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Abstract

1. For the purpose to clarify the relationship between the structural change and lipid composition of isolated rat liver mitochondria, lipid composition and swelling rate of mitochondria obtained from the rat of 3'-Me-DAB feeding and raised in cold room are measured, and the following results were obtained. 2. The mitochondria obtained from the liver of 3'-Me-DAB-fed rat and of rat raised in cold room show a low rate of swelling by addition of Na-oleate accompanied by the decrease in highly unsaturated fatty acids (C18:3 and C20:3or 4) and with the increase in saturated fatty acids (C16 and C18). 3. Activation energy for the mitochondrial swelling is about 16.2 Kcal in the mitochondria obtained from normal rat liver, but requires 19.7 Kcal in the mitochondria that show a low rate of swelling. The fatty acid composition, especially in glycerophosphatides which occupy about 80 per cent of total lipids, is a structural component of mitochondrial membrane, undergoes the change from former to latter in the following fashion: C16:0 21.73→32.10, C16:1 3.37→2.96, C18:0 25.0→29.75, C18:1 13.75→17.40, C18:2 23.90→16.0 and C20:3 or 4 12.23→1.79. 4. At the time of low rate swelling of mitochondria isolated from 3'-MeDAB- fed rat liver, there could be observed a marked increase of the acetone soluble lipid (simple lipids) in the total liver lipids and in the fatty acid distribution of the acetone-soluble lipids, oleic acid was markedly increased (0.838→3.81%/dry liver), despite the fact that in the acetone-insoluble fractions or in the mitochondria there are no marked changes in the oleic acid contents (1.84→2.56% or 0.212→0.246%/dry liver).

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ON THE RELATION BETWEEN THE FATTY ACID COMPOSITION AND THE SWELLING RATE IN RAT LIVER MITOCHONDRIA

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In the recent years, the role of lipids in mitochondrial function has been postulated by many investigators. [1-8] FLEISCHER *et al.* [6] have observed that phospholipids play a vital role in the electron-transport system of mitochondria. It is well known that the phospholipids of rat liver mitochondria contain a high proportion of classically essential fatty acid and the lipids of mitochondria generally are highly unsaturated. [5,8,9] This fact of unsaturation has a considerable importance in biochemical and physicochemical properties of membrane system and hence the behavior of mitochondrial membrane might be affected by the properties of these fatty acids. Thus, it is necessary to analyse the fatty acid component in the membrane system of mitochondria for the over-all understanding of its physiological meaning on the structure and function of mitochondria.

The swelling rate and the fatty acid composition of mitochondria, as a rule, differ according to the kinds of organs and to the species of animals. [9,10] Mitochondria isolated from the cancer cells show a lower swelling rate than those from the normal cells. [14,15] In rat liver mitochondria and microsomes the swelling rates fall in the course of liver carcinogenesis caused by aminoazodyes. [11,12,13] Swelling of normal rat liver mitochondria is closely correlated to the phosphorylation, which is known to be linked with electron transport. [8,15,16,17] Therefore, it is suggested that the metabolism of mitochondria might be regulated by swelling. [22]

In the present study relationships between the fatty acid composition and the swelling rate in rat liver mitochondria were investigated in the course of 3'-Me-DAB feeding.

MATERIALS AND METHODS

Male albino rats weighing 160-200g were used, and fed *ad libitum* on the polished rice containing 0.06 per cent of 3'-Me-DAB (3'-methyl-4-dimethylamino-

azobenzene) for the experimental group of liver carcinogenesis and on polished rice and "Oriental Kobo Kogyo Co.'s semisynthetic rat diet" for the control. Five to ten rats were killed as an experimental group every week or ten days.

Rat liver mitochondria were isolated by the modified method of HOGEBOOM[18]: 0.25 M and 0.34 M sucrose solutions containing 40 μM EDTA and 50 μM tris-HCl buffer (pH 7.4) were used. The mitochondria were washed twice with 0.25 M sucrose solution.

