EFFECTS OF DIETARY FAT ON LIPID COMPOSITION OF SERUM AND ERYTHROCYTES OF THE SWINE AND IN VITRO INCORPORATION OF FATTY ACIDS INTO ERYTHROCYTE MEMBRANES

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Changes in fatty acid patterns of lipids in serum and erythrocytes induced by dietary fats and in vitro incorporation of fatty acids into erythrocyte membranes were investigated with pigs. On feeding various diets, it was found that fatty acid composition of serum and erythrocytes could be modified and altered toward the fatty acid pattern of the diet. In vitro, the incorporation of labeled fatty acids into erythrocyte membranes was accelerated by the addition of cofactors such as lysolecithin, CoA and ATP. Dietary fats also had certain effects on the incorporation of fatty acids into erythrocyte membranes. Erythrocytes, collected from the blood of pigs fed corn oil, incorporated and also released more labeled linoleate than those of pigs fed hydrogenated soybean oil. Palmitic acid was more slowly incorporated into erythrocyte membranes than linoleic acid in the pigs fed both a commercial chow and scheduled meals, indicating selective esterification of fatty acids in the erythrocyte membranes.

Although the mechanism involved in fat transport in the body has been vigorously investigated, it is still incompletely understood. It has been demonstrated that after ingestion of different fats, the lipid and fatty acid compositions of animal sera (1, 2) and erythrocyte membranes (3, 4) change toward those of fat ingested. It has been established that the membrane of an erythrocyte is not merely an envelope but is a living organization (5, 6) which carries out various metabolic processes. Since the red cell membrane is constructed in part with lipids, transfer of various plasma components across this membrane may be affected by chemical changes in its lipids. The red cell membrane readily exchanges its cholesterol with serum lipoproteins and, in contrast to most other tissues, it has virtually no capacity to synthesize fatty acids; it incorporates fatty acids into phospholipids.

1 佐藤晋昭
The structural phospholipids which are most abundant in animal erythrocytes may be important in the maintenance of their viability and function, and they are continuously renewed while the blood cells circulate. Oliveira and Vaughan (7) and Van Deenen and coworkers (8, 9) noted the incorporation of ¹⁴C-labeled fatty acids into washed erythrocyte ghosts in the presence of ATP, CoA and MgCl₂. Mulder et al. reported an influence of dietary fat (10) on the incorporation of fatty acids into animal erythrocytes and other investigators (11, 12) on the importance of cofactors and nutrients in this phenomenon. This report describes a study of the effect of dietary fat on the lipid composition of sera and erythrocyte in the swine, and the incorporation and release of fatty acids in vitro.

EXPERIMENTAL

1. Experimental animals and dietary plan. Ten pigs of both sexes of Yorkshire (about six months of age) which had been fed the commercial chow, were used for the analysis of fatty acid patterns of serum and erythrocytes.

For the study with scheduled diets, 1 1/2 year old pigs of a crossbreed of Yorkshire and Duroc were allotted to one of the four high fat diets, fat constituting 44% of the total calorie. Four dietary regimens were prepared, using partially hydrogenated soybean oil and corn oil, and each fat diet was combined with two dietary protein levels of 15% and 5%. The partially hydrogenated soybean oil analysed by the method described by Swern et al. (13) was found to contain 48±2% of its fatty acids in the trans configuration. These two protein levels were employed in order to study whether or not the dietary protein has any effect on fatty acid composition. Prescription of experimental diets is shown in Tables 1A and 1B.

2. Materials and methods. Labeled fatty acids, 1-¹⁴C-palmitic and 1-¹⁴C-linoleic acids, were purchased from Amersham-Searle Corporation, Chicago, Illinois 60005, U.S.A. or Daiichi Kagaku Co., Tokyo. ATP, CoA, snake venom and lysolecithin were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Lysolecithin was prepared by the enzymatic removal of unsaturated fatty acids from the 2 position of egg lecithin, using snake venom as the source of enzyme.

Blood was collected into heparinized or citrated bottles from the swine by heart puncture at the time of slaughter and also taken from the anterior vena cava by a heparinized syringe at various times during the feeding study. Fresh blood samples were kept in the ice box to prevent oxidation of lipids.

Solution of fatty acid salts were prepared (15), by adding a slight excess of 1/10 N NaOH to each labeled and nonlabeled fatty acid which had been dissolved in absolute ethanol. The fatty acid salts were dissolved in a small amount (1–2 ml) of boiling saline and were combined with ten times amount of albumin by weight as described by Goodman (16).

