42 STUDIES ON THE METABOLISM OF LIPIDS UNDER FAT-DEFICIENCY

BY

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INTRODUCTION

In recent years much has been observed on the mechanism of lipid metabolism in mammals and its outline has become partly clear. It has been observed in vivo that the lipid was synthesized from carbohydrate. Recently Stellen and Boxer [1] reached an important conclusion that fatty acid synthesis was a more important pathway in the utilization of D-glucose than the formation of glycogen. Massaro et al[2] fed mice with glucose which was uniformly labeled with C^{14} and clearly demonstrated the importance of lipogenesis as a pathway in D-glucose utilization. There exists a relation between fatty acid synthesis and the diet, that is, the liver activity of converting D-glucose and acetate into fatty acids is also dependent upon the intake of diets of the animal. A diet rich in carbohydrate apparently provides optimum conditions for hepatic lipogenesis.

As described above animals are suffered from deficiency disease by breeding with fat-deficient diets and several abnormal symptoms appear. These phenomenas occur from the deficiency of essential fatty acids.

The author studied the metabolism under fat-deficiency to make clear the functions of lipid of metabolism.

It has been already shown that aminals suffered from fat-deficiency by breeding fat-free diets and this phenomenon disappeared by administration of adequate amounts of the essential fatty acids. But the mechanism from which fat deficiency appears, that is tosay, the mechanism in which the lipids, including the essential fatty acids concern is not yet clear. To be clear above functions of lipids is very important and urgent for the study of animal metabolism. Therefore the author determined the influences of fat-deficiency on body fats, on the activities of liver enzymes and on the lipids of liver mitochondria, and wished to be clear the special activity of lipids.

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CHAPTER 1. INFLUENCES OF FAT-DEFICIENCY ON BODY FAT.

The rats which had been fed on a fat-deficient diet for a long period presented a syndrome of deficiency. Usually the rats are inspected periodically to detect symptoms of essential fatty acid deficiency. The severity

of symptoms is judged by feet-scaliness, scaliness of tail and the roughness of hair coat.[3,4] Besides these symptoms several abnormalities such as prolonged period of pregnancy,[4,6,7] still-births,[8] excessive bleeding at birth and high mortality ratio of unweaned rats were obsrved.[9] Kummerow et al.[8] observed that there were several differences in the constitution of body fat between normal and fat-deficient rats. The deficient syndromes disappear after administrating an adequate amount of linoleate, but there is no agreement on the amount to be administrated among workers. The mechanism by which the essential fatty acids act in the fat-deficiency symptoms is not yet to be certain. The author intended to clarify the mechanism in which the essential fatty acids were concerned.

Experimental.

Animals.

Female albino Wister weaning rats which were 40 day old were used and their body weights were 75–90 g. Each animal was kept in a separated metal cage. Feeding period was about 11 weeks.

Diets.

The diets were shown in Table 1. The diets given were 20 g. per rat per day. In case of the fat-deficient diet, sugar was substituted for soy bean oil. Linoleate (Iodine value was 161.3) was added up to an extent of 0.5% and 0.1% respectively and linolenate (Iodine value was 273.8) was the same. Diets and water were given ad libitum. The vitamin mixture contained B_1 0.1 mg., B_2 0.1 mg., niacin 0.2 mg., Ca-pantothenate 0.4 mg., B_6 0.05 mg., biotin 0.002 mg., folic acid 0.004 mg., B_{12} 0.0004 mg., inositol 2.0 mg., and choline chloride 3.0 mg. per rat per day and vitamin A 10.000 I. U. and D 1.000 I. U. per rat per week.

Determination of liver carbohydrate and total lipids.

Animals were killed by a blow on the head at the end of the feeding period and the livers were immediately taken out. One g. of each liver was hydrolyzed at 100° C for 3 hours with 5 ml. of N-H_2SO_4, neutralyzed with N-NaOH and made to 25 ml. Five ml. of neutralyzed solution was deproteined with 5 ml. of 10% $ZnSO_4$ solution and 5 ml. of 0.5 N-NaOH, then centrifuged. Reducing

TABLE 1. DIETS.

	Sucrose	Casein	Salt	Soy Bean Oil	Linoleic Acid	Linolenic Acid
	%	%	%	%	%	%
1	74	20	6			
2	74	20	6		0.5	
3	74	20	6		0.1	
4	74	20	6			0.5
5	74	20	6			0.1
6	69	20	6	5		

Beside above components vitamin mixture was administered as noted in text.

sugars were determined by the Hanes method[10] making use of those supernatants. Total lipids were estimated by ether extraction from livers dried in vacuum over P_2O_5.

Extraction of body lipids.

Total body lipids, free fatty acids, neutral lipids, phospholipids were extracted from the carcases according to the following methods. (1) Carcases were saponified with two volumes of 30% alcoholic KOH solution and lipids were separated by the usual method. (2) All carcases were blended with the same amount of anhydrous sodium sulfate and two volumes of petroleum ether. The lipids dissolved in petroleum ether were isolated by the usual methods and separated into neutral lipids and phospholipids with acetone. All solvents were removed under CO_2 starem.

Separation of unsaturated fatty acids from saturated fatty acids.

Unsaturated fatty acids were separated from saturated fatty acids as follows. Two g. of fatty acids were dissolved in 20 ml. of alcohol, and 20 ml. of hot alcoholic solution containing 10% Zinc acetate was added. The precipitate formed was filtered after 24 hours and 5 ml. of the fatty acids mixture (2 g. of stearic acid and 2 g. of palmitic acid were dissolved in 80 ml. of alcohol) was added into filtrate. The precipitate formed was filtered after 24 hours. Additional 5 ml. of fatty acid mixture was added into the filtrate and treated as before. All of the precipitate was collected, suspended in 20 ml. of water, and acidified with N-hydrochloric acid. The saturated fatty acids were extracted with ether. The filtrate, from which saturated fatty acids

were removed was acidified with N-hydrochloric acid and unsaturated fatty acids were extracted with ether.

Determination of fatty acids.