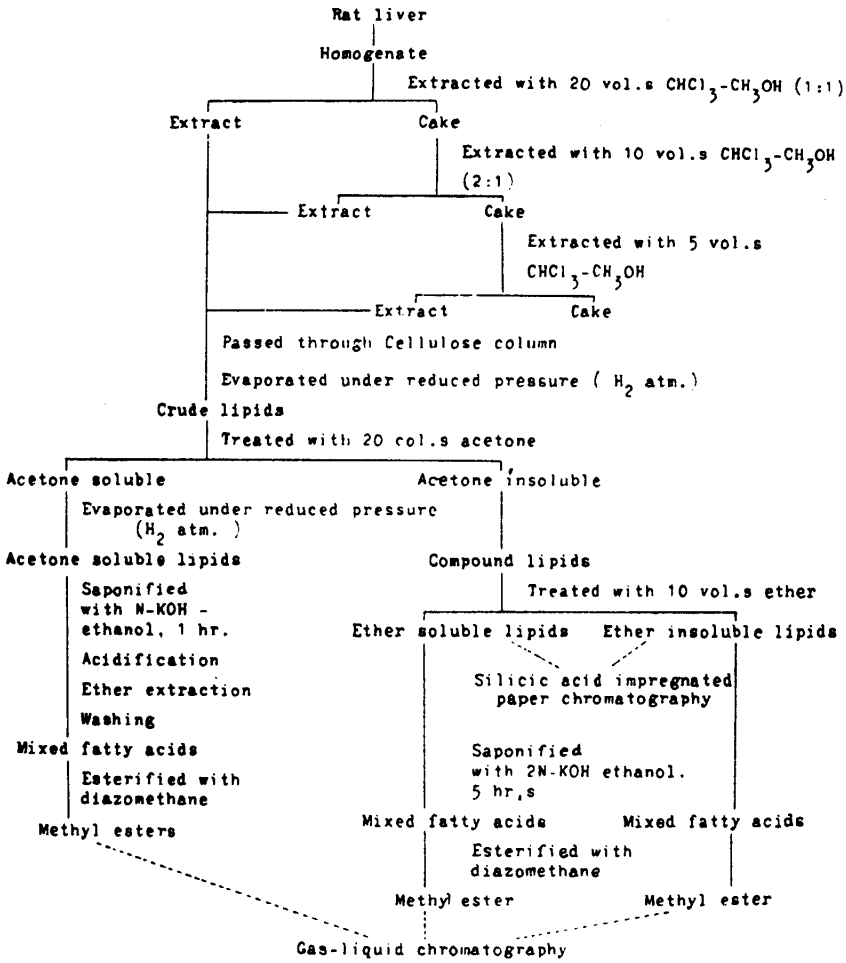


Fig. 1 Procedure for the Extraction of Lipid from Rat Liver and the Determination of the Composition

The procedures for the extraction and isolation of lipids from liver are shown in Fig. 1, and those from mitochondria are as follows: mitochondrial

lipids were extracted at room temperature with $CHCl_3$-MeOH (2 : 1 v/v) twenty volumes of the packed mitochondrial pellets. Non-lipid contaminants were eliminated through the cellulose column, and the solvents were evaporated under reduced pressure. Then, the lipids were saponified with 2N KOH ethanol for five hours and esterified by diazomethane in ether. The esters were analyzed by means of the gas-liquid chromatography using "Shimadzu Model GC-2B" under the conditions shown in Table 1. The area per cent for each component was determined by triangulation.

Table 1 Condition of Gas-Liquid Chromatography and Retention Time of the Fatty Acid Methyl Esters

Apparatus	Shimadzu Gas Chromatography GCB
Column	Cu (3,000 mm × 4 mm) × 2
Column Packing	Ethyleneglycol Succinate Polyester
Carrier Gas	H_2, 50 ml/min.
Bridge Current	110 mV
Column Temp.	200°C
Chart Speed	10 mm/min.
Span	4 mV
Sample	2—3.5 μl

Carbon Number	16	16	18	18	18	18	20	20	22
No. of Double Bond	0	1	0	1	2	3	0	4	0
Retention Time (min.)	4.4	4.9	7.2	7.8	8.9	10.3	13.0	17.6	22.5

For analysis of phospolipids, chromatography was carried out on silicic acid impregnated paper, according to the modified method of MARINETTI. [21]

