MECHANISMS OF THE FATTY LIVER PRODUCED
BY THE ADMINISTRATION OF ANTIBIOTICS

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A considerable number of clinical and experimental investigations have been reported concerning the liver dysfunctions\textsuperscript{1,2,3} and the fatty degeneration of the livers\textsuperscript{4,5,6} produced by large doses of aureomycin. The mechanisms of the fatty degeneration due to the antibiotics, however, have remained obscure and have been speculated only by several investigators\textsuperscript{7,8,9}. On the other hand, there have been many studies on the biochemical mechanisms of the development of fatty accumulation in the liver following carbon tetrachloride poisoning. According to Dianzani\textsuperscript{10,11,12}, the development of the fatty liver following carbon tetrachloride poisoning may depend on the primary loss of mitochondrial function in the hepatic cells. Recent investigations\textsuperscript{13,14,15} suggest that the fatty accumulation in the liver may be attributable to the inhibition on the mechanism of triglyceride secretion in the liver by carbon tetrachloride.

This paper is concerned with the investigation of some of the mechanisms of the fatty degeneration in mouse livers produced by antibiotics which are administered parenterally in relatively large doses at intervals of over five days, with special relation to the fatty acid metabolism. And the discussion is presented on the comparison of these mechanisms with that of the fatty liver following carbon tetrachloride poisoning.

EXPERIMENT

Materials

The male d.d.-strain mice, weighing 20 to 25 g each, were used throughout the experiments. They were fed with commercial diet ("Oriental pellet") without supplement and divided into four groups.

The first group (Aureomycin group) was given daily intraperitoneal dosis of 1 mg (40 to 50 mg per kg of body weight) of aureomycin, dissolved in 0.1 ml of isotonic sodium chloride solution, for five days. The second group (Chloramphenicol group) was given 2 mg (80 to 100 mg per kg of body weight) of chloramphenicol intraperitoneally for five days. The third group (Penicillin group) was given 1,000 units (40,000 to 50,000 units per kg of body weight) of procaine penicillin intraperitoneally for five days. The fourth group (CCl\textsubscript{4} group) received an intramuscular injection of 0.01 ml of CCl\textsubscript{4} (0.4 to 0.5 ml per

Received for publication March 14, 1962.
kg of body weight), dissolved in 0.1 ml of olive oil. A control group received a daily intraperitoneal injection of 0.1 ml of isotonic sodium chloride solution for the same period.

**Analytical Procedures**

The animals were sacrificed by decapitation 1, 2, 3, 5 and 7 days after the final administration of each drug, and the livers were removed for the purpose of various determinations.

1. For the estimation of the total lipid content, the liver was homogenized with a homogenizer of the Potter-Elvehjem type and extracted in Bloor solution (ethanol-ether, 3:1) and the total lipid content was determined by evaporation of the lipid extracts and weighing of the residue.

2. The value of liver phospholipids was determined by the method of Fiske-Subbarow.

3. Free and esterified cholesterols were estimated by the colorimetric method of Sperry-Webb.

4. The succinic dehydrogenase activity of mouse liver homogenate was measured by the method of Schneider and Potter. The liver was homogenized in cold 0.154 M KCl isotonic solution (mixed at the rate of 19 ml of the solution to one gm of liver).

5. The activity of fatty acid oxidase of liver mitochondria was determined by the method of Lehninger using octanoate. The preparation of mitochondria from mouse liver were made according to the method of Schneider and Hogeboom using 0.25 M sucrose as the medium at 0–4 °C. The medium had the following composition (final concentration): phosphate buffer (0.02 M, pH 7.4), ATP-K (0.0015 M), MgCl₂ (0.0075 M), octanoate (0.001 M, pH 7.4), cytochrome C (0.000015 M). The center well equipped with alkali and filter paper roll. To each vessel, 0.5 ml of suspension of mouse liver mitochondria in 0.25 M sucrose was added. This addition, which brings the total volume to 2.5 ml, started the reaction. Oxygen uptake was checked every 10 minutes at 25 °C.

6. Further investigations of the direct antibiotic inhibition to these two enzyme activities were carried out by adding 20 μg of aureomycin, 20 μg of chloramphenicol or 2 units of penicillin dissolved in 0.1 ml of distilled water, respectively, to each reaction system.

7. Microscopic changes of the livers following the administration of the drugs were also studies by staining with H. E., Sudan III and PAS.

8. The fatty acid composition of the liver lipids was determined by gas-liquid chromatography (GLC) using the methyl esters derived from the liver lipids. The methyl esters of the hepatic fatty acids were prepared according to the method of Böttcher et al. with some modifications: extraction of lipids from the liver was carried out with refluxing methanol-ether, and saponification of the lipids extracted was obtained by refluxing with methanolic 0.2 N KOH for 60 minutes. After the removal of the unsaponifiable, the fatty acid mixtures obtained by acidification were methylated with dry methanolic HCl, which was prepared by 100 times dilution of saturated methanolic HCl,
with methanol (so as to render the dry solution 0.16 N), refluxing for 120 minutes. The methyl esters of fatty acid mixtures thus obtained were used as samples for GLC. GLC was carried out essentially as described by James and Martin \(^{22}\) using GC-1A type of gas chromatograph made by Shimadzu Seisakusho Co. Ltd. with a katharometer. Three 750 mm stainless steel columns 6 mm in inside diameter, containing 80 to 100 mesh Celite 545 coated with 20 per cent succinic acid-diethylene glycol polyester were used for the regular packed column. Polyester was synthesized according to the procedure of Herb, Magidmann and Riemenschneider \(^{23}\) using succinic acid and diethylene glycol. Temperatures employed were: column 200\(^\circ\) C, detector cell 200\(^\circ\) C, and flash heater 260\(^\circ\) C. The helium carrier gas pressure was 0.9 atm at column inlet with an outlet flow of 90 ml per minute. The bridge was operated at 175 mA from a 12 volt storage battery. The recorder was operated on the 2 or 4 mV range, depending on the sample size. The sample was dissolved in ether and was applied to the column, using a syringe. By referring to the work of Stoffel *et al.* \(^{24}\), the retention times had been calculated and decided by the preliminary analysis carried out repeatedly on the purified samples of methyl esters of various fatty acids at the same conditions. The percentage composition of fatty acids was obtained by measuring the area under each peak with a planimeter.