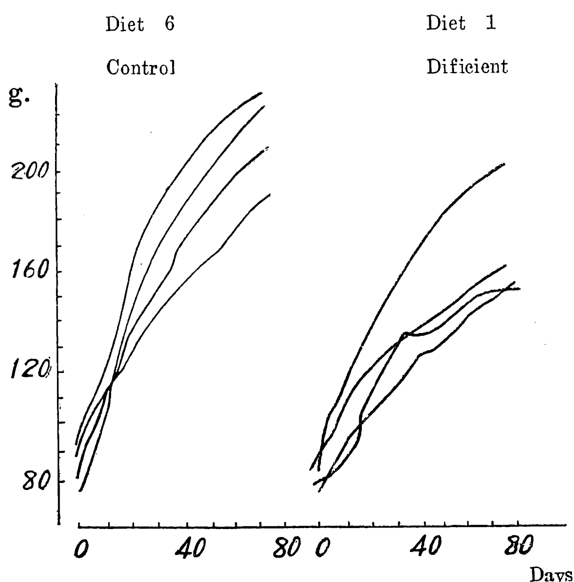
Linoleate, linolenate and arachidonate in neutral lipids were determined spectrophotometrically,[11] namely about 10 mg. of lipids was isomerized in 5g. of 21% KOH-glycol solution at 180° C for 15 minutes under a nitrogen stream and their extinction coefficients were determined at wave lengths of 233, 268, 315 and 346 mμ. Unsaturated fatty acids were calculated from the extinction coefficients using the following expressions.

Linoleic acid, % $= 1.092K_{233} - 0.573K_{268} - 0.259K_{315} - 0.033K_{346}$

Linolenic acid, % $= 1.105K_{268} - 0.879K_{315} + 0.190k_{346}$

Arachidonic acid, % $= 1.650K_{315} - 1.667K_{346}$

FIG. 1. GROWTH CURVES. (1)



Results.

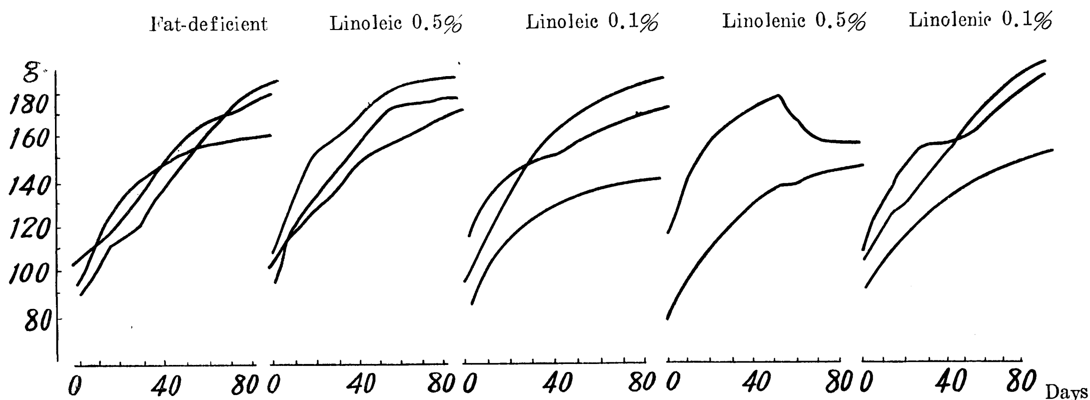
In spite of the fact that all animals were fed for 11 weeks, no distinctive deficiency symptoms appeared except roughness of hair coats, but in 2 or 3 animals the scaliness of the tail could be observed. Figure 1 showed the body weight curves of the deficient group and the control which were fed on the diets of Table 1. The increase in body weight of the deficient group appeared later than that of the control group. The average value of body weight increase in the deficient group was only 71% compared to the control group and a difference between the two groups was statistically significant.

The livers serve a very important function in the metabolism of animals. The half time required for body fat conversion is considered to be 16 or 17 days for saturated fatty acids and 19 or 20 days for unsaturated acids, but in the liver it is considered that only one day is needed for saturated acids and about two days for unsaturated acids. It is considered that the liver is influenced in various ways by the abnormality of nutrition and the liver is very active in metabolism. For example, under such conditions enzyme activity of the liver seems to differ from normal; this will be discussed later. Table 2 showed the weight of livers. The ratio of liver weight to body weight differed between the two groups. The variance ratio (F value) found was 7.2 and this

TABLE 2. THE INFLUENCE ON THE WEIGHT OF LIVSERS.

Diet	Complete Group			Deficient Group		
	Liver wt.	Body wt.	Ratio	Liver wt.	Body wt.	Ratio
	6.6g	145g	4.6	6.1g	137g	4.5
	6.0	132	4.6	4.5	109	4.1
	5.8	139	4.2	5.9	145	4.1
	4.4	120	3.7	6.2	163	3.8
	5.4	115	4.7	6.9	140	4.9
	6.6	158	4.2	6.1	140	4.4
	6.1	147	4.2	6.1	135	4.5
	5.4	128	4.2	5.6	139	4.0
	5.5	125	4.4	4.3	100	4.3
	4.4	104	4.2	8.9	175	5.1
	5.3	95	5.6	6.5	120	5.4
	6.0	120	5.0	6.8	120	5.7
	6.5	140	4.6	7.3	182	4.0
	6.1	177	3.4	7.0	170	4.1
	6.2	157	3.9	5.8	125	4.6
	6.5	142	4.6	4.9	116	4.2
	5.6	124	4.5	4.3	89	4.8
	6.5	160	4.1	5.5	115	4.8
	6.3	149	4.2	4.3	120	3.6
	6.7	190	3.5	6.2	175	3.5
	7.2	150	4.8	5.8	140	4.1
A.V.			4.34			4.55
F Value			7.21			

FIG. 2. GROWTH CURVES. (2)



difference could be measured between the two groups with a level of significance 0.05.

The amounts of total lipids and carbohydrates in livers were shown is Tabl 3. The total lipids content was 3.4% in the complete group and 4.1% in the deficient group. But differences between control and fat-deficient group could not be found statistically.

From the above results it was shown that fat-deficiency had an influence on the increase of body weight and normal growth or livers. Therefore linoleate and linolenate were administered under such conditions. Each acid was mixed in the diet in the amount of 0.5% and 0.1%. Figure 2 showed the influence of these fatty acids on the increase of body weight. The activity of linoleate in the increase of body weight could not be found to be th same as that of linolenate.

Next, body lipids should be taken into consideration. Table 4 showed the contents of body lipids. The total content of body lipids in the fat-deficient group was identical with that of the control group, but the total content of unsaturated fatty acids in the deficient group was smaller than that in the control. In the neutral lipid fraction the contents of unsaturated fatty acids were caused to become somewhat higher by the administration of linoleate, but not by linolenate. However, in general, the increase in contents of unsaturated fatty acids in neutral fats was extremely small so that it was impossible to justify the conclusion that it was effected by linoleate administration.

TABLE 3. THE CONTENTS OF LIPIDS AND CARBOHYDRATES IN LIVERS OF FEMALE RATS.