3. Fatty acid analysis. After centrifugation of the blood at 2,000 rpm for
10 min, plasma was subjected to chloroform-methanol extraction as described by FOLCH (17). The extracted lipid was methylated by heating in a sealed ampule with 6% \( \text{H}_2\text{SO}_4 \) in methanol at 90°C for 2 hr and was subjected to gas-liquid chromatography (18). The erythrocytes, after washing three times with 0.9%

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Hydrogenated fat diet</th>
<th>Corn oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein content, %</td>
<td>15%</td>
<td>5%</td>
</tr>
<tr>
<td>Hydrogenated soybean oil</td>
<td>26.05</td>
<td>26.05</td>
</tr>
<tr>
<td>Corn oil (refined)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Soybean meal (49.5% CP)</td>
<td>27.1</td>
<td>9.1</td>
</tr>
<tr>
<td>Corn meal</td>
<td>30.1</td>
<td>10.0</td>
</tr>
<tr>
<td>Starch</td>
<td>—</td>
<td>38.0</td>
</tr>
<tr>
<td>Oat hulls</td>
<td>8.8</td>
<td>8.3</td>
</tr>
<tr>
<td>Solka floe</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Mineral supplement(a)</td>
<td>1.66</td>
<td>2.22</td>
</tr>
<tr>
<td>Trace-mineralized salt(b)</td>
<td>.37</td>
<td>.37</td>
</tr>
<tr>
<td>Vitamin supplement(c)</td>
<td>.055</td>
<td>.055</td>
</tr>
</tbody>
</table>

\(a\) 10.8% monosodium phosphate, 44.6% ground limestone and 44.6% dicalcium phosphate for high protein diets, and 33.3% monosodium phosphate, 33.3% ground limestone, and 33.3% dicalcium phosphate for low protein diets.

\(b\) Contributed the following per kg diet: NaCl 3.5 g; Zn, 37 mg; Mn, 30 mg; Fe, 0.8 mg; Cu, 3.7 mg; I, 0.4 mg; and Co, 0.4 mg.

\(c\) Contributed the following per kg diet: vitamin A, 1815 IU; vitamin D\(_2\), 181.5 IU; riboflavin, 0.6 mg; calcium pantothenate, 3.3 mg; nicotinic acid, 9.1 mg; choline chloride, 90.7 mg; vitamin B\(_2\), 9.7 µg; and vitamin E (d-alpha-tocopheryl acetate) 12 IU.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Hydrogenated fat diet</th>
<th>Corn oil diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed supplied, kg/head/day</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Metabolizable energy consumed, kcal/head/day(d)</td>
<td>9085</td>
<td>9240</td>
</tr>
<tr>
<td>Energy requirement, kcal/head/day(e)</td>
<td>5760</td>
<td>5760</td>
</tr>
<tr>
<td>Protein requirement, g/head/day(f)</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>Protein content, % (analyzed)(g)</td>
<td>15.7</td>
<td>5.1</td>
</tr>
</tbody>
</table>

\(d\) 7.55 kcal/g of oils, 3.40 kcal/g corn, 3.54 kcal/g starch after DIGGS et al. (42).

\(e\) Energy requirement = 93 x body mass, kg\(^{3/4}\), where body mass is 250 kg.

\(f\) Based on an average wt. of 221 kg and 0.88 m\(^{3/4}\) or on experimental results of BAKER et al. (43).

\(g\) Analysis for nitrogen (44).
saline, were hemolyzed with 1–2 volumes of distilled water. The hemolysate was added dropwise to 6 volume of methanol with agitation, and this mixture was added to 12 volume of chloroform. After thorough extraction, the extracted erythrocyte lipids were saponified with 0.5–1 ml of 1 N NaOH in 90% methanol by refluxing for 2 hr at 80°C. The fatty acids recovered from the aqueous phase by acidifying with 1 N HCl and extraction with n-hexane. The fatty acids were then methylated as above. Duplicate runs of gas-chromatography were performed for each sample.

4. Incubation procedure. The erythrocytes were washed three times with 0.9% saline, the supernatant leucocyte-containing buffy coat and the one tenth of the red cell column was discarded after each centrifugation at 2,000 rpm for 10 min. Contaminating leucocytes were counted in a Neubauer counting chamber, and found to be 1–2 thousand per cmm of the packed cells. Reticulocyte count was less than 0.01% of erythrocyte.

The packed cells were resuspended in Krebs-Ringer solution containing cofactors, such as ATP, CoA and Mg++, and incubated at 37°C with constant agitation.

Erythrocyte ghosts were prepared by centrifugation of hemolyzed erythrocytes (19) at 10,000×g for 15 min, three washings with deionized water and decantation of the supernatant.

The intact red cells after incubation with 14C-labeled fatty acids, were washed five times with cold 0.9% saline, hemolyzed by addition of 1 volume of distilled water, and the ghost cells were also washed three times with 5 ml of distilled water followed by centrifugation at 10,000×g for 15 min as previously described. Lipids were extracted from the cells according to the above method (17).

Neutral lipids were removed on a silicic acid coated thin-layer plate with petroleum ether-ether-gracial acetic acid (85:15:1) and phospholipids were separated into subclasses by two dimensional thin-layer chromatography (20). To check the contamination by tailing or poor separation, several areas (1/2×1 cm) surrounding each phospholipid spot was scraped off into scintillation vials to determine the radioactivity. It was negligible as compared with the radioactivity of the phospholipid spot.