**RESULTS**

1. *Gross and Microscopic Studies*

In many instances, the livers of the mice receiving aureomycin and chloramphenicol parenterally were apparently pale yellow in colour on the 3rd or 5th day after the last administration and, thus, easily differentiated from the livers of the penicillin and control groups which had normal appearances.

Microscopically, almost all livers of the aureomycin group showed slight degenerative findings in the hepatic parenchymal cells. On the 3rd day, vacuolation appeared in the cytoplasm of the hepatic cells located in the center of the lobule. Fat staining indicated that the vacuoles were occupied by neutral fat. On the 5th day, the infiltration of fat droplets became significant in the degree and diffuse in the extent in the livers, which consequently showed the typical microscopic findings of fatty liver. But these changes became somewhat milder on the 7th day than on the 5th day.

Almost the similar microscopic changes were observed in the livers of the chloramphenicol group, but the degree of fatty accumulation was slight as compared with that in the livers of the aureomycin group. Furthermore, in nearly all instances of the chloramphenicol group there was no degeneration of hepatic cells except in the livers examined on the 1st day.

The livers of the penicillin group revealed no microscopic alterations almost in any cases. A few droplets of neutral fat appeared in some livers only on the 7th day, but these slight changes occasionally appeared even in the livers of the control group in the late stage of the experiments. The majority of the control group exhibited minimal or no changes in the livers.
The livers of the CCl₄ group exhibited the maximal centrilobular deposition of neutral fat with some of the primary necrotic changes and swelling of hepatic parenchymal cells on the 1st day after the intramuscular injection. Thereafter, the fatty infiltration in the livers was gradually depleted to the complete recovery on the 5th day. Some of the regenerative findings in the hepatic cells already appeared on the 5th day after the injection.

2. Changes in the Total Lipid Content in the Livers
The changes in the total lipid content in the livers is presented in Fig. 1. After the last administration of aureomycin, the total lipids in the liver were kept at almost normal level on the 1st and 2nd day, started to increase on the 3rd day, reached the maximum (141% of the normal value) on the 5th day and remained at a high level on the 7th day. The changes in the total lipid content of the chloramphenicol group exhibited a similar tendency to that of the aureomycin group, but the increase of fat (127%) was slighter in the former than in the latter. The livers of the penicillin group showed minimal or no alterations in the total lipids except the slight increase on the 7th day. On the other hand, the administration of CCl₄ caused a marked elevation of the lipid content in the liver (180%) within 24 hours, and a gradual fall with a recovery to the normal level on the 5th day. As shown above, distinct differences of the changes in the total lipids in the livers were observed between the antibiotic groups and the CCl₄ group.

3. Changes in the Phospholipid Content in the Livers
Liver phospholipids of the three antibiotic groups and the CCl₄ group, as represented in Fig. 2, showed relatively similar fluctuations in the changing pattern with some differences in the degree among drugs. Namely, the phospholipid level, in general, was slightly elevated in the early stage of the
experiments and then fell off to a significant degree in the middle stage and rose again in the late stage.

The phospholipids of the aureomycin group decreased to the minimum (63.3%) on the 5th day. Those of the chloramphenicol group were declined to the minimum (66.7%) on the 3rd day, and those of the penicillin group showed minimal alterations. The phospholipid level of the CCl₄ group was somewhat elevated on the 1st day, fell down to lowered levels (73.3%), and again was elevated (120%) on the 7th day. The decrease of the phospholipids of the CCl₄ group in the early stage of the experiments was not so significant, but the ratio of phospholipids to total lipids may indicate an evidence of a relative decrease in the phospholipids of the livers.

4. Changes in the Total and Esterified Cholesterol Contents in the Livers

As shown in Fig. 3, the total cholesterol level in the liver of the aureomycin or chloramphenicol group in the present experiments exhibited a remarkable elevation already on the 1st day after the last injection, a fall on the 5th day, and a considerable rise again on the 7th day. Only a transitory elevation of the total cholesterol level of the penicillin group was observed on the 3rd day. On the contrary, the changes in the total cholesterol level in the liver of the CCl₄ group were slight as compared with those of the antibiotic groups in the early stage, but the marked increase were observed in the middle stage.

The esterified cholesterol level of the aureomycin group showed a significant elevation corresponding to a marked increase of the total cholesterol level on the 1st day, thereafter remained at lowered levels and again showed a slight elevation on the 7th day. The chloramphenicol group showed a decline of the esterified cholesterol content in the middle stage and a late gradual elevation. The penicillin group showed minimal changes within the normal
range of fluctuations. The esterified cholesterol level of the CCl₄ group remained rather at lowered levels during early three days after the injection and subsequently showed a tendency to increase.

![Graphs showing changes in total and esterified cholesterol contents](image)

**FIG. 3. Changes in the total and esterified cholesterol contents in the livers**

5. **Alterations in the Succinic Dehydrogenase Activity in the Livers**

These alterations are represented in Fig. 4. The succinic dehydrogenase activity in liver homogenate of the aureomycin group remained at lowered levels throughout the experiments, reaching the minimum (54.3%) on the 3rd day. The depression of this enzyme activity preceded both the increase of total lipids and the decrease of phospholipids in the liver. The enzyme activity of the chloramphenicol group showed a change similar to that of the phospholipid level, and that of the penicillin group exhibited lowered levels and reached the minimum (63%) on the 5th day. In the CCl₄ group, the enzyme activity remained depressed throughout the experiments. It decreased day after day, and showed the minimum value (45.2%) on the 3rd day.