Diet	Lipids		Cardohydratos	
	Control	Deficient	Control	Deficient
	3.0%	4.1%	0.58%	1.00%
	3.5	3.2	0.59	0.70
	3.7	3.3	1.18	0.30
	2.7	4.8	1.43	0.96
	3.5	4.2	1.49	0.84
	4.3	5.7		
	3.1	3.2		
Average	3.4	4.1	1.05	0.76
Ratio	100.0	120.6	100.0	72.3
F Value	2.64		1.66	

Table 5 showed that the iodine values of body fat of the fat-deficient group were smaller than those of the control group. The same relationships in iodine values were observed in respect to total fatty acids, and neutral lipid fractions. F. A. Kummerow et al.[8] found that the contents of oleic acid in rats which had been fed on corn oil was higher than in the fat-deficient group, this being in agreement with the present author's results.

There was no difference between the groups which were fed on linoleic acid and the fat-deficient gronup.

TABLE 4. THE CONTENTS OF BODY FAT OF FEMALE RATS.

	Total Lipid	Total Fatty Acid	Unsaturated Fatty Acid In Total Fatty Acid	Neutral Fat	
				Fatty Acid	Unsaturated Fatty Acid In Fatty Acid
Fat-deficient	7.8%	6.0%	27.0%	76.0%	30.2%
Linoleate 0.5%	7.3			76.0	33.6
Linoleate 0.1%	7.5			70.0	33.8
Linolenate 0.5%	5.5			78.0	31.5
Linolenate 0.1%	6.8			78.0	30.9
Control	6.8	6.4	38.0		

On the other hand, a considerable difference could be found in the fatty acid constitution of body fat. Carcases were blended with petroleum ether, lipids were extracted and linoleate, linolenate and arachidonate of neutral lipid fraction were determined spectrophotometrically. The results are shown in Table 6. In the fat-deficient

TABLE 5. THE IODINE VALUES OF BODY FATS.

	Total Fatty Acid	Fatty Actd in Neutral Fat
Fat-deficient	60.0	65.0
Linoleate 0.5%		66.0
Linoleate 0.1%		66.0
Linolenate 0.5%		66.0
Linolenate 0.1%		67.0
Control	81.0	81.0

group the content of unsaturated fatty acids in neutral lipids was very small, especially the content of linoleate was only one-seventh of the control group. Linolenate was one-third and arachidonate half of the control group. F. A. Kummerow et al.[8] recognized 6 times as large a content of linoleate in fat-deficient group after feeding corn oil. In the present experiments the content of linoleate in the neutral lipid fraction of the group which was administered linoleate was about 30% higher than that of the fat-deficient group, being about the same with that of 0.5% and 0.1% administered groups.
On the other hand when 0.5% linolenate was administerd, it showed the same activity as linoleate, but did not show activity in case of 0.1% linolenate. The contents of linolenate ande arachidonate in neutral lipids were hardly affected by theadministration of linoleate and linolenate.

TABLE 6. THE CONSTITUTION OF BODY FATS OF FEMALE RATS.

	Linoleate	Linolenate	Arachidonate
	%	%	%
Fat-deficient	1.39	0.57	0.21
Linoleate 0.5%	1.89	0.60	0.24
Linoleate 0.1%	1.95	0.45	0.38
Linolenate 0.5%	1.81	0.43	0.87
Linolenate 0.1%	1.37	0.64	0.34
Control	9.24	1.48	0.44

Discussion

The results of experiments to decide the period at which fat-deficient symptoms appear are not identical among various workers. Ralph T.Holman and Sheldon I. Green-berg[4] reported that in all animals being fed on fat-deficient diets for 3 or 4 weeks the first visible symptoms of fat-deficiency appeared, and at the end of 2 months moderate severe symptoms appeared. H. O Kunkel and J. N. Williams[12] successfully obtained fat-deficient symtoms after 13 weeks. F. A. Kummerow et al.[8] and O. S. Privett et al.[13] recognized symptoms after 17 weeks and 40 weeks respectively. The present author's results concerning this point do not agree with these data, but it is convinced that the animals suffered from fat-deficiency because of their increase in body weight and liver growth. As above noted, liver is a very important organ for the metabolism of animals, and liver is considered to be sensitive to the change of nutrition. As the metabolism of carbohydrate and lipid is closely related with each other, it is expected that fat-deficiency will influenced on the content of these two components in the liver. Therefore the contents of carbohydrate and lipids in the liver and the liver weight were determined.

The liver weight of the fat-deficient group was higher than that of the control, but in the case of the contents of carbohydrate and lipids in the liver the statistical difference could not be found between control and fat-deficient groups.

As shown in Figure 1, the increase of body weight of the fat-deficient group was only 71% of the control group, so linoleate and linolenate were administered to the fat-deficient group. The amount of linoleate to be administered is yet not been decided among workers, i. e., Harry J. Daul[14] added 100mg. of linoleate per day, O. S. Privett[13] about 1 % and P. G. Tulfpule[15] 670mg. Daul[14] has observed the influences of linoleate and linolenate on the pregnancy, survival and growth of young rats from females on a fat-free diet, and concluded that the optimum amount of linoleate to be administered was 100 mg. per day per rat. From Daul's conclusion the author administered 100 mg. and 20mg. of linoleate per day per rat.

It is interesting to note that only the administration of linoleate exerted effects on the linoleate content in body fat while linolenate and arachidonate were almost not affected. James F. Mead[16] administered sodium-1-

C^{14}-acetate to rats and after analysis of body fat fractions presumed that arachidonate could be synthesized by the condensation of acetate and a 18-C precursor. Gunther Steinberg and James F. Mead[17] recognized that linoleate or its derivative was incorporated with acetate into arachidonate after administeration of methyl linoleate-l-C^{14}) to the rats. They presumed that the arachidonate would be formed from linoleate by addition of acetate in the carboxyl end of the linoleate, which was derived from other linoleate, and further-more recognized that the rate of arachidonate formation from linoleate to be relatively low. In the above described experiments the contents of arachidonate in body fat was not effected by the administration of linoleate inspite of the increase in linoleate content. It was thus considered that the contents of arachidonate did not incresse on account of the low efficiency of the linoleate to be incorporated into arachidonate.