RESULTS

A. Feeding study

1. Fatty acid patterns of serum and erythrocyte lipids in pigs fed commercial chow

Fatty acids of serum and erythrocyte lipids in ten pigs fed the commercial chow are shown in Fig. 1. The fatty acid pattern of serum is similar to that of red cells. Percentage of palmitate in serum was lower than that in red cells, and linoleic acid was lower in erythrocytes than in serum. The coefficient of correlation in fatty acid composition between serum and Rbc in these pigs was 0.973.
2. **Fatty acid patterns of serum and erythrocyte lipids during long term scheduled feeding** (Fig. 2)

Analyses were made on the fatty acids of serum and erythrocyte lipids in pigs kept feeding more than one year with four dietary regimens. The linoleic acid was the highest both in the serum and erythrocyte lipids of pigs fed the corn oil diets, and the oleic acid was the highest in those of pigs fed the soybean oil diets. In the lipids of Rbc from pigs maintained on corn oil diet, linoleic acid was relatively low and, in contrast, palmitic acid was high, compared to serum lipids. Thus, the fatty acid composition of Rbc was not exactly the same as that of
serum. The coefficients of correlation in fatty acid composition between serum and Rbc were 0.996, 0.979, 0.884 and 0.814 in the pigs fed soybean oil-high protein diet, soybean oil-low protein diet, corn oil-high protein diet and corn oil-low protein diet, respectively, showing lower correlation in corn oil-fed animals.

Myristic acid (C14:0) in the lipids of both serum and Rbc from pigs fed the scheduled diets was minimal, compared with pigs fed commercial chow.

Dietary protein does not seem to produce a significant change in the fatty acid pattern.

3. Effect of short term feeding on fatty acid composition of serum and erythrocyte lipids

Pigs were fasted for 18 hr, then fed, and blood was withdrawn before and 4 hr after the feeding. Figure 3 shows the fatty acid patterns of serum and erythrocyte lipids. No remarkable changes were observed. It is interesting, however, that a reciprocal movement of oleic acid was noted between serum and erythrocytes except in the pigs fed corn oil-high protein diet in which percentage of palmitic acid increased somewhat in erythrocytes.

Decrease in % linoleic acid of erythrocytes from pigs fed corn oil-high protein diet seems to be compensated for by a relative increase in percentage of palmitate and oleate, compared with the corresponding serum of pigs.

Fig. 3. Effects of short term feeding on relative concentration of fatty acids in pigs fed on hydrogenated soybean oil with low protein (A) or high protein (B), and in pigs on corn oil with low protein (C) or high protein (D). Animals were fasted for 18 hr and then fed. Blood was taken before (0) and 4 hr after feeding.
SERUM AND RED CELL LIPIDS AFTER FAT FEEDING

B. In vitro study

1. Distribution and time course of radioactivity of lipids incorporated into erythrocytes (Figs. 4 and 5)

To examine the existence of a pathway other than the acylation of lysolecithin by fatty acids, 20 µCi of Na$_{32}$PO$_4$ and 1 µCi of $^{14}$C-palmitate were incubated with 1.5 ml of Rbc suspension and no $^{32}$P radioactivity was found in erythrocyte lipids while $^{14}$C-palmitate was incorporated into triglyceride and phospholipids. In another set of experiment, 40 µCi of Na$_{32}$PO$_4$ and 1 µCi of $^{14}$C-linoleate were incubated with 1 ml of Rbc and the results were essentially the same. When $^{14}$C-palmitate was incubated with Rbc, about 98% of the radioactivity in the erythrocyte was found in the free fatty acids and neutral lipid fraction after 7 hr, followed by the phosphatidyl choline and phosphatidyl ethanolamine fractions in decreasing order (Fig. 4).

![Graph showing the distribution of radioactivity in erythrocyte lipids](image)

Fig. 4. Percentage of radioactivity taken up by swine erythrocyte lipids after incubation with $^{14}$C-palmitate. FFA, free fatty acid; NL, neutral lipid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PL, phospholipids; other, other than PC and PE.

As shown in Fig. 5, $^{14}$C-linoleate was rapidly incorporated into phospholipids of erythrocyte membranes, especially into phosphatidyl choline by one hour, at a slower rate thereafter. The incorporation of palmitic acid was slow. Incorporation into triglyceride was about one tenth of that of phospholipids.

2. The effect of cofactors on fatty acid incorporation into erythrocyte membranes

Labeled linoleate was used. Difference in radioactivity brought about by
withdrawal of various cofactors is shown in Table 2.

The removal of ATP and CoA from media seems to reduce the fatty acid incorporation to a greater extent than the removal of Mg++.  

Table 2. The effect of cofactors on the incorporation of labeled linoleate into phospholipids of membranes from swine erythrocytes.