These changes of the enzyme activity were in relatively good agreement with the changes of the cellular degeneration and the phospholipid content in the livers.

6. **Alterations in the Fatty Acid Oxidase Activity in the Livers**

The octanoate oxidase activity of mitochondria prepared from the mouse liver treated by aureomycin was slowly declined (60% of the normal activity) until the 3rd day after the last injection, and was moderately elevated on the 5th (126.7%) and the 7th day (141.7%), as shown in Fig. 5. The enzyme
FATTY LIVER DUE TO ANTIBIOTICS

FIG. 4. Alterations in the succinic dehydrogenase activities in the liver homogenates

FIG. 5. Alterations in the octanoate oxidase activities of the liver mitochondrion

activity of the chloramphenicol group showed a tendency of the change similar to that of the aureomycin group except on the 7th day, while that of the penicillin group decreased on the 1st and 5th day. The oxidase activity of the CCl₄ group was markedly reduced by 33.3% of the normal within 24 hours, and recovered to above the normal already on the 2nd day and remained at higher levels thereafter. Recovery of the enzyme activity in all cases occurred almost simultaneously with the depletion of fatty deposition in the hepatic cells and with the recovery of the elevated lipid levels in the livers.

7. The Direct Action on the Enzyme Activities in the Liver (Fig. 6)

The direct inhibitory actions of antibiotics on the enzymes were observed in vitro adding these three antibiotics to each reaction system of Warburg
apparatus. By adding aureomycin to the reaction system, the succinic dehydrogenase activity was intensively inhibited (by 56.3%), but the activity of octanoate oxidase was somewhat less intensively inhibited (by 68.5%) than that of the former. By adding other antibiotics, both enzyme activities were observed to be lowered to some degree. However, the inhibitory actions of these three antibiotics on enzyme activities manifested some discrepancies between succinic dehydrogenase and octanoate oxidase in each experimental group.

8. Changes in the Proportional Composition of Fatty Acids in the Liver Lipids

The composition of fatty acids in mouse liver was determined using GLC and expressed as percentage of the total fatty acids. The major components of fatty acids detected by GLC were C₁₈, C₁₉, C₂₀, and C₂₂ fatty acids, and all alterations in the proportion of these fatty acids in the livers are summarized in Table 1. The fatty acid composition of the diet lipid (Oriental pellet) was also determined. The composition of the polyunsaturated fatty acids derived from the liver lipids of normal mice were as follow: diene 15.60%, triene 1.01%, tetraene 6.73%, pentaene less than 1.0%, and hexaene 6.77%.

a. Aureomycin Group (Fig. 7): The sum of the fatty acids with the same length of the carbon chain expressed as percentage of the total showed the changes presented in Fig. 7a. The sum of C₁₈-fatty acids moderately rose (113.2%) in the early stage of the experiments and returned later to the normal. C₂₀-fatty acids were slightly elevated on the 1st day, were significantly declined (80.5%) thereafter, and returned to the normal on the 7th day. C₂₂-fatty acids were declined (81.9%) in the early stage, and were remarkably elevated (136.2%) on the 7th day.
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Fatty acid composition of the lipids in the liver of the aureomycin group

**FIG. 7 a.** Changes in the sum of the fatty acids with the same length of the carbon chain expressed as percentage of the total.

**FIG. 7 b.** Changes in each C_{18}-fatty acid of the liver lipids.

**FIG. 7 c.** Changes in highly unsaturated fatty acids of the liver lipids.

The changes in each of the saturated and unsaturated C_{18}-fatty acids are illustrated in Fig. 7b. Fa in this report represents the number (a) of double bonds (F) in the fatty acid; for example, C_{18F2} indicates linoleic acid which consists of the chain of 18 carbon atoms and has two double bonds in its carbon chain. C_{18F2}-acid (=stearic acid) markedly increased (166.9%) in the early stage of the experiments. C_{18F1}-acid (=oleic acid) significantly decreased (82.5%) on the 1st day, while C_{18F2}-acid (=linoleic acid) markedly increased (131.0%). Contrary to the relatively high levels of C_{18F1} and C_{18F2}-acids, the low concentration (1% or less of total fatty acids) of C_{18F3}-acid (=linolenic acid) showed minimal changes.

The changes in C_{20F4}-fatty acid (=arachidonic acid) and C_{22F6}-acid (=docosahexaenoic acid) are summarized in Fig. 7c. Both acids were markedly declined (by about 68%) through all experimental period except the elevation (about 120%) on the 7th day. It is of interest that the alterations in the main components of highly unsaturated fatty acids is inversely related with the changes in total lipids and is roughly parallel to the changes in phospholipids in the liver.

*b. Chloramphenicol Group* (Fig. 8): The changes in the sum of the fatty acids with the same length of the carbon chain are shown in Fig. 8a. C_{20}-fatty acids remained at considerably high levels throughout the experiments, especially in the early stage with a remarkably high level (148.1%). C_{22}-acids also showed a marked increase (135%) in the early stage of the experiments.

Fig. 8b represents the variations in each C_{18}-fatty acid. C_{18F2}-fatty acid showed an extreme increase (253.5%) within 24 hours after the last adminis-
FATTY LIVER DUE TO ANTIBIOTICS

Fatty acid composition of the lipids in the liver of the chloramphenicol group

\[\text{\%50} \quad \text{\%25} \quad \text{\%10} \quad \text{\%5} \quad \text{\%1} \quad \text{\%0.5} \quad \text{\%0.1} \]

\[\text{C}_{18} \quad \text{C}_{16} \quad \text{C}_{14} \quad \text{C}_{12} \quad \text{C}_{10} \quad \text{C}_{8} \quad \text{C}_{6} \]

\[\text{Normal} \quad 1 \quad 2 \quad 3 \quad 5 \quad 7 \quad \text{day} \]

FIG. 8 a

FIG. 8 b

FIG. 8 c

Changes in the sum of the fatty acids with the same length of the carbon chain expressed as percentage of the liver total.