CHAPTER 2. LNFIUENCES OF FAT-DEFICIENCY ON LIVER ENZYME ACTIVITIES

It has been already shown that the normal metabolism of animals were influenced by their diets, and especially liver enzyme activities might be markedly altered by dietary conditions. Such enzymes as D-amino acid oxidase, arginase, xanthine oxidase, rhodanase, and adenosine pyrophosphatase have been shown to be lost or decreased in dietary protein and amino acid insufficiency[18—25] Burr and Beeber[26] reported that rats suffering from fat-deficiency had a much higher metabolic rate than the controls. Kunkel and Williams[12] found that both cytochrome C oxidase and endogenous respiration of the livers of fat- deficient rats were significantly higher than normal rats. Recently Klein and Johnson[27] reported that liver mitochondria from fat-deficient rats esterified less high energy phosphate per mole of oxygen taken up during the oxidation of certain intermediate of the tri-carboxylic acid cycle. Burr and Beeber[26] considered that uncoupled phosphosphorylation could account for the high metabolic rate, the high endogenous respiration, and the high cytochrome C oxidase of essential fatty acid deficient rats.

Tulfpule and Patwardhan[28] have obserbed a reduction in liver glutamic dehydrogenase activity of essential fatty acid or vitamin B_6-deficient rats, which was further decreased by a double deficiency of these dietary factors. Tulfpule and Williams[15] reported that succin ic oxidase activity of rats was unaffected by the deficiency of essential fatty acid or B_6 and it was the same by a double deficiency of these factors, but on the same dietary condition Antimycin-A sensitive factor, which denotes a hypothetical electron transport component between succinic dehydrogenase and cytochrome C, was significantly increased. This increase in Animycin-A sensitive factor was not reversed by ad libitume feeding of the control diet for 2 additional weeks, and deficiencies of above factors depressed phosphate esterification coupled with DPNH or reduced cytochrome C oxidation.

It is considered that the nutrition of animals, especially the deficiency of essential fatty acids, may influence on activities of liver enzymes. So the author determined activities of lipase, choline oxidase, succinic-cytochrome C oxidase, succinic dehydrogenase, cytochrome C oxidase and caprylic acid oxidase with use of rat liver homogenate and liver mitochondria.

It has been already shown that fat was hydrolyzed to fatty acids and glycerol in vivo and resynthesized to fat globe after absorbed into the circulatory system. Therefore liver lipase was assayed from the view point that the nutrition of animal influenced on the activity of lipase.

The synthesized fat globe which was absorbed into circulatory system combines with a choline to form the phosphatide and circulate in the body. The enzyme which oxidizes choline has been found in rat livers[29] and it is considered to be a protein complex combined with the yellow enzyme. This enzyme seems to cooperate with cytochrome C[30] and is named choline oxidase. Therefore both choline and choline oxidase are considered to be closely related with the metabolism of lipid, and so choline oxidase activity was assayed.

Recently the metabolic breakdown of fatty acids has become more clear. A portion of fatty acids is broken down by β-oxidation and the active acetate which was derived from long chain fatty acids are oxidized to CO_2 and H_2O through the Krebs' cycle, and another portion is synthesized into the higher fatty acid and acetoacetate. These reactions are performed mainly in the liver and kidney and it is considered that the rate of the above reaction is influenced by the metabolic condition of the animal body.

Electron transport system is a very important process for the oxidation of active acetate through the Krebs' cycle. So it must de determined whether fat-deficiency influence on the function of liver or not. From the above view point, activities of oxidative breakdown of fatty acid and the succinic oxidase system were determined.

Exprimental.

Animals.

Male and female albino Wister King weaning rats which were fed with stock diet for a month were used. Each animal was kept in a metal cage. After 20–23 weeks breeding animals were sacrificed with a blow on the head, and livers were removed immediately and chilled in cracked ice.

Diets.

The components of experimental diets were shown in Table 7. Each diet was given 20g. per rat and water and diets were given ad libitum. Cane sugar was fed for the experiments of lipase, choline oxidase and caprylic acid oxidase. But as the increase of body weight of sucrose administered rats was not good after 13 weeks' breeding, starch was substituted for sugar in the experiments of succinic-cytochrome C oxidase, succinate dehydrogenase and cytochrome C oxidase.

The vitamin mixture contained B_1 0.1mg., B_2 0.1 mg., niacine 0.2mg., Ca-pantothenate 0.4mg., B_6 0.05mg., biotin 0.002mg., folic acid 0.004mg., B_{12} 0.0004mg., inositol 2.0mg., and choline chloride 3.0mg., per rat per day and vitamin A 10.000 I. U., D 1.000 I. U. and α-tocopherol 1.4mg. per rat per week.

TABLE 7. THE DIETS.

	Sucrose or Starch	Casein	Soy Bean Oil	Salt
	%	%	%	%
Control Diet	68	20	6	6
Deficient Diet	74	20	0	6

In addition to the above components vitamin mixture was administered as noted in the text.

Determination of lipase activity.

Livers of the rats were washed free of blood in ice cold water, weighed and homogenized with a Potter-Elvehjem glass homogenizer in two volumes of ice cold water. The incubation mixture consisted of M/15 phosphate buffer (pH 7.0) 10ml., tri-n-butylin 0.5ml., 3% gum arabic 1.0ml., and homogenate 2ml. After 1 hour incubation at 37° C, 30ml. of alcohol was added into the mixture to stop the enzyme activity, and titrated with N/ 20 alcoholic sodium hydroxide. The milliters of alkail solution which are consumed represented the enzyme activity.

Determination of choline oxidase activity.

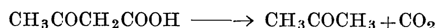
Livers were washed in M/15 phosphate buffer. Five g. of liver was homogenized with 25ml. of ice cold M/15 phosphate buffer (pH 6.36), centrifuged at Gx 12000 for 15 minutes and the supernatant was discarded. The precipitate was washed with 5ml. of M/15 phosphate buffer (pH 6.36), suspended in phosphate buffer (pH 7.34) and was made up 10ml. Enzyme activity was assayed by measuring O_2 uptake for 60 minutes with a Warburg's manometer. The following mixture was used for incubation at 37°C. Homogenate 1.0ml. was added in main compartment and 2% choline chloride 0.5ml. in side arm.

Oxidative breakdown of caprylic acid.

Two g. of liver were washed in 0.85% sodium chloride and made up to 5ml. The O_2 uptake was measured at 30°C for 40 minutes with Warburg's manometer. The incubation mixture consisted of M/15 phosphate buffer (pH 7.4) 0.9ml., veronal buffer (pH 7.4) 0.8ml., ATP 6mg., cytochrome C 0.1mg., magnesium chloride 0.56 mg., caprylate (pH 7.4) 0.432 mg., and homogenate 0.5ml.