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>Phospholipids incorporating labeled fatty acid</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC</td>
<td>Radioactivity</td>
</tr>
<tr>
<td></td>
<td>Radioactivity</td>
<td>%</td>
</tr>
<tr>
<td>Complete system</td>
<td>$\times 10^8$ cpm/ml RBC</td>
<td>%</td>
</tr>
<tr>
<td>without CoA</td>
<td>110.0</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>78.2</td>
<td>71.1</td>
</tr>
<tr>
<td></td>
<td>71.3</td>
<td>64.8</td>
</tr>
<tr>
<td></td>
<td>52.6</td>
<td>47.8</td>
</tr>
<tr>
<td></td>
<td>46.2</td>
<td>42.0</td>
</tr>
</tbody>
</table>
To study the influence of plasma on the incorporation of fatty acid, 2 ml of red blood cell suspension were incubated in two different media. (A) 4 ml of Krebs-Ringer solution containing 4 μmole of ATP, 0.4 μmole of CoA and 0.4 μCi of 14C-palmitate. (B) 2 ml of Krebs-Ringer solution containing same amount of cofactors, 0.4 μCi of 14C-palmitate and 2 ml of corresponding plasma. Radioactivities of phosphatidyl choline were compared at each 1 hr interval up to 5 hr. The erythrocytes took up less radioisotope when incubated in the presence of plasma (Fig. 6).

3. Influence of contaminating leucocytes on fatty acid incorporation into red cells
One μCi of 14C-linoleate was incubated with erythrocytes remained by varying numbers of leucocytes which were purposely increased by gathering leucocyte-containing buffy coat after centrifugation. The cofactors consisted of 4 μmoles of ATP, 0.4 μmole of CoA, 4 μmoles of MgCl₂ and 0.2 ml of 0.1 M glucose. Figure 7 shows the interrelation of erythrocytes and leucocytes with the incorporated radioisotope. A linear increase was found in the radioactivities of phospholipids and triglyceride as the numbers of leucocytes increased, while a linear decrease was seen with increasing numbers of erythrocytes (Fig. 7A).

When phospholipids were separated and counted, the radioactivity of phosphatidyl ethanolamine did not change with the increase of blood cell number. However, the radioactivity of phosphatidyl choline showed a linear reciprocal relationship between erythrocyte and leucocyte numbers (Fig. 7B).

4. The effect of lysolecithin added and dietary fat on fatty acid incorporation into intact erythrocytes
One and a half ml of erythrocytes of pigs fed hydrogenated soybean oil or corn oil were incubated in 1.0 ml of Krebs-Ringer solution containing 4 μmoles of ATP, 0.4 μmole of CoA and 0.75 μCi of 14C-linoleate-albumin complex, with or without lysolecithin. Table 3 demonstrates that the effect of added lysolecithin
was much greater in the corn oil-fed swine.

Without lysolecithin, incorporation into phosphatidyl choline of erythrocytes was smaller in pigs fed corn oil than those fed hydrogenated soybean oil. With lysolecithin, the incorporation rate of linoleic acid into phosphatidyl choline of erythrocytes was higher in pigs fed corn oil than in those fed hydrogenated soybean oil.

Table 3. The effect of lysolecithin on the incorporation of labeled linoleate into erythrocytes of pigs fed either corn oil or hydrogenated soybean oil.

The incubation mixture consisted of 1.5 ml of RBC, 1.0 ml of Krebs-Ringer solution containing 4 μmoles of ATP, 0.4 μmole of CoA and 0.75 μCi of 14C-labeled linoleate, with (+) or without (−) 0.025 mg of lysolecithin. 100 percent for the radioactivity of phosphatidyl choline in the lipids of RBC of pigs incubated with lysolecithin. PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; S.D., standard deviation.

<table>
<thead>
<tr>
<th>Type of diet</th>
<th>Phospholipids incorporating labeled linoleate</th>
<th>Addition of lysolecithin</th>
<th>Radioactivity $\times 10^6$ cpm/ml RBC</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil</td>
<td>PC</td>
<td>+</td>
<td>30.6±1.8 (S.D.)</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>−</td>
<td>15.4±1.5</td>
<td>50.3</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>+</td>
<td>4.4±0.5</td>
<td>14.4</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>−</td>
<td>3.5±1.1</td>
<td>11.4</td>
</tr>
<tr>
<td>Hydrogenated soybean oil</td>
<td>PC</td>
<td>+</td>
<td>26.7±4.7</td>
<td>87.3</td>
</tr>
<tr>
<td></td>
<td>PC</td>
<td>−</td>
<td>20.3±0.7</td>
<td>66.3</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>+</td>
<td>5.2±0.1</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td>PE</td>
<td>−</td>
<td>5.6±0.3</td>
<td>18.3</td>
</tr>
</tbody>
</table>
To study the difference in incorporation rate of different fatty acids, $^{14}$C-linoleate and $^{14}$C-palmitate were incubated with erythrocytes from pigs fed corn oil and commercial chow. The results in Table 4 revealed a greater incorporation of linoleate than palmitate into both phospholipids and triglyceride fraction of erythrocytes from pigs on each diet.