Changes in each C18-fatty acid of the liver lipids.

Changes in highly unsaturated fatty acids of the liver lipids.

Fatty acid composition of the lipids in the liver of the chloramphenicol group

C18-fatty acid showed fully inverse alterations as compared with C18F1-acid. C18F1-acid showed a tendency of gradual increase.

C20-fatty acid markedly increased (136.6%) on the 3rd day and again increased (136.6%) on the 5th day. C18F1-acid showed fully inverse alterations as compared with C18F1-acid. C18F1-acid showed a tendency of gradual increase.

C18F1-fatty acid markedly increased (151.8%) on the 1st day, returned thereafter to the normal percentage, and again moderately increased (121.1%) on the 7th day, as shown in Fig. 8 c. C18F1-acid was considerably elevated (134.8%) in the early stage, followed by a moderate decrease (85.8%) on the 3rd day (Fig. 8 c). The changes in these highly unsaturated fatty acids were roughly similar to those in phospholipids except a marked increase in the latter on the 7th day.

c. Penicillin Group (Fig. 9): The changes in the sum of the fatty acids with the same length of the carbon chain are shown in Fig. 9 a. C18-fatty acids exhibited a detectable depression through the whole period of the experiments. C20-acids were moderately elevated (124.2%) in the early stage and significantly (141.6%) on the 5th day. C20-acids showed a rise throughout the experiments, especially as high as 134.6% of the normal level on the 7th day.

Fig. 9 b summarizes each fluctuation of C18-fatty acids. The variations in C18F1-and C18F2-fatty acids were found to have an inverse correlation between them. Both C18F1- and C18F2-acids showed minimal or no alterations. C18F1-fatty acid was slightly elevated (108.3%) in the early stage and was moderately decreased (85.3%) later, and C18F1-acid showed an elevation (122.8%) to some degree in the late stage (Fig. 9 c).
Fatty acid composition of the lipids in the liver of the penicillin group.

**FIG. 9 a.** Changes in the sum of the fatty acids with the same length of the carbon chain expressed as percentage of the total.

**FIG. 9 b.** Changes in each C18-fatty acid of the liver lipids.

**FIG. 9 c.** Changes in highly unsaturated fatty acids of the liver lipids.

Fatty acid composition of the lipids in the liver of the carbon tetrachloride group

**FIG. 10 a.** Changes in the sum of the fatty acids with the same length of the carbon chain expressed as percentage of the total.

**FIG. 10 b.** Changes in each C18-fatty acid of the liver lipids.

**FIG. 10 c.** Changes in highly unsaturated fatty acids of the liver lipids.

**d. Carbon Tetrachloride Group (Fig. 10):** The changes in the sum of the fatty acids with the same length of the carbon chain are shown in Fig. 10a.
FATTY LIVER DUE TO ANTIBIOTICS

C_{16}-fatty acids showed the slight fluctuations within their normal limits. C_{18}-acids slightly increased in agreement with a marked increase of total lipids observed in the early stage of the experiments. C_{20}-and C_{22}-acids showed a linear elevation from remarkably low levels (49.8%, 47.1%) on the 1st day to significantly high levels (125.9%, 146%) on the 7th day, respectively.

Fig. 10 b represents the alterations in each of C_{18}-fatty acids. C_{18F1}-fatty acid markedly decreased (57.7%) on the 1st day and increased significantly on the 3rd day (147.2%). C_{18F2}-acid showed a marked increase (138.4%) on the 7th day, directly correlating to the changes in total lipids in the liver. C_{18F3}-acid was markedly elevated (127.5%) in the middle stage of the experiments. C_{18F4}-acid showed a definite change in the liver.

C_{19F1}-and C_{19F2}-fatty acids showed similar alterations and were markedly depleted (53.2%, 40.2%) corresponding to a significant increase of total lipids in the early stage of the experiments, and were remarkably elevated (134.2%, 133.2%) concomitantly with an increase of phospholipids in the late stage (Fig. 10 c).

DISCUSSION

Several reports in the literature suggested that mice tolerate aureomycin well. Harned and his co-workers showed that mice tolerated a single dose of 200 mg per kg of body weight of aureomycin given intraperitoneally. At the initial attempt of the present study, d.d.-strain mice were given injections of the single dose of 2 mg (80 to 100 mg per kg of body weight) of aureomycin for five days intraperitoneally. Majority of the mice died by the fifth day. Therefore, the dose was reduced to 1 mg (40 to 50 mg per kg), which was injected to mice once a day for five days.

Aureomycin has been reported to produce hepatic abnormalities in patients and in experimental animals. On the other hand, beneficial results with the use of this antibiotic have been reported in patients with hepatic coma and in animals fed with diets producing hepatic necrosis. It has also been reported that no significant hepatic abnormality follows the administration of aureomycin to human volunteers and experimental animals. It is of interest in view of conflicting reports on effects of antibiotics on liver lipids. Coniglio and Bell reported that the results of radioisotopic analysis of hepatic fatty acids showed significantly greater total radioactivity in hepatic fatty acids of aureomycin-fed animals within 24 hours while the amount of fat in the liver was normal.

From the present experiments done on mice, it was evident that daily intraperitoneal injection of aureomycin or chloramphenicol for five days in relatively large doses was capable of producing gross and microscopic changes of fatty degeneration to varying extent in the livers from the 3rd day after the last administration, while penicillin injection revealed no changes in the liver except a few droplets of neutral fat in the centrilobular hepatic cells only on the 7th day. On the other hand, a single subcutaneous injection of CCl_{4} in a relatively small dose induced a very intensive deposition of neutral
fat in the liver within 24 hours after the injection as observed by Dianzani. Since the fatty infiltration induced by CCl₄ injection was so intensive and rapid that the initial histological alterations, that should have taken place before the onset of hepatic fatty accumulation, could not be detected in the present study. However, the histological findings of fatty infiltration in the livers were well consistent with the fluctuations in the total lipid contents of the livers receiving either antibiotics or CCl₄.