Determination of acetoacetate.[31–33]

After incubation the flasks of the above experiment were chilled in ice water, and 0.25 volume of 2 N hydrochloric acid was added to stop the reaction. The incubation mixture was centrifuged and acetoacetate was determined as CO_2 using the supernatant at 25°C for 30 minutes with Warburg's manometer. A mole of CO_2 is released from a mole of acetoacetate.



$$CH_3COCH_2COOH \longrightarrow CH_3COCH_3 + CO_2$$

The reaction mixture used for the determination of acetoacetate consisted of each 3.0ml. of supernatant, 0.5ml. of 2 N hydrochloric acid., 0.5ml. of 50% citric acid and 1ml. of anilin-citric solution.

Succinate-cytochrome C oxidase system.

One gram of each chilled liver was weighed, homogenized in M/30 phosphate buffer (pH 7.38) with a glass homogenizer and was diluted to 10ml. Enzyme activity was assayed by measuring O_2 uptake by means of Warburg's manometer at 37°C for 60 minutes.

Incubation mixture consisted of M/15 phosphate buffer (pH 7.36) 1.5 ml., cytochrome C 0.1 mg., calcium chloride 1.7 mg., aluminium chloride 0.17 mg., succinate (pH 7.3) 19 mg., and homogenate 1.0 ml.

Preparation of mitochondria.

Each liver was chilled in 0.25 M sucrose solution, weighed, and mitochondria was separated by a modified Schneider's method.[34] Each liver was homogenized in a volume of 0.25 M sucrose solution by a Potter-Elvehjem glass homogenizer. Homogenate was centrifuged for 10 minutes at G x 1000. Supernatant was discarded. This treatment was repeated additional two times. The fat clinging to the tubewall was removed fully. The precipitate was taken up in 10ml. of sucrose solution and homogenized. All the above procedures were carried out at 2°C.

Determination of succinic deydrogenase activity

Enzyme activity was assayed by measuring O_2 uptake using mitochondria suspension at 37°C for 60 minutes with Warburg's manometer. The incubation mixture in the flask was as follows: 0.18 M phosphate buffer (pH 7.4) 2.3ml., 0.01 M methylen blue 0.3 ml., 0.1 M potassium cyanate (pH 7.3) 0.3ml., mitochondria suspension 0.2ml., and 0.4 M succinate (pH 7.0) 0.2ml.

Determination of cytochrome C oxidase

Enzyme activity was assayed as with succinic dehydrogenase. The incubation mixture in the flask was as follows: hydrochinone 3.0mg., 0.4% cytochrome C 0.2 ml., 0.1 M semicarbazide (pH 7.1) 0.3ml., M/15 phosphate buffer (pH 7.1) 1.6 ml., and mitochondria suspension 0.5 ml.

Determination of nitrogen.

Nitrogen of mitochondria suspension was determined with usual micro Kjeldahl method. Nitrogen was calculated as milligram per ml. of suspension.

Results.

Liver lipase activity.

The results were given in Table 8. In both male and female rats, the mean value of lipase activity in the fat-deficient group was low but the difference between ontrol and deficient groups was not statistically significant from the results of analysis of variance.

Liver choline oxidase activity.

The results were shown in Table 9. In Table 9 each value was variable and the difference between fat-deficient and control groups were not significant at a level of significance 0.1 (Rats which were used for the experiments of lipase and choline oxidase were bred with experimental diet for 13 weeks after one day of fasting.)

Oxidative degradation of caprylic acid.

Table 10 showed O_2 uptake when the substrate was caprylic acid. In general O_2 uptake of the fat-deficient group was large. In male rats a difference between fat-deficient and control groups was found only with a level of significance 0.2, but in female rats a difference could be found with a level of significance 0.05.

TABLE 8. LIVER LIPASE ACTIVITIES.

	Male		Female	
	Control	Deficient	Control	Deficient
	ml.	ml.	ml.	ml.
	38.5	52.0	67.2	61.7
	21.9	14.8	70.7	55.0
	22.6	14.2	21.2	13.5
		20.5	45.0	22.5
			31.6	12.5
Average	27.7	25.4	47.1	33.0
F. Value	3.063		1.28	

Above values mean ml. of N/20-NaOH solution for 60 minutes per gm. liver.

TABLE 9. LIVER CHOLINE OXIDASE ACTIVITIES.

	Male		Female	
	Control	Deficient	Control	Deficient
	μl 45.7	μl 139.8	μl 55.2	μl 76.0
	116.6	129.5	67.4	11.9
	110.5	87.2	57.4	81.1
			79.7	99.8
			47.3	136.4
			57.2	
Average	90.9	118.8	60.7	81.0
F. Value	1.004		1.857	

Above values mean μl. of O_2 uptaken for 60 minutes per 100mg. liver.

TABLE 10. OXIDATIVE DEGRADATION OF CAPRYLIC ACID OF RAT LIVERS.

	Male Rats		Female Rats	
	Control	Deficient	Control	Deficient
	μM 4.8	μM 8.5	μM 2.8	μM 6.7
	3.6	3.5	6.7	10.0
	3.4	7.0	1.8	3.4
	2.8	4.5	2.1	14.0
				6.4
				11.0
				5.0
				7.2
Average	3.7	5.9	3.4	7.9
F. Value	3.29		5.70	

Above values mean μM of O_2 uptaken per gm. liver·

Acetoacetate which was a product of oxidation of caprylic acid was determined as CO_2.

$$CH_3COCH_2COOH \longrightarrow CH_3COCH_3 + CO_2$$

The results were shown in Table 11. In both male and female rats the formation of acetoacetate was higher in fat-deficient group than in the control group and a difference between the two groups could be seen with a level of significance 0.05.

From the results of Table 10 and 11, a relation could be found between oxidation activity of caprylic acid and the formation of acetoacetate. Table 12 showed the ratio of CO_2 to O_2. In all comparisons except the oxidation of male rats differences could be found with a level of significance 0.05, therefore the ratio CO_2 to O_2 was worth considering. In Table 12 the ratio of CO_2 to O_2 in the fat-deficient group was higher than in the control group. This may mean that acetoacetate was accumulated in the fat-deficient rat. Especially this tendency was large in female rats.

TABLE 11. FORMATION OF ACETOACETATE OF RAT LIVERS.

	Male Rats		Female Rats	
	Control	Deficient	Control	Deficient
	μM 1.4	μM 3.4	μM 1.7	μM 2.9
	0.74	2.3	0.7	2.9
	1.3	3.3	1.7	6.3
	2.4	4.0	2.2	4.6
		2.0		
Average	1.46	3.0	1.56	4.17
F.Value	8.87		8.88	

Above values mean μM of CO_2 released for 30 minutes per gm. liver.