### Table 4A. Incorporation of labeled linoleate and palmitate into erythrocyte lipids of pigs fed chow ad libitum.

<table>
<thead>
<tr>
<th>Type of labeled fatty acid</th>
<th>Lipid incorporating labeled fatty acid</th>
<th>Radioactivity $\times 10^4$ cpm/ml RBC</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C-Linoleate</td>
<td>Phospholipids</td>
<td>250.0 ± 11.2 (S.D.)</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>168.6 ± 17.8</td>
<td>67.4</td>
</tr>
<tr>
<td></td>
<td>Lecithin</td>
<td>98.6 ± 15.2</td>
<td>39.4</td>
</tr>
<tr>
<td></td>
<td>Phosphatidyl ethanolamine</td>
<td>71.0 ± 5.4</td>
<td>28.4</td>
</tr>
<tr>
<td>$^{14}$C-Palmitate</td>
<td>Phospholipids</td>
<td>173.8 ± 13.4</td>
<td>69.5</td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>66.7 ± 6.7</td>
<td>26.7</td>
</tr>
<tr>
<td></td>
<td>Lecithin</td>
<td>73.4 ± 7.8</td>
<td>29.4</td>
</tr>
<tr>
<td></td>
<td>Phosphatidyl ethanolamine</td>
<td>31.8 ± 4.0</td>
<td>12.7</td>
</tr>
</tbody>
</table>

### Table 4B. Incorporation of labeled linoleate and palmitate into erythrocyte lipids of pigs fed a scheduled diet.

The incubation mixture consisted of 1.0 ml of RBC from pigs fed either ad libitum or a corn oil-low protein diet, 0.5 ml of Krebs-Ringer solution containing 4 $\mu$moles of ATP, 0.4 $\mu$mole of CoA, 1.0 $\mu$Ci of fatty acid salt and 0.025 mg of lysolecithin per ml of RBC. Figures are mean radioactivities from four parallel experimental samples. S.D., standard deviation.

<table>
<thead>
<tr>
<th>Type of labeled fatty acid</th>
<th>Lipid incorporating labeled fatty acid</th>
<th>Radioactivity $\times 10^4$ cpm/ml RBC</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C-Linoleate</td>
<td>Phospholipids</td>
<td>298.9 ± 10.0 (S.D.)</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>48.4 ± 2.2</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td>Lecithin</td>
<td>126.5 ± 10.6</td>
<td>42.3</td>
</tr>
<tr>
<td></td>
<td>Phosphatidyl ethanolamine</td>
<td>107.5 ± 9.7</td>
<td>36.0</td>
</tr>
<tr>
<td>$^{14}$C-Palmitate</td>
<td>Phospholipids</td>
<td>134.9 ± 7.6</td>
<td>45.1</td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>9.8 ± 1.3</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>Lecithin</td>
<td>44.5 ± 6.0</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>Phosphatidyl ethanolamine</td>
<td>45.2 ± 6.2</td>
<td>15.1</td>
</tr>
</tbody>
</table>

5. **Fatty acid incorporation into erythrocyte ghosts**

The results in Table 5 shows a much greater incorporation of $^{14}$C-labeled linoleate into phosphatidyl choline and phosphatidyl ethanolamine in the erythrocyte ghosts in the swines fed corn oil than in those fed hydrogenated soybean oil. The erythrocyte ghosts of corn oil-fed swines took up twice as much $^{14}$C-linoleate as those of soybean oil-fed ones into phosphatidyl choline and slight greater radioactivity into phosphatidyl ethanolamine.
Table 5. Fatty acid incorporation into erythrocyte ghosts of pigs fed corn oil and hydrogenated soybean oil.

The incubation mixture consisted of 1.0 ml of erythrocyte ghosts, 1.0 ml of Krebs-Ringer solution containing 4 μmoles of ATP, 0.4 μmole of CoA, 0.025 mg of lysolecithin and 1.5 μCi of linoleate-albumin complex per ml of RBC.

<table>
<thead>
<tr>
<th>Phospholipid, incorporated labeled fatty acid</th>
<th>Type of diet</th>
<th>Radioactivity (\times 10^2) cpm/ml RBC</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidyl choline</td>
<td>Corn oil</td>
<td>490.8 ± 26.7 (S.D.)</td>
<td>100.0</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>Hydrogenated soybean oil</td>
<td>227.9 ± 16.9</td>
<td>46.4</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>Corn oil</td>
<td>37.5 ± 1.7</td>
<td>7.6</td>
</tr>
<tr>
<td>Phosphatidyl ethanolamine</td>
<td>Hydrogenated soybean oil</td>
<td>29.0 ± 1.0</td>
<td>5.9</td>
</tr>
</tbody>
</table>

6. Specificity of fatty acid incorporation with respect to structural position of erythrocyte phospholipids

Enzymatic degradation of phospholipids was carried out with the aim to elucidate the position where the labeled fatty acid is incorporated according to the method described by LONG and PENY (21). \(^{14}\)C-palmitate and \(^{14}\)C-linoleate were separately incubated with swine erythrocytes in the mixture containing necessary cofactors.