The changes in the total lipid content were almost inversely correlated to those in the phospholipid content in the liver of the aureomycin group. Moreover, the total cholesterol level in the liver showed only a moderate alteration. Calculating the above results, it can be concluded that a significant amount of accumulated fat in the liver consists of neutral fat. Abodi-Djourabtschi and Hartmann also reported that the moderate increase of neutral fat with the significant reduction of phospholipids following perioral administration of aureomycin for 15 days.

Both the total and esterified cholesterol levels in the liver of the aureomycin group were almost parallel in the alterations and exhibited also a similar pattern of fluctuation to phospholipids. Consequently, there were undoubtedly no significant variations in the ester : total cholesterol ratio that is one of the indices of hepatic functions.

Since the cholesterol and phospholipid levels in the liver of the aureomycin group increased in the early stage of the experiments and the total lipids increased later, the elevation of the total lipids in the later stage must be mainly consisted of triglycerides, and it may be possible that the aureomycin treatment impairs the metabolism of cholesterol and phospholipids more rapidly than that of triglycerides.

The present investigation also revealed that the administration of chloramphenicol was also able to produce fat accumulation in mouse liver, although the degree of fat infiltration was less than in the livers of the aureomycin group. Although no reports have been available which chloramphenicol administration in vivo produces accumulation of fat in the liver, Lépine et al. reported that in tissue cultures of chick embryo cells, the cells showed numerous vacuoles and fatty degeneration when chloramphenicol was added, and suggested that the action was similar to that of aureomycin.

The alterations in the phospholipid and total cholesterol levels of the chloramphenicol group were almost similar to those of the aureomycin group, although the esterified cholesterol level of the former group elevated on the 5th day. As compared to the aureomycin group, in which the elevation of the total lipids and the depression of the phospholipids are well corresponded to each other, the changing patterns of the phospholipid and cholesterol contents in the liver of the chloramphenicol group, as shown in Fig. 2 and Fig. 3, tend to precede somewhat the changing pattern of the total lipids and, as a whole, to be inversely correlated to the latter. This discrepancy of the changing patterns of lipid fractions may be derived from the differences in biochemical actions on lipid metabolism between aureomycin and chloramphenicol.

Histological changes of the liver and the alterations in all lipid fractions
of the liver of the penicillin group were the slightest of all groups. The literatures with regard to the toxicity of penicillin have not suggested that the penicillin administration induces fatty degeneration in the liver. Although the alteration in lipid fractions in the liver was revealed to occur to some degree, its causes can not be clearly explained with the present knowledge. One of the possible causes is that the interferences of lipid metabolism in the liver are induced through the resulting development of the deficiency in vitamins and coenzymes when penicillin is administered in large doses.

The total lipid content in the liver of the mouse treated with a single injection of CCl$_4$ showed very marked elevation within 24 hours, and was followed by a gradual fall to the normal level. On the other hand, the phospholipid content showed slight decrease rather than increase during the whole experimental period except on the 7th day, and the cholesterol levels, both total and esterified, showed minimal alterations for the experimental period. Consequently, accumulated fat in the liver in the early stage of the experiments consists almost entirely of neutral fat as many workers have reported. The present results are in good accordance with the results of Kasbekar et al. that there is a nearly two fold increase in the fat content of the liver and a slight decrease in hepatic phospholipids at 48 hours after CCl$_4$ treatment. Richter et al. also reported a reduction of lipid phosphorus in the liver in the very early stage following CCl$_4$ treatment.

Effects on Enzyme Activities

Many reports which are concerned with aureomycin have demonstrated that this antibiotic may act upon some enzyme systems. In the present investigation, fatty acid oxidase was chosen for the study of antibiotics on enzyme systems. Fatty acid oxidation is generally carried out by way of the β-oxidation sequence to convert the straight long chain of fatty acid to two carbon units, namely acetyl-CoA. This end-product of fatty acid oxidation enters into the TCA cycle and is oxidized to CO$_2$ and water, coupling with the synthesis of ATP from ADP and inorganic phosphate. Thus, acetyl-CoA is the link between the two main metabolic cycles in mitochondria, namely the TCA cycle and the fatty acid oxidation cycle. For the purpose of observing the enzyme activity involved in both the TCA cycle and the fatty acid oxidation system, the succinic dehydrogenase activity was also investigated, since succinic acid is one of intermediates in the TCA cycle in mitochondria.

The in vivo administration of aureomycin showed the depression of the succinic dehydrogenase activity in all livers of mice examined throughout the period of experiments, and especially, a marked depression was seen on the 3rd day after the last injection. It is likely that this depression precedes both the increase of the total lipid level and the decrease of the phospholipid level in the liver. The in vivo administration of chloramphenicol revealed indefinite alterations in the succinic dehydrogenase activity. The in vivo administration of penicillin showed a gradual depression of the succinic dehydrogenase activity until it reached a maximal depletion on the 5th day. It is also likely that this depletion precedes the slight elevation of the total lipid level noted in the late
stage of the experiments. On the other hand, the \textit{in vivo} administration of CCl\textsubscript{4} exhibited a depression of succinic dehydrogenase activity throughout the experiments. It seemed that the maximal depression of the enzyme activity following CCl\textsubscript{4} injection took place two days after the marked elevation of the total lipids in the aureomycin and penicillin groups.

As a whole, it may possible that the \textit{in vivo} interference of the antibiotics with the succinic dehydrogenase activity intensifies the degree of fat accumulation in the livers, since the depression of this dehydrogenase activity precedes the elevation of the total lipid levels in the livers. On the contrary, it seems that the succinic dehydrogenase is secondarily interfered by some metabolic derangements responsible for the fatty degeneration in the liver, since the marked depletion of the enzyme activity takes place concomitantly with the recovery of the fatty liver following CCl\textsubscript{4} poisoning.