TABLE 12. THE RATIO BETWEEN OXIDATIVE DEGRADATION ACTIVITY OF CAPRYLATS AND ACETOACETATS FORMATION

		O_2 Uptaken	CO released	CO_2/O_2
Male Rats	Control Group	μM 3.76	μM 1.46	0.394
	Deficient Group	5.90	3.00	0.508
Female Rats	Control Group	3.46	1.56	0.458
	Deeicient Group	7.96	4.17	0.528

Succinate-cytochrome C oxidase system.

From the above results that acetoacetate was accumulated in fat-deficient rats the abnormality of the electron transferinng system was expected. Therefore the succinate-cytochrome C oxidase activity of male rats

was assayed. Results were given in Table 13. Succinate-cytochrome C oxidase activity of the fat-deficient group was relatively lower than that of the control group, and a difference between two groups could be found with a level of significance 0.01.

Succinic dehydrogenase and cytochrome C oxidase.

In all above experiments rat liver homogenate was used as enzymes, but in these two enzyme experiments male rat liver mitochondria suspension was used. In the case of succinate oxidase, enzyme activity was assayed in association with the cytochrome system and so it was difficult to determine in what place of the electron transport system the restriction of activity occurred. Mitochondria which was used in the experiment was washed sufficiently to remove substrates. Results were shown in Table 14. The mean value of succinic dehydrogenase activity in the fat-deficient group was considerably higher than in the control group and remarkable difference could be found between control and deficient groups with a level of significance 0.01 as a result of statistical analysis.

TABLE 13. ACTIVITIES OF SUCCINATE-CYTOCHROME C OXIDASE SYSTEM OF MALE RATS.

	Control Group	Deficient Group
	μM 32.0	μM 24.2
	32.0	25.3
	25.4	23.6
	30.6	22.1
	27.0	13.2
	34.6	
Average	30.2	21.7
F. Value	11.78	

Above values mean μM of O_2 uptaken for 60 mimutes per 100 mg. liver.

Cytochrome C oxidase.

The results of cytochrome C oxidase activity, which was shown in Table 15, was the same with the succinic dehydrogenase, that is, mean value of activity in the fat-deficient group was higher than that of the control group, and each value was high variable. But a statistical difference could be found between the fat-deficient group and the control group with a level of significance 0.01. The difference in cytochrome C oxidase activity was larger than in succinic dehydrogenase activity.

TABLE 14. SUCCINIC DEHYDROGENASE ACTIVITIES OF MALE RATS.

	Control Group	Deficient Group
	μl. 99.0	μl. 61.4
	81.4	74.0
	20.0	112.0
	63.0	76.5
	30.9	64.7
	51.2	
	54.7	
	12.7	
Average	51.57	77.72
F. Value	13.94	

Above values mean μl. of O_2 uptaken per mg. mitochondria nitrogen.

TABLE 15. CYTOCHROME C OXIDASE ACTIVITIES OF MALE RATS

	Control Group	Deficient Group
	μl. 128.0	μl. 43.9
	102.0	148.0
	18.4	110.0
	77.1	98.5
	73.2	80.8
	67.5	
	56.0	
	33.7	
Average	69.43	96.24
F. Value	31.5	

Above values mean μl of O_2 uptaken per mg. mitochondria nitrogen.

Discussion.

Kunkel[12] divided rats into four groups and these groups were fed their respective diets. One group recieved only the fat-free basal diet, and a second group was fed the basal diet supplemented with 100 mg. of pure methyl linoleate. The two remaining groups were fed diets containing 5% corn oil and 3% tetrabromostearic acid, respectively. And then choline oxidase, succinate oxidase and cytochrome C oxidase activities were assayed. Kunkel observed in his experiment that choline oxidase appeared to be elevated about 10% in all animals except those fed the corn oil diet, the activity of cytochrome C oxidase was markedly increased in the fat-deficient group, and the activity of succinate oxidase of each group was the same. Tulfpule[15] reported that succinate oxidase was not in-fluenced by fat-deficiency but the activity of cytochrome C oxidase was reduced considerably by the deficiency of essential fatty acids and vitamine B_6.

In the author's experiment lipase and choline oxidase activities were not so distinctly influenced by fat-deficiency. In Kunkel's results, choline oxidase activity of the deficient group was only 10% higher than the control group and the variability of each value was large. Therefore it is considered that choline oxidase is not influenced by fat-deficiency.

From the results of oxidative breakdown of caprylic acid the following processes are considered; one or more of the steps, in which fatty acids are broken down into C_2- units and oxidized into CO_2 and H_2O through Krebs' cycle, is inhibited, the recovery of energy become insufficient, other active acetate can not enter into Krebs' cycle and as a result ketone bodies are accumulated.

In the above experiment the O_2 uptake in oxidative degradation of caprylic acid in the deficient group was larger than in the control. It was the same with Kunkel's results. Consequently the electron transport system was inhibited in some places and oxygen which was absorbed was consigned in other places. In order to study the above consideration, succinate oxidase activity was assayed. As shown in Table 13, the activity of the fat-deficient group was lower. Then succinic dehydrogenase and cytochrome C oxidase activites were assayed to make clear in what place of that enzyme complex system the abnormality was. Succinic dehydrogenase and cytochrome C oxidase activities were found to be higher in the fat-deficient group, which was not expected. Why were different results obtained with succinate oxidase and succinic dehydrogenase? It was considered that some factors which connected the succinic dehydrogenase with the cytochrome system might be inhibited or might be deficient. This hypothesis is discussed in the following chapter.

CHAPTER 3. LNFIUENCES OF FAT-DEFICIENCY ON THE MITOCHONDRIAL LIPIDS.

The application of phase electron microscopy to the examination of mitochondria has provided valuable information on the internal structure and arrangement of the mitochondria. Palade[35] has examined the fine structure of sectioned animal mitochondria in the electron microscope and found a characteristic pattern of organization irrespective of the species of origin and the cell type. Similar studies have been made by Sjöstrand and Rhodin[36-37] and by Weinred and Jarman.[38-39] By their work it is clear beyond doubt that mitochondria have highly organized and regular internal structure. A great number of fine lamelae are visible which seem to extend in a very regular fashion partly or all the way through the mitochondrial body in a plane perpendicular to the long axis of the mitochondrion. Mitochondria consists from soluble and insoluble particles, which are closely connected with the solubility and insolubility of enzymes in mitochondrion. It is considered that many enzymes bind with the axis of insoluble structure of mitochondria, and these are ATP-ase, succinic dehydrogenase, cytochrome C oxidase, DPN-cytochrome C reductase, uricase and cytochrome C. On the other hand the enzymes which are considered to act with combined enzymes may be soluble. Glutamic dehydrogenase, adenylatekinase and fumarase are contained in this group.[40]

Green et al.,[41-42] who has studied mitochondria, introduced a folloing hypothesis; mitochondria had a membrane and cristaes. The inner structure of these two materials is the same essentially. All the

dehydrogenase of the Krebs' cycle and DPN except the succinic dehydrogenase are contained inside of the membrane. The membrane has the units of electron transport system and oxidative phosphorylation. DPN and soluble material in mitochondria moved out of the cell after the splitting of the membrane by any stimulus and a little fragment of membrane forms a particle which contains considerable amounts of lipids.