Half ml of ether containing lecithin separated by thin-layer chromatography was pipetted into each of four test tubes (125 mm × 15 mm), and 0.01 ml (1 μg) of aqueous snake venom (Crotalus adamanteus) solution (containing 1 ml of 5 mM CaCl₂/mg venom) was added. The contents were mixed while shaking vigorously and tubes allowed to stand at room temperature for 30 min. Enzyme activity was stopped by adding 2 ml of ester-free ethanol and dried by nitrogen stream. The precipitated lipids were dissolved in chloroform to apply the TLC.

Table 6. Degradation with snake venom of phospholipids isolated from swine erythrocytes.

The erythrocytes from four different pigs fed ad libitum were incubated in the phosphate buffer containing 4 μmoles of ATP, 0.4 μmole of CoA, 4 μmoles of MgCl₂ and 1.0 μCi of \(^{14}\)C-labeled fatty acids. \(^{14}\)C radioactivity was determined in fatty acid, phospholipid and in the lysocompound of the phospholipid after enzymatic degradation and separation by thin layer chromatogram. FA, fatty acid; PC, phosphatidyl choline; LL, lysolecithin; PE, phosphatidyl ethanolamine; L-PE, lysocompound of phosphatidylethanolamine.

Labeled fatty acid, incorporated into phospholipid

<table>
<thead>
<tr>
<th>14C Radioactivity in</th>
<th>Phosphatidyl choline</th>
<th>Phosphatidyl ethanolamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>PC</td>
<td>LL</td>
</tr>
<tr>
<td>FA</td>
<td>PE</td>
<td>L-PE</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>14C Linoleate</th>
<th>14C Palmitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm</td>
<td>351</td>
</tr>
<tr>
<td>%</td>
<td>100</td>
</tr>
</tbody>
</table>

FA, fatty acid; PC, phosphatidyl choline; LL, lysolecithin; PE, phosphatidyl ethanolamine; L-PE, lysocompound of phosphatidylethanolamine.
plates. Separated fatty acids, lecithin and lysolecithin were submitted to radioactivity counting by the liquid scintillation counter. Phosphatidyl ethanolamine was also similarly separated and the radioactivities were counted. Radioactivities of separated lipids after enzymatic degradation are shown in Table 6. Most of the radioactivities of degraded phospholipids were found in the fatty acid fraction and 10 to 20% of the radioactivity was detected in phospholipids and their lysocompounds when \(^{14}\text{C}\)-palmitate was used, while all radioactivity was found in the fatty acid fraction when \(^{14}\text{C}\)-linoleate was used.

7. Release of phospholipid fatty acid from swine erythrocytes

To study the effect of dietary fat on the catabolism of erythrocyte phospholipids, erythrocytes of pigs fed two kinds of diet were labeled with radioactive fatty acid (\(^{14}\text{C}\)-linoleate) in vitro. The labeled cells were then incubated in the serum of the original blood and loss of radioactivities from the cells into plasma was examined. Release of phospholipid fatty acid from the erythrocytes of pigs fed corn oil diet was measured and compared with the results in pigs fed hydrogenated soybean oil diet. As demonstrated in Fig. 8, the release was somewhat greater in the former, compared with the latter.

![Fig. 8. The release of \(^{14}\text{C}\)-linoleic acid from swine erythrocyte.](image)

**DISCUSSION**

It is generally thought that lipid composition of serum and erythrocytes can be modified by manipulation of dietary fat (1-4, 23-25). Present study has been designed to investigate the direct and indirect effect of dietary fat on fatty acid composition of serum and erythrocytes of pigs by feeding either hydrogenated...
soybean oil or corn oil, combined with varying protein levels. Major fatty acids in the soybean oil and corn oil examined by gas-liquid chromatography were C₁₆:₀ 13.3%, C₁₈:₀ 14.1%, C₁₈:₁ 72.6%, for partially hydrogenated soybean oil, and C₁₆:₀ 11.2%, C₁₈:₀ 1.8%, C₁₈:₁ 25.9%, C₁₈:₂ 61.1% for corn oil.

Although KUMMEROW et al. (14) demonstrated that dietary protein influenced the cholesterol-depressing effect of corn oil in the chicks, the present study did not show any difference in fatty acid composition. This might be due to the fact that these pigs consumed so much calories and proteins in excess of their requirements.

Evidence has been virtually presented in this paper that fatty acid patterns of sera and erythrocytes from pigs are changed toward those of dietary fat ingested, especially in the serum (Fig. 2A, 2B). A greater variation was noted in the fatty acid composition of erythrocytes, compared with serum fatty acids.