In order to detect the behavior of mitochondrial membrane, mitochondrial swelling was measured by the method of LEHNINGER. [19,20] 0.4 ml of 0.1 M K-phosphate or of 0.05 per cent Na-oleate, and of 0.15M KCl-0.02M tris-HCl buffer as the control were added to 3.5 ml solution of mitochondria (equivalent of 0.05g liver tissue) suspended in 0.15 M KCl-0.02 M tris-HCl buffer (pH 7.4), and the mixtures were incubated at 25 °C for 30 minutes. The volume changes of mitochondria were measured by the optical density at 520 mμ using "Shimadzu Spectrophotometer MR-31". The swelling rate of mitochondria at 5 min. after the above-stated treatments was indicated by the following formula :

$$\text{Swelling rate} = \frac{A-B}{B} \times 100 \text{ (per cent)}$$

A : Optical density of mitochondrial suspension under the medium of KCl-tris-HCl buffer.

B : Optical density of mitochondrial suspension under the treatment of K-phosphate or Na-oleate.

The activation energy required for the swelling was estimated as follows: initial rates of swelling were estimated, referring to the swelling curve at 0°, 10°, 15°, 20°, 25°, 30° and 35°C, and activation energy was calculated according to Arrhenius equation.

RESULTS

Rat liver lipids were extracted and separated according to the method shown in Fig. 1. The lipid compositions and the fatty acid contents are summarized in Table 2. The acetone-insoluble, ether-soluble part occupying about 80 per cent of the total weight of lipid was composed of a large quantity of phosphatidylcholine-plasmalogen and phosphatidylethanolamine, and of a small quantity of phosphatidylserine, glycerophosphatidylinositide and sphingolipid. As shown in Table 2, the highly unsaturated fatty acids such as linoleic, linolenic and arachidonic acids were chiefly located in the glycerophosphatide. It is a well-known

Table 2 Lipid Composition and its Fatty Acid Contents in Rat Liver

		Acetone Soluble Fraction	Acetone Insoluble Fraction	
			Ether Soluble Fr.	Ether Insoluble Fr.
Per Cent to Total Lipid		11.3	79.4	9.3
Main Component		Sterol Sterol esters Glyceride Fatty acids V-A	Lecithin Phosphatidylethanolamine Phosphatidylserine Phosphatidylinositide Lysophosphatide Sphingolipid	Cerebroside (Sphingolipid) Saturated lecithin
Yield of Fatty Acid to Each Fraction (%)		85.6	61.2	6.83
Fatty Acid (Area %)	$C_{16:0}$	29.4	31.0	32.9
	$C_{16:1}$	7.62	4.6	4.75
	$C_{18:0}$	3.36	24.8	22.1
	$C_{18:1}$	39.1	15.2	25.7
	$C_{18:2}$	15.8	10.5	14.8
	$C_{18:3}$	—	1.6	—
	$C_{20:3\&4}$	—	11.9	—

fact that 30～35 per cent of the dry weight of mitochondria represents lipids, and that almost 90 per cent of the lipids is occupied by glycerophosphatide.[8]

The quantitative alteration of rat lipids was observed in the course of 3'-Me-DAB feeding. As shown in Table 3, the proportion of acetone-soluble liver lipids to acetone-insolubles has gradually changed: the former has risen, while the latter fallen. The changes of fatty acid compositions in two parts of the lipids, the simple lipids and the compound lipids, are shown in Table 3. In the

Table 3 Changes of the Fatty Acid Composition of Rat Liver Lipid in the Course of 3'-Me-DAB Feeding

A) Acetone Soluble Fraction (Simple Lipids)

Days of 3'-Me-DAB Feeding	%/Total Lipid	Fatty Acid Content (Area %)					
		$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2\&3}$	$C_{20:3\&4}$
0	24.5	24.0	8.8	1.9	23.1	38.1	4.1
6	30.5	30.1	6.2	1.85	40.7	19.1	2.0
13	34.4	24.9	8.3	2.0	46.0	17.5	—
20	35.9	24.6	11.6	—	50.7	12.3	—
30	38.6	22.9	10.0	—	55.0	11.3	—
38	28.6	27.8	6.9	4.9	46.8	13.1	—

B) Acetone Insoluble Fraction (Compound Lipids)