The octanoate oxidase activity of the mitochondria obtained from the liver of the aureomycin group was markedly decreased prior to the rise in the total lipid level in the liver, and then elevated with a concomitant accumulation of fat. By the \textit{in vivo} administration of chloramphenicol, it was also decreased prior to the elevation of the total lipid concentration in the liver, and recovered almost simultaneously with development of fatty liver. By the \textit{in vivo} administration of penicillin, this enzyme activity also showed a similar trend in its depression before slight elevation of the total lipid level, and its recovery occurred in accordance with the rise in the total lipid level in the liver. However, some differences in octanoate oxidase activity were noted between the CCl\textsubscript{4} group and the antibiotic groups. A marked depression of the octanoate oxidase activity was seen in the liver of the CCl\textsubscript{4} group, concomitantly with the marked rise in the total lipid level within 24 hours, and a plateau of the activity was maintained during the gradually decreasing period of the total lipid level.

The obtained result that the depression of the fatty acid oxidase activity in the livers treated with antibiotics precedes the rise in the total lipid content suggests that the interference of the antibiotics with this oxidase may be the cause of fat accumulation in the livers. On the other hand, it is difficult to determine whether or not the interference of CCl\textsubscript{4} with octanoate oxidase is directly responsible for the fat accumulation in the liver, because the remarkable fat accumulation and the marked depression of this oxidase activity appeared simultaneously within 24 hours. However, the accumulated fat in the liver is successively oxidized and is gradually depleted by the rapidly restored oxidase activity.

As for the direct inhibitory actions of the antibiotics on succinic dehydrogenase and on octanoate oxidase, the \textit{in vitro} administration of aureomycin intensively inhibited succinic dehydrogenase, and also significantly octanoate oxidase. The \textit{in vitro} effects of chloramphenicol and of penicillin were also inhibitory on both enzyme activities to the same degree, but their effect was less intensive than that of aureomycin.

These results obtained in the present study on the enzyme activities offer some support to the concept of Loomis\textsuperscript{7)}, of Van Meter and his collaborators\textsuperscript{40} (40)
and of Zimmerman\textsuperscript{9}, that the antibiotic action of those drugs is their inhibitory effect on various enzyme activities. More recently, several investigations have been reported on the inhibitory effects of antibiotics upon the riboflavin enzyme activities\textsuperscript{47-50}, which are necessary for both TCA cycle involving succinic dehydrogenase\textsuperscript{4} and fatty acid oxidation sequence\textsuperscript{4} \textsuperscript{40}. Thus, aureomycin \textit{in vitro} apparently inhibits not only endogenous respiration and oxidative phosphorylation\textsuperscript{7} \textsuperscript{8} \textsuperscript{9} \textsuperscript{40}, but when added to homogenate and washed mitochondria it directly depresses succinic dehydrogenase and octanoate oxidase as well as choline oxidase and choline dehydrogenase\textsuperscript{8}.

On the other hand, the \textit{in vivo} inhibitory effects on the enzyme systems by prolonged administrations of the antibiotics demonstrated some delay of onset and a progressive enhancement in the present study. The above fact may possibly be explained as follows: the primary inhibition of antibiotics on these enzyme systems secondarily causes many interrelated metabolic impairments, such as less formation of ATP following the inefficiency of oxidative phosphorylation\textsuperscript{46}, inactivation of riboflavin enzymes\textsuperscript{47-50}, and lack of the protein fraction in synthesizing enzymes\textsuperscript{51-52} \textsuperscript{53}, which, in turn, result in the apparent delayed inhibition on the \textit{in vivo} enzyme activities.

In fatty acid oxidation, DPN is required as a specific electron acceptor of \(\beta\)-hydroxylacyl dehydrogenase which is also one of the enzyme members of fatty acid oxidation sequence. In addition, DPN is necessary for many reactions connected with the metabolism of fatty acid; (1) the oxidation of \(\beta\)-hydroxybutyrate to acetoacetate, (2) 3 steps of the TCA cycle: (a) oxidation of \(\alpha\)-isocitrate to \(\alpha\)-ketoglutarate, (b) oxidation of \(\alpha\)-ketoglutarate to succinate, (c) oxidation of malate to oxaloacetate. Thus, it is clear that an eventual damage of the TCA cycle by DPN-deficiency represents an obstacle to the normal oxidation of fatty acids. There are many reports concerning the decrease of pyridine nucleotides in fatty liver\textsuperscript{10} \textsuperscript{44} \textsuperscript{55}. Calvert and Brody\textsuperscript{56} showed that DPN had the ability to restore depressed phosphorylation as well as oxidation in the liver treated with CCl\textsubscript{4}. Recently Dianzani and Marinari\textsuperscript{57} demonstrated that when DPN was added together with ATP to the octanoate oxidation system in mitochondria, significant stimulation was seen in cases of fatty liver whereas the addition of ATP alone caused smaller oxidation rates.

It has been shown that the enzyme system for fatty acid oxidation is located within mitochondria\textsuperscript{44} \textsuperscript{55} \textsuperscript{60}, and that the oxidation takes place by way of the reaction which includes CoA and ATP\textsuperscript{61} \textsuperscript{61}: \textit{R-COOH} + SH-CoA + ATP \rightarrow R-CO-S-CoA + AMP + PP. Its function depends upon oxidative phosphorylation\textsuperscript{44} \textsuperscript{45} \textsuperscript{46}. Dianzani\textsuperscript{14} thought that uncoupling of oxidative phosphorylation results in a loss of utilization of energy and in a deficient synthesis of ATP, and suggested that a decrease in concentration of ATP within the cell produces a deficient oxidation of fatty acids\textsuperscript{10}. Mitochondrion from fatty livers are considerably swollen\textsuperscript{45} \textsuperscript{46} and show a high degree of uncoupling of oxidative phosphorylation\textsuperscript{10} \textsuperscript{45} \textsuperscript{47}, as well as activation of ATP ase\textsuperscript{10} \textsuperscript{31}. If aureomycin, \textit{in vitro} and \textit{in vivo}, causes uncoupling of oxidative phosphorylation in liver mitochondria as Loomis\textsuperscript{7} showed previously, and if the mitochondria in the fatty liver produced by aureomycin exhibits the same enzymatic responses
as that produced by CCl₄ or distilled water, the depression of ATP-formation and the activation of ATPase might cause a marked decrease of ATP concentration in the liver.

