and the decrease of 16:0 was marked.

In phosphatidic acid, the concentration of 16:0, 16:1, 18:1 and 18:2 increased and the concentration of 18:0 and 20:4 decreased.

**Fig. (7) Fatty Acid Composition of Phospholipid**

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**Ligation of the thoracic duct was accomplished**

**The thoracic duct was intact**

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**Fig. (7) Fatty Acid Composition of Phospholipid**

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**Fig. (7) Fatty Acid Composition of Phospholipid**
Fig. (7) Fatty Acid Composition of Phospholipid

DISCUSSION

It was revealed in this investigation that the lung contained phosphatidyl choline, phosphatidyl ethanolamine, phosphoinositol, sphingomyelin and lysolecithin, that the amounts of phosphatidyl serine were very small in the lung, and that a phosphatidic acid was discovered in the lung after the administration of fats.

It is of interest that a new phospholipid subfraction was discovered in the rat lung after the administration of fats, and that this
subfraction was considered to be a phosphatidic acid judging from its Rf value, fatty acid composition and two-dimensional thin-layer chromatographic behavior.

There are reports that a phosphatidic acid is revealed in the lung of normal rats but the amount of phosphatidic acid is so small in the lung that it is not detected by the analytical method used in the present investigation.

Kennedy et al\(^7\) presented data which they regarded as an evidence that a phosphatidic acid intermediated in phospholipid metabolism.

Since Chibnall and Channon\(^8\) isolated a phosphatidic acid from cabbage leaves in 1927, Dawson\(^9\) and Marinetti\(^20\) investigated whether a phosphatidic acid was present in animal tissue, and were of the opinion that there was not a phosphatidic acid but was a closely related substance.

On the other hand, Hokin and Hokin\(^21\) investigated whether a phosphatidic acid was present in animal tissues in vivo, and found that acetylcholine stimulated the incorporation of P into a highly labelled lipid of brain and pancreas slices which was presumed to be a phosphatidic acid since it yielded glycerophosphate by the hydrolytic method of Dawson.

And Hokin and Hokin arrived at the conclusion that a phosphatidic acid was present in vivo and that it was not only an intermediary in phospholipid biosynthesis but also played a role in the mechanism of active transport across cell membranes.

On the basis that a phosphatidic acid plays such an important role, it is very interest that the amounts of phosphatidic acid increased in the lung when fats were administered. This finding indicates that the lung also has an ability of lipid synthesis.

It was observed in the preliminary investigation that the amount of neutral lipids, cholesterol and phospholipids did not increase in the lung when tripalmitin or tristearin was administered to the rats. However, the amounts of phosphatidic acid in the present investigation even if tripalmitin was administered.

The increase of phosphatidic acid must indicate that lipid synthesis increases in the lung even if tripalmitin was administered.

In order to resolve this gap, the rate of increase of a phosphatidic acid must be measured when tripalmitin or trilinolein was administered. As the rate was not measured at the present investigation, this problem remains unresolved.

The appearance of a phosphatidic acid was delayed in the rats in which ligation of the thoracic duct was accomplished. In the rats in which the thoracic duct remained intact, a phosphatidic acid was revealed already 3 hours after the administration of trilinolein. In the rats in which ligation of the thoracic duct was accomplished, however,
a phosphatidic acid was revealed 6 hours after the administration of trilinolein.

This result indicates that the lung removes chylomicrons passing through the thoracic duct and may suggest particularly the significance of the thoracic duct.

The results obtained from the investigation of fatty acid composition indicate that the lung also plays a role in fatty acids synthesis.

The increase of the administered fatty acid (18:2) was not observed in each phospholipid subfraction, but the results obtained from the investigation of fatty acid composition of phosphatidyl ethanolamine or the unknown spot suggest that the conversion or elongation of fatty acid occurs in the lung.

The results obtained from the investigation of fatty acid composition one hour after the administration of trilinolein showed that the concentration of 20:4 increased markedly at one hour rather at 6 hours, and suggest that the elongation or utilization of chylomicrons occurs already in the early stage.

It was resulted by ligation of the thoracic duct that the concentration of 16:0 increased and the concentration of 18:1, 18:2 and 20:4 decreased. This finding might indicate that the lung can not utilize well lipid in the rats in which ligation of the thoracic duct was accomplished, and might be related with the fact that lung removes and utilizes chylomicrons passing through the thoracic duct.

The effect of ligation of the thoracic duct on fatty acids occurred in phosphatidyl ethanolamine and the unknown spot but did not occur in phosphatidyl choline.

Relation between phosphatidic acid and inositol metabolism is well established and an increased phosphatidic acid should be reflected in phosphoinositol if the established pathway for phosphatidic acid and phosphoinositol is applied to the lung. But phosphoinositol could not be separated by the analytical method used in the present investigation and was mixed with phosphatidyl ethanolamine. However, the unknown spot showed an interest finding. The concentration of 18:2 (the administered fatty acid) increased only in the unknown spot one hour after the administration of trilinolein. This finding might indicate the close relation between phosphatidic acid and the unknown spot.

In generaly it was concluded that phosphatidyl ethanolamine and the unknown spot were active in lipid metabolism although phosphatidyl choline was stable.

It was revealed in the present investigation that the lung also could have an ability of lipid synthesis. Of course, the lung can not be given the first situation in lipid metabolism but can have a role as a safety valve to prevent a temporary overwork of the liver in lipid metabolism.
absorption. The idea that the lung might have a role as a safety valve may be promoted by the finding that the lung removes chylomicrons passing through the thoracic duct and by the clinical observation that fatty liver at a greater rate in patients with pulmonary tuberculosis.

CONCLUSION

It is an important finding that a phosphatidic acid was discovered in the lung of rats only when fats were administered. This finding indicates that the lung has an ability of lipid synthesis. Furthermore, the appearance of a phosphatidic acid in the lung was delayed by ligation of the thoracic duct. This observation suggests that the lung removes chylomicrons passing through the thoracic duct.

The results obtained from the investigation of fatty acids indicate that the lung is active in fatty acid synthesis and that phosphatidyl ethanolamine is most active among phospholipid subfractions although phosphatidyl choline is stable.

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