MULDER et al. (10) demonstrated a selective incorporation of fatty acids into rabbit erythrocyte phospholipids, relating that, in contrast to linoleic acid, ingestion of large amount of lauric acid failed to elevate the content of this fatty acid in the red cell phospholipids in spite of notable elevation of lauric acid level in plasma, while the percentage of linoleic acid was doubled within 10 days after replacing the lauric acid-containing coconut oil by corn oil rich in linoleic acid. Increase in percentage of palmitic acid might indicate relative increase in its incorporation or synthesis by erythrocytes, as demonstrated by PITTMAN and MARTIN (22).

The present study casted the focus on the fatty acid incorporation into erythrocyte membrane in connection with dietary fat. Incorporation of unesterified fatty acids in the external medium into the phospholipids of erythrocyte membranes was first noted by OLIVEIRA and VAUGHAN (7). They found, using erythrocyte ghosts as experimental models, that the energy dependent incorporation was primarily into phosphatidyl choline.

Subsequently, MULDER et al. (27) suggested that two biochemical pathways could be operative in the final stage of the incorporation. These pathways are; (A) Insertion of fatty acids (activated as acyl CoA) into preformed lysolecithin by the pathway originally described by LANDS (28). (B) Transesterification between two lysolecithin molecules to form one phosphatidyl choline, as described by ERBLAND and MARINETTI (29) as well as SHOHET et al. (26).

The in vitro incubation study (Fig. 4) showed that more than 98% of the radioactivity in the erythrocyte membranes was still in the free acid-neutral fat fraction and that about 2% of the radioactivity in the membranes was incorporated into phospholipid fraction in 7 hr of incubation. The author, however, admits that the radioactivity derived from the fatty acid-neutral fat fraction might contain radioactive free fatty acid on the cell surface which had not been completely washed off.

Incubation of Na₂¹⁵PO₄ with swine erythrocytes revealed absence of pathway
other than one reported above, representing Kennedy and Greenberg pathways.

The results of the present study agree with those of DONABEDIAN and KAR-
MEN (12) in that most of the radioactive fatty acids were incorporated into erythro-
cyte phospholipids and triglyceride in the presence of the energy producing system. 
As shown in Table 2, ATP markedly enhanced fatty acid incorporation, parti-
cularly when the experiment was carried out with hemolysate.

WINTERBOURN and BATT (30) also observed a very active incorporation of 
fatty acids into leucocytes, mainly into phospholipids and triglyceride. As shown 
in Fig. 7, with higher concentrations of leucocytes, apparent decrease was found 
in the amount of radioactivity incorporated into the erythrocyte lipids. In calcu-
ating the radioactivities of lipids taken up by both blood cells, a white blood 
cell appeared to incorporate about 85 times radioactive fatty acids into phospho-
lipids, and about 280 times more into triglyceride, compared with a red blood cell. 
Judging from the life span and activity of cellular metabolism of Rbc and Wbc, it 
is expected that Wbc took up the fatty acid far more than Rbc. And when in-
cubated together, fatty acid incorporation into phosphatidyl choline per a red 
cell would become less and less as the number of Wbc and Rbc increased. As 
for fatty acid incorporation into phosphatidyl ethanolamine, no distinct increase 
of radioactivities was found with the increase of cell number both in Wbc and 
Rbc, which means proportional decrease of the incorporation per a cell. The 
author speculates that much greater incorporation of fatty acid into phosphatidyl 
choline of both blood cells brought about the decrease in the uptake per each cell 
into phosphatidyl ethanolamine as the cells increased. In other experiments, 
effort was made to remove leucocytes as much as possible, but it was not feasible 
to remove them completely. The erythrocyte preparation contained 0.001% to 0.002% of leucocytes.

A significant difference was observed in the rate of fatty acid incorporation 
into erythrocytes when incubated in plasma or Krebs-Ringer solution. Although 
plasma contains various cofactors and nutrients, the incorporation was less ac-
tive, which might indicate that plasma inhibited the removal of the fatty acid from 
albumin before energy-requiring acylation of fatty acid takes place. In fact, 
GOODMAN (16) noted a strong binding of serum albumin and fatty acid anions, 
and postulated three classes of binding sites between them.

Synthesis of lecithin by acylation of lysolecithin was first demonstrated by 
LANDS (28) in liver microsomes. In the studies of acyltransferase from this source, 
LANDS and MERKEL (31) compared the utilization of several fatty acyl CoA com-
pounds with acylglycerophosphoryl choline as an acceptor. CoA esters of un-
saturated fatty acids reacted more rapidly than did those derivatives of saturated 
fatty acids. In the present study, more linoleic acid than palmitic acid was in-
corporated into erythrocyte lipids. A similar specificity of acyltransferase in 
erythrocytes might be responsible for the preferential incorporation of linoleic 
acid because more of 14C-labeled linoleate was incorporated than labeled palmitate
whereas erythrocytes of pigs fed commercial chow was richer in palmitic acid than in linoleic acid, and it seems that the difference in the incorporation between both fatty acids was greater in the pigs fed a scheduled diet than those fed the commercial chow.