Thus, it seems possible that the lack of coenzymes is the reason for the decreased oxidation in fatty livers, since ATP, pyridine nucleotides, CoA, and cytochrome c, all of which are required for fatty acid oxidation, are decreased. Accordingly, both the direct inhibition of antibiotics upon fatty acid oxidation and the secondary depression of this oxidation in consequence of the lack of available stock of these substances owing to the prolonged administration of large doses of antibiotics, may possibly produce fatty accumulation in the livers in the late period of the experiments. Furthermore, the delayed depression of enzyme activities due to the resultant lack of available enzyme protein and flavoprotein or FAD responsible for the inefficiency of the synthesis as quoted above, may be also regarded as one of the factors producing fatty accumulation in the livers.

The data obtained from the present investigation have not revealed whether the primary attack point of CCl₄ is in the phase of fatty acid oxidation or not, since fat had already accumulated in the liver to a marked degree within 24 hours following CCl₄ injection. In his study on the mechanism of action of CCl₄, Dianzani suggested that the key lesion in toxic fatty liver involved a loss of mitochondrial function, leading primarily to a lowering of the supply of ATP, and secondarily to a failure of the fatty acid activating reaction, thus accounting for the increase in liver fat on the basis of a failure of fat oxidation.

However, Recknagel et al. recently made a question upon these interpretations regarding to the fat accumulative action of CCl₄, on the basis of their detailed study on the correlation between the time of the first appearance of mitochondrial degeneration and the time of onset of fatty accumulation, and led to the formation of a new simple hypothesis. This hypothesis is as follows: the liver is constantly secreting large quantities of triglycerides into the plasma. CCl₄ poisons the secretory mechanism and as a result triglycerides accumulate in the liver. If the increase of fat is due ultimately to loss of the phases of cellular metabolism sustained by the mitochondria, then clear cut evidence of the failure of the relevant mitochondrial functions should appear before the increase of fat. However, without failure of fat oxidation or uncoupling of oxidative phosphorylation, the direct demonstration of a major derangements in the metabolism of triglycerides by the liver, coincident in time with the attainment of the peak concentration of CCl₄ in the liver and with the rapid early rise in hepatic triglyceride content, provides a new insight into the problem of the pathogenesis of CCl₄ fat accumulation and has offered the formulation and testing of the new simple hypothesis. Thus, according to the hypothesis, the formation of triglycerides by the liver is not interfered with in the CCl₄-poisoned animal, but the hepatic triglyceride secretory mechanism is inhibited or destroyed. As a result, triglycerides accumulate in the liver. The data of the present study that the delayed enhancement of depression of the succinic dehydrogenase activity following CCl₄ poisoning was demonstrated.
two days after the maximal fat accumulation in the livers, may also offer an indirect support for this hypothesis.

Effects on Fatty Acid Composition

The polyunsaturated fatty acid composition of the liver lipids of the normal mouse demonstrated in the present investigation was in relatively good accordace with that of the liver lipids of the male rat obtained by Swell et al.\textsuperscript{71} using gas chromatography. According to our experiences, the proportion in hepatic fatty acid composition of mouse is very similar to that of rat, although the content of highly unsaturated fatty acids is somewhat less in mouse liver than in rat liver. The fatty acid spectrums of the liver lipids of the normal rat have been reported by Okey and Harris\textsuperscript{72}, Swell et al.\textsuperscript{71}, and Kirschman and Coniglio\textsuperscript{73}, and there are some discrepancies among their data. It is possible that differences among them are due largely to different extractive and analytical techniques.

\textbf{a. Fatty Liver resulted from CCl\textsubscript{4} poisoning:} The increase of C\textsubscript{18} fatty acids and the depletion of C\textsubscript{20} and C\textsubscript{22} fatty acids were found simultaneously with a significant elevation of the total lipids, especially of triglycerides, in the CCl\textsubscript{4}-poisoned liver. The compositional increase of the sum of C\textsubscript{18} fatty acids is well interpreted as the consequence of the large proportion of C\textsubscript{18} fatty acids contained in the diet.

As compared with the proportion of fatty acids in other lipid fractions, the low proportion of C\textsubscript{18} fatty acid and the high proportion of C\textsubscript{18} fatty acids of the liver\textsuperscript{71} lead us to a reasonable interpretation that the depletion of C\textsubscript{18} fatty acid and the elevation of C\textsubscript{18} fatty acid in the fatty liver may be the manifestation of a remarkable increase of triglycerides among the increased lipid fractions. On the other hand, it is impossible to explain the causes of a marked increase of C\textsubscript{18} fatty acid within 24 hours after the CCl\textsubscript{4} injection on the basis of the fatty acid compositions either in Oriental pellet taken as diet or in each lipid fraction of the liver which has been recently analysed by several investigators. Hence, some specific involvements in metabolic processes of the acids might possibly be one of the causes.

The proportion of C\textsubscript{18} fatty acids in the fatty liver declined significantly, inversely relating to a marked rise in triglycerides. These present results can be well understood, if one considers the fact that far larger quantities of tetaeae and hexaene are contained in the fatty acids of phospholipids than triglycerides and cholesterol esters\textsuperscript{71}. With the progress of the investigation, the changes in each fatty acid composition returned gradually to the normal values, and the marked decline in proportion of C\textsubscript{18} and C\textsubscript{22} within 24 hours showed a gradual elevation with an inverse correlation to the total lipid content in the liver.

\textbf{b. Fatty Liver resulted from Aureomycin Administration:} The changes in fatty acid composition, as a whole, were not so remarkable in the liver of the aureomycin group as those of the CCl\textsubscript{4} group.