In general mitochondria contains lipids in considerable amounts.[43] The enzyme, which catalyze the terminal oxidation of fatty acids and the intermediate of the Krebs' cycle, is in intracellular particles which especially contain lipids.

Crane et al.[44] separated neutral fats, phospholipids, cholesterol, sphingolipid, tocopherol and etc. from mitochondria. Also Crane broke the little particle which was derived from the mitochondrial membrane with fat soluble reagents such as cholic acid, deoxycholic acid, butanol and amylalcohol and presumed that lipids or lipo-proteins connected the components of the electron transport system.

Edwards and Ball[45] reported that large volume of phospholipids was contained in the succinic oxidase complex, from which acid soluble phosphorus was separated by phospholipase of the venom of Clostridium welchii and in consequence succinic dehydrogenase activity decreased.

As above described, the lipids, especially phospholipids, which are contained in mitochondria, are considered to play a very important role in electron transport system. Therefore in this chapter the lipids and their phospholipid fraction in fat-deficient rat liver mitochondria were determined.

Experimental.

Animals.

Male albino Wister King weaning rats were used. Each animal was bred in metal cage for 23 weeks.

Diets.

These were the same with the former chapter.

Preparation of liver mitochondria.

Mitochondria was prepared by a modified method of Schneider.[34] From a liver 10 ml. of mitochonria suspension was made of 0.25 M sucrose solution.

Extracion of total lipids.

Five ml. of mitochondria suspension was mixed with 4 volumes of a 3 to 1 alcohol-ether mixture and the mixture was refluxed for one and a half hour. The mixture was filtered through a glass filter. The precipitate was extracted with chloroform in a Soxhlet apparatus for 6 hours. The above filtrate and chloroform extract were mixed and evaporated under 40°C in vacuum under a CO_2 stream. The residue was dissolved in chloroform and made up 10 ml. The chloroform solution which was prepared was mixed with 2 ml. of 0.25 M magnesium chloride in centrifuge tube, was shaken vigorously, and emulsion was completed. After standing at room temperature for several hours, the emulsion was broken by freezing at-10°C, centrifuged and the supernatant was pippeted out. The chloroform solution obtained was dehydrated with sodium sulfate and was evaporated to dryness at 40°C in vacuum under a CO_2 stream and the residue was weighed as lipid.

Separation of phospholipids from liver mitochondria.

Twenty-five ml. of 10% trichloracetic acid was added to 1 ml. of liver mitochondria suspension, and the mixture was centrifuged. The precipitate was washed with 2.5ml. of 95% alcohol and was centrifuged. The washed residue was refluxed with a 3 to 1 alcohol-ether mixture for 3 minutes. This procedure was repeated three times and each supernatant was mixed with the above cold alcohol extract and evaported in vacuum under a CO_2 stream at 40°C and made up to 10 ml. with an alcohol- ether mixture.

Determination of phosphorus.

A portion of above extract solution containing 0.01-0.05mg. phosphorus was pipetted into a Kjeldahl flask containing 2ml. of 60% perchloric acid. The solution was gently heated over a micro burner until the soultion cleared. The solution was neutralized with sodium hydroxide.

The neutralized solution was put into a 25ml. volumetric flask and 2ml. of 60% perchloric acid,

1ml. of 4% sodium sulfite and 1ml. of 7% sodium molybdate were added. After shaking the flask, 1ml. of 0.4% stannous chloride was added and diluted to 25ml. The absorption at 660 mμ was measured. The mg. of phosphorus was calculated from the standard absorption curve which was determind previously.

Determination of nitrogen.

Nitrogen was determined by the micro Kjeldahl method, and calculated as mg. per ml. of mitochondria suspension.

Results.

The increase of body weight in the fat-deficient group was considerably lower than that in the control group. In the rats which were used for the extraction of total lipid in liver mitochondria, the mean value of increase in control group was 271.2 g. but it was only 199.5 g. in fat-deficient group. Increase of body weight was the same as in the rats which were used for the extraction of phospholipids. In this case the control group was 245.5 g. and the fat-deficient group was 198.3 g. These results were shown in Table 16 and 17. In rats which were fed a fat-deficient diet, roughness of hair coat could be observed. In general the rats were considered to be fat-deficient from the body weight increase and from the results obtained in experiments of chapter 1 and 2.

TABLE 16. THE INCREASE OF BODY WEIGHT OF RATS WHICH WERE USED FOR THE EXTRACTION OF TOTAL LIPIDS IN LIVER MITOCHONDRIA.

Control Group			Deficient Group		
Initial Weight	Final Weight	Increase	Initial Weight	Final Weight	Increase
60 g.	335 g.	275 g.	80 g.	301 g.	221 g.
70	330	260	75	280	205
60	305	245	75	265	190
65	270	305	95	290	195
60	331	271	70	255	185
			80	295	215
			80	275	195
			45	235	190
A. V.		271.2			199.5

Total lipids in liver mitochondria.

As above described considerable amounts of lipids were contained in mitochondria and the important roles in the electron transport system of mitochondria were expected to be caused by the lipids. Therefore lipids in liver mitochondria, especially phospholipids were determined. The results were shown in Table 18.

TABLE 17. THE INCREASE OF BODY WEIGHT OF RATS WHICH WERE USED FOR THE EXTRACTION OF PHOSPHOLIPIDS IN LIVER MITOCHONDRIA.

Control Group			Deficient Group		
Initial Weight	Final Weight	Increase	Initial Weight	Final Weight	Increase
80 g.	330 g.	250 g.	60 g.	275 g.	215 g.
80	250	270	70	265	195
90	355	265	55	220	165
80	315	235	45	245	200
65	245	180	35	250	215
80	270	190	80	280	200
90	355	265			
70	330	260			
80	375	295			
A. V.		245.5			198.3

Total lipids which were contained in liver mitochondria was 1.68mg. per mg. mitochondria nitrogen in the control group, and on the other hand 1.09mg. in fat-deficient group. The differences between these two groups were significant at level of significance 0.01

Phosphorus which was contained in the above total lipids was 2.41% in the control group and 1.83% in the fat-deficient group. The statistical difference could be seen with a level of significance 0.1.