As the lipid composition of plasma and erythrocytes varies with species (32, 33), differences in the rate of fatty acid incorporation into animal erythrocytes have also been noted. MULDER et al. reported that incorporation of fatty acids into phosphatidyl choline of red cells of the rat, rabbit, and man was greater than those of the ox and sheep which have a very low lecithin content. Similar observations were made by OLIVEIRA and VAUGHAN (7), who also reported a higher uptake of labeled linoleate into phosphatidyl ethanolamine of bovine red cells when compared with rabbit erythrocytes. Thus, various observations suggest that distribution among lipid fractions of the incorporated fatty acids is related only indirectly to the known differences in phospholipid composition between the ruminants and nonruminants studied.

Furthermore, it has been found that these erythrocytes differ in permeability, as modified by manipulation of dietary fat (33, 34), and in the binding of phospholipids (35), indicating that altered fine structure of erythrocyte membrane induced by difference in phospholipids and their fatty acid compositions might influence its function.

The results of in vitro studies with labeled fatty acids showed that the fatty acids are directed to the specific ester sites. In general, it had been thought that unsaturated fatty acids are incorporated at 2nd or β position of phosphatidyl choline whereas saturated fatty acids at 1st or α position, and a more recent study by WAKU and LANDS (36) has indicated that in human erythrocytes the acyltransferase activity measured with 2-acylglycerol phosphoryl choline was much lower than with 1-acylglycerol phosphoryl choline. On the other hand, OLIVEIRA and VAUGHAN (7) reported that in human erythrocyte ghosts palmitic acid was esterified like linoleic acid mainly at 2 position of phosphatidyl choline.

In rabbit erythrocytes, as studied by MULDER and Van Deenen (8), linoleic and oleic acids were esterified at 2 position whereas palmitic acid was preferentially incorporated into 1 position.

Furthermore, the experiment by RAY et al. (45), using E. coli, demonstrated the incorporation of stearic acid, saturated fatty acid, into 2 position of diacylglycerophosphate.

The present study revealed that the linoleic acid was incorporated into 2 position and that palmitic acid mostly into the same position but also into 1 position of lecithin and phosphatidyl ethanolamine. The result is not comparable with those already reported because swine erythrocytes have not yet been studied in this regard.

Thus, fixed selectivity of fatty acid incorporation into the phospholipids is somewhat betrayed. As shown in the studies of several other investigators, the
results varied with the experimental conditions, such as concentration and type of substrates (46), acceptors (47, 48), pH (49), temperature (50), species, and so forth.

Furthermore, it is generally said that fatty acids at 2 position of glyceryl-phospholipids are easily transferred to 1 position. The author speculates that the palmitic acid incorporated into 2 position might be transferred to 2 position during long (8 hr) incubation, because the fatty acid was incorporated into both positions while linoleic acid was incorporated exclusively into 2 position. These facts also seem to indicate the existence of different types of acyltransferase, as shown by Cronan et al. (50). At present, the multiplicity of the enzyme action needs further investigation.

In the experiments in which synthetic lysolecithin was used, a much greater incorporation of linoleic acid than palmitic acid was observed as expected, because the lysolecithin preparation used had the 2 position unesterified. Thus, fatty acid composition may be predicted to a certain extent by comparison between relative rates of acyl transfer to 2 position and composition of red cell lipids.

The fatty acid pattern of erythrocytes is determined by an equilibrium between the fatty acids taken up and their degradation or release through the membranes. This in vitro study revealed a greater incorporation and removal of linoleic acid in the erythrocytes of swines fed corn oil than in the erythrocytes of those fed hydrogenated soybean oil. It may be a reasonable finding, provided that lipid content of erythrocyte membranes and serum are not increasingly changed and keep relatively constant levels after a certain equilibration is attained.

Furthermore, Nichaman et al. (41) reported that a diet high in linoleic acid stimulated the conversion of the acid to carbon dioxide both in normal and hyperlipemic subjects, and they speculated that high linoleate feeding may contribute to the control of hyperlipemia by diverting more dietary fatty acids toward oxidative pathways.

There is some question of whether or not the fatty acid incorporation observed in these in vitro studies represented a mechanism for in vivo renewal of fatty acids in erythrocyte phospholipids (8). It seems to be the case, and presumably the acyltransferase reactions proceed normally at about the same relative rates as those observed for the exchange of phospholipids between plasma lipoproteins and erythrocyte membrane (37).

Several workers have investigated phospholipid alterations in various disease states (38–40). In such disorders, the erythrocyte membranes seems to be a good monitor for similar change occurring in the membranes of other tissue cells. And hopefully, what is learned about the structure and function of erythrocyte membranes may be applied directly to the membranes of other tissue cells or their intracellular organelles in relation with their lipid composition.
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REFERENCES