The increase of C\textsubscript{18} fatty acids and C\textsubscript{18} fatty acids and the decline of C\textsubscript{18} fatty acids were
observed during the early period of the experiments. Since no increase of triglycerides was yet demonstrated in the liver, the increase of C_{18:2} acid may probably be due to the increase of the phospholipid and cholesterol fractions\(^{70}\). However, it is impossible to explain the metabolic response for the increase of C_{18:2} in terms of the increase of the latter two lipid fractions. Consequently, this increase may be concerned with some impairments of the metabolic processes of the exogenous linoleic acid. For example, the possible involvements in the synthesis of highly unsaturated fatty acids from the linoleates in diet may result in an accumulation of this acid in the liver.

Since the relatively marked depletion of C_{20:4} and C_{22:6} can be explained as a relative depletion due largely to the increase of triglycerides in the developed fatty liver, it may be concluded that the hepatic interconversion of the polyunsaturated, especially of the highly unsaturated fatty acids, is significantly impaired by large doses of aureomycin. It is interesting that a similar tendency in fluctuation is observed between the proportion of the highly unsaturated fatty acids such as C_{18:2} and C_{20:4} and the phospholipid content in the liver. Particularly, C_{20:4}-fatty acid fluctuates with almost the same pattern as the phospholipids do, and begins to decline somewhat before increase of the total lipids.

Until recently, few reports were available of detailed studies on the fatty acid composition in specific lipids. MacFarlene et al.\(^{75}\) have analysed the fatty acid composition of cephalin and lecithin fractions from mitochondria and microsome of rat liver and shown that linolenic acid was not present in either cephalin or lecithin and about 45% of the fatty acids were "essential" fatty acids, calculated as the sum of linoleic and C_{18:2} and C_{20:4} polyenoic acids. Moreover, according to Okey's study\(^{72}\), up to 27% of the total in the phospholipid fatty acids in rat liver were tetraenoic acids. Thus, the fact that the proportion of tetraenoic acids is extremely high in the composing fatty acids of phospholipid molecules suggests an important and essential role of arachidonic acid in functional activities in the liver cells, and it is quite possible that lecithin and cephalin are unable to function without a large proportion of highly unsaturated acids in the molecules.

In the present studies, the important role of the highly unsaturated fatty acids is also affirmed by demonstrating that the decline of C_{20:4} and C_{22:6} is followed by the depression of the hepatic enzyme activities, and subsequently leads to the accumulation of fat in the liver. Presumably the phospholipid turnover in the liver is decreased, on account of inability to synthesize phospholipids following the lack of the available polyunsaturated fatty acids with result that fat metabolism\(^{76}\) and fat transport\(^{77}\) are blocked. Consequently, fatty acids accumulate in this organ. Thus, the development of fatty liver are readily explained by a failure in the biosynthesis of phospholipids in the absence of the highly unsaturated fatty acids which are needed for incorporation into the molecules.

c. The Liver treated with Chloramphenicol: In the early stage of the experiments, the increase of C_{18:3}, C_{20:4} and C_{22:6} and the decrease of C_{18:1} may
be explained by the increase of phospholipids\(^7\). However, the increase of phospholipids obtained by the present studies is, in fact, so slight that it offers no probable explanation for these alterations. Since the depletions of the highly unsaturated fatty acids and the phospholipids were already marked before the changes in total lipids, these depletions may be regarded as one of the factors that lead to the following slight development of fatty liver.

\(d\). The Liver treated with Penicillin: The changes in the fatty acid composition in the liver were generally slight and the causes of the changes in C\(\text{18:0}\) and C\(\text{18:1}\) in the early stage of the study are not clear. Comparing with the alterations in lipid fractions in the livers treated with other drugs, these slight changes are well consistent with the present result that no significant alterations were observed in the lipid fractions of the liver treated with penicillin. The metabolic processes of fatty acids in the liver are involved in the slightest degree with the penicillin administration among three antibiotics.

**SUMMARY**

1. A considerable accumulation of fat was observed in the mouse livers after the administration of some antibiotics, but there were significant differences in the time of onset and in the degree of fatty accumulation, between those livers and the fatty liver produced by CCl\(_4\).

2. Before the accumulation of fat in the livers, gradual inhibitory actions on the activities of both succinic dehydrogenase and fatty acid oxidase were demonstrated \(in\) \(vivo\) by the prolonged administration of relatively large doses of antibiotics.

3. By the addition of antibiotics \(in\) \(vitro\), the activities of both succinic dehydrogenase and fatty acid oxidase were directly depressed to a large extent.

4. The fatty acid composition in liver lipids of the mice treated with antibiotics and CCl\(_4\) was analysed by gas-liquid chromatography. There were definite differences in the fatty acid composition of the hepatic lipids of the fatty livers between the antibiotics administered groups and the CCl\(_4\) group.

5. The proportional changes of the highly unsaturated fatty acids, especially of arachidonic acid were well consistent with the changes in the phospholipid content in the liver lipids.

6. The depletion of the highly unsaturated fatty acids was followed by the depression of hepatic enzyme activities, which resulted in the accumulation of fat in the livers.

7. These evidences suggest that the increase of liver lipids following administration of antibiotics is the result of derangements of the lipid metabolism which are induced by both the direct inhibition of antibiotics on the liver enzyme activities and the secondary depression of the biochemical functions in the liver due to the lack of available stock, such as the highly unsaturated fatty acids and the phospholipids.

The writer is deeply indebted to Prof. Shingo Aoyama, Assist. Prof. M. Matsubara and Lecturer Dr. S. Kikuchi for their hearty advices; to Assist. Prof. K. Fukuzumi and
Dr. T. Takagi of the Department of Applied Chemistry, Faculty of Engineering, Nagoya University, for their valuable assistances on gas-liquid chromatography; and to the members of the research group on lipid metabolism for their cooperations.

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