Days of 3'-Me-DAB Feeding	%/Total Lipid	Fatty Acid Content (Area %)*					
		$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2\&3}$	$C_{20:3\&4}$
0	75.5	25.1	—	16.1	13.5	30.5	14.8
6	69.5	25.9	7.4	23.3	16.8	18.0	8.6
13	65.6	31.2	—	18.6	24.3	16.1	9.8
20	64.1	26.6	4.9	28.3	20.6	16.4	3.2
30	61.4	28.1	5.0	26.9	19.8	15.8	3.7
38	71.4	30.8	6.2	15.4	30.8	16.2	—

* Fatty acid content was determined with gas-liquid chromatography.

Table 4 Changes of the Fatty Acid Contents of Mitochondria Isolated from 3'-Me-DAB-Fed Rat Liver

Days of 3'-Me-DAB Feeding	Lipid Content to Dry Mitochondria %	Fatty Acid Content to Dry Mitochondria %	Fatty Acid (Area %)					
			$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2\&3}$	$C_{20:3\&4}$
0	29.7	11.15	15.0	6.9	15.7	19.0	34.8	9.6
6	32.1	11.20	28.1	5.3	22.2	17.2	23.0	4.2
13	26.8	9.41	25.3	5.1	20.5	19.0	23.6	6.4
20	33.7	10.42	31.0	4.4	20.8	19.5	21.7	2.0
30	36.3	11.70	25.6	3.0	27.1	21.0	19.4	3.0
38	25.5	12.35	25.2	4.3	21.6	23.0	20.5	4.3

acetone-soluble lipids, palmitic, palmitoleic and oleic acids increased, remarkably, especially in the last. In the acetone-insoluble lipid fraction, the contents of highly unsaturated fatty acids over $C_{18:2}$ decreased in about 20 days. The changes of the mitochondrial fatty acid contents in the course of 3'-Me-DAB feeding are given in Table 4, and they are similar to those of the acetone-

insoluble fraction in the rat liver lipids, but in mitochondria the increase in palmitic and stearic acids was marked.

A marked swelling (phase II)[28] of rat liver mitochondria can be induced in the medium of 0.15 M KCl-0.02 M tris-HCl buffer (pH 7.4) by addition of Na-oleate or inorganic phosphate[15,19] and its swelling rate varied in the course of 3'-Me-DAB feeding (Figs. 3 and 4). The changes of this swelling rate in the course of 3'-Me-DAB feeding appear to have an exact parallel relationship with those of the saturated fatty acid content in the mitochondria (Fig. 2). Namely,

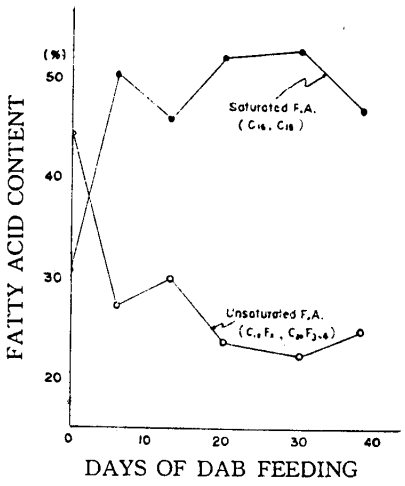
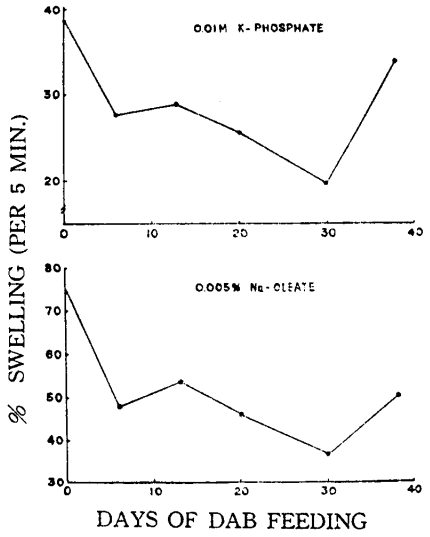


Fig. 2 Changes of the Saturated and Unsaturated Fatty Acids Contents in Rat Liver Mitochondria During 3'-Me-DAB Feeding