Table 19 showed the phosphorus of the phospholipids fraction which was determined directly with liver mitochondria. The results obtained was the same with Table 18, that is, the phospholipids fraction of the fat-deficient group was smaller than that of the control and the statistical difference could be seen with a level of significance 0.1.

TABLE 18. CONTENTS OF TOTAL LIPIDS IN MITOCHONDRIA AND CONTENTS OF TOTAL PHOSPHORUS IN LIPIDS.

	Total Lipids		Total Phosphorus in Lipids			
	Control	Deficient	Control		Deficient	
	mg.	mg.	mg.	%	mg.	%
	2.05	1.18	0.0680	3.32	0.0273	2.31
	1.74	0.92	0.0324	1.86	0.0157	1.72
	1.27	1.10	0.0264	2.07	0.0252	2.29
	1.68	0.79	0.0421	2.51	0.0125	1.58
	1.68	1.44	0.0342	2.03	0.0217	1.51
		1.08			0.0195	1.81
		0.97			0.0155	1.60
		1.23			0.0226	1.84
Average	1.68	1.09	0.0406	2.41	0.0200	1.83
F.Value	22.96		4.538 %			

Above values mean mg. of lipid and phosphorus per mg. mitochondria nitrogen, and % means the phoshorus content in total lipids.

TABLE 19. PHOSPHORUS OF PHOSPHOLIPIDS FRACTION IN LIVER MITOCHONDRIA.

	Control Group	Deficient Group
	0.017 mg.	0.014 mg.
	0.047	0.021
	0.033	0.015
	0.024	0.014
	0.016	0.013
	0.028	
	0.018	
Average	0.0261	0.0154
Phospholipid Calculated	0.660	0.399
F. Value	4.28	

Above values mean mg. of phosphorus per mg. mitochondria nitrogen.

Discussion.

Recently many workers studied the mechanim of the electron transport system, and the roles of phospholipid in that system were observed. Cliffe al.[46] determined the lipids in succinate and reduced DPN oxidase preparation of beef heart muscle mitochondria and reported that the phosphorus content was 1.5 % of dry enzyme preparation which was calculated 38 % as phospholipids. Marinett et al.[47] observed that the amount of lipids in pig heart muscle cytochrome C oxidase preparation was 33 % which consisted of 14.7 % of phospholipids, 12.8 % of neutral fat, 1.02 % of free cholesterol and etc. Also Marinett[48] reported that the pig heart cytochrome b-cytochrome C_1 preparation contained lipids about 16 % of dry weight in whichphos pholipids were one third.

Tho relation betweenthe content of lipids in mitochondria and fat-deficiency has not been reported. In the author's experiments both total lipids in mitochondria and phospholipids in total lipids were small in the fat-deficient group. That is, the total lipids of the control group was 1.68 mg. per mg. of nitrogen and 1.09 mg. in the fat-deficient group. The phosphorus in total lipids was 0.0406 mg. (2.41 %) in the control group and 0.0200 mg. (1.83 %) in the fat deficient group.

The phosphatide fraction in mitochondria was calculated by direct determination of 0.660 mg. per mg. mitochondria nitrogen in the control group and 0.399mg. in the fat-deficient group. These amounts corresponded to 39.3 % and 36.6 % of tatal lipids on Table 18 respectively. The percentage of phosphatide in the total lipids of the fat-deficient rat liver mitochondria was almost the same with that of the control, but the total amount of phosphatide in fat-deficient rat liver mitochondria was smaller than the control.

Nygard and Sumner[49] observed that succinate oxidase of rat liver homogenate and mitochondria was inactivated by pancreas lecithinase A but cytochrome C oxidase and succinic dehydrogenase was not influenced under the same conditions. Then they presumed that lecithin was one of the components which connected the succinic dehydrogenase with cytochrome C. Tookey[50] and Nason[51] reported similar results about beef heart succinate oxidase and considered that the place which lipids served in this system was near the cytochrome C.

It has been already shown that oxidative phosphorylation was essential for the biosynthesis of phosphatides in the initial steps. But liver mitochondria from fat-deficient rats esterify less high energy phosphate per mole of oxygen taken up during the intermediate oxidation in Krebs' cycle, therefore the content of phosphatides in fat-deficient liver mitochondria is considered to decline.

From the above data the low succinate oxidase acitivity of fat-deficient rat liver homogenate can be

accounted for. The lipids which are components connecting the succinate dehydrogenase with the cytochrome oxidase system are deficient or incomplete, and in consequence the enzyme activty decreased.

Summary.

Rats were bred with fat-deficient diets and suffered from fat-deficiency. The growth of weaning rats was inhibited by administration of a fat-deficient diet and the normal growth of livers was also inhibited.

The contents of lipids and carbohydrates in livers were not influenced by the fat-deficiency.

The growth of fat-deficient rats was not increased by the aministration of linoleic or linolenic acid.

The iodine values of body fat in the deficient group was considerably smaller than that of the control group, and the content of unsaturated fatty acids, especially that of linoleic acid, was found to be low.

The content of linoleate in body fat increased after the administration of linoleate but the increase was not observed in the case of arachidonate. Linolenate had the same effects as linoleate only when a large quantity was administered.

Lipase and choline oxidase activities of fat-deficient rat livers did not differ from the control, but acetoacetate tended to be accumulated in the fat-deficient rat livers.

Succinic-cytochrome C oxidase activity of the deficient group was lower than that of the control, but both succinic dehydrogenase and cytochrome C oxidase activities of deficient rats liver mitochondria were higher than the control. The contents of total lipids and phospholipids in fat-deficient rat liver mitochondria were smaller than control. The content of total lipids of the control group was 1.68 mg. per mg. mitochondria nitrogen, and that of the fat-deficient group was 1.09 mg.

The phospholipids content of the control group was 0.660mg. per mg. mitochondria nitrogen and that of the fat-deficient group was 0.399 mg.

The relation between the activity of succinate oxidase and the content of lipids in mitochondria was discussed. Considerable amoumts of lipids, especially phospholipids, are contained in the electron transport system of an animal, and lipid is considered to act the important function when the electron is transported from succinate to cytochrome C. That is, it is hypothesized that phospholipids cement some factors which exist between succinate and cytochrome C. The above hypothesis was proved partly by the author's experiments which showed a low content phospholipids and low activities of the electron transport system in fat-deficient rat liver mitochondria. The relation between fat-deficiency and the function of the electron transport system was made clear by this work, and it is believed that a contribution was given toward the development of the chemistry of fat metabolism.

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