Fgi. 3

Fig. 4

Figs. 3 and 4 Changes of Swelling Rate of Mitochondria Isolated from the 3'-Me-DAB-Fed Rat Liver. Induced by 0.005 per cent Sodium Oleate and 10 mM K-phosphate in the Medium of 0.15 M KCl-0.02 M Tris-HCl buffer (pH 7.4) at 25°C

despite the fact that the increase of saturated fatty acids in the liver is not so marked, when the saturated fatty acid content in mitochondria increases, the swelling rate of mitochondria falls. In the rat liver at the period where a low rate swelling could be observed (on the 30th day) acetone-soluble lipid was increased, and in the fatty acid components a marked increase (0.838→3.81%/dry liver) of oleic acid was seen.

Thus, in analysing fatty acid compositions of the mitochondria at the same time calculating the activation energy required in inducing the swelling, it has been found that this activation energy required in the mitochondria which show a low rate swelling is 19.2 Kcal as compared to the normal of 15.6 Kcal,

showing distinct rise (Fig. 5). The composition of fatty acids in these mitochondria, as shown in Table 5, reveals a decrease in the unsaturated fatty acids such as linoleic and arachidonic acid, while an increase in stearic acids, as compared with the normal.

It is not reasonable to discuss about the relationship of structural changes to the fatty acid composition of mitochondria solely on the basis of the foregoing results, because it is well known that fatty acid compositions of animal tissue are dependent upon the lipid composition of feed stuff. By chance, having noticed that the degree of swelling-shrinkage of rat liver mitochondria in winter is less than what it is in summer time, the authors made a comparative study on the rate of liver mitochondrial swelling with two groups of rats, raised in summer and fed on the same "Oriental" semisynthetic rat diet for 3 weeks by keeping

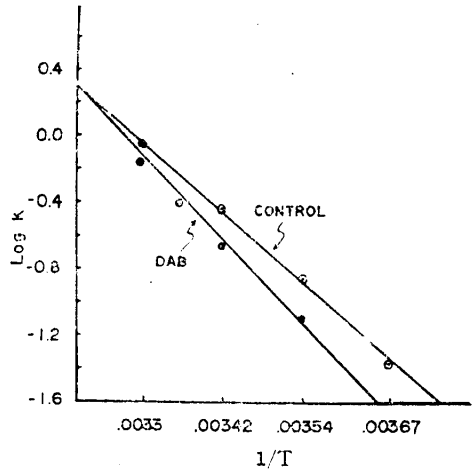


Fig. 5 Arrhenius Plats for Kinetics of Swelling of Normal Rat and 3'-Me-DAB -Fed Rat Liver Mitochondria in 0.15 M KCl-0.02 M Tris-HCl Buffer (pH 7.4)

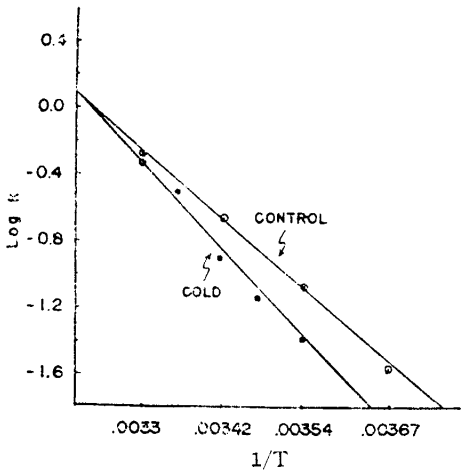


Fig. 6 Arrhenius Plats for Kinetics of Swelling of Mitochondria Isolated from Rat Liver Raised in Cold (5°C) and Room Temperature. They were Measured in 0.15 M KCl-0.02M Tris-HCl Buffer (pH 7.4)

Table 5 Relation between the Activation Energy for the Mitochondrial Swelling and the Fatty Acid Composition

	Activation Energy of Swelling	Fatty Acid (Area %)						
		$C_{16:0}$	$C_{19:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:3\&4}$
Control*	15.6 Kcal.	23.9	6.17	25.1	12.35	15.65	1.83	11.1+3.9
3'-Me-DAB	19.2 Kcal.	31.03	4.37	20.8	19.5	21.7	---	2.0

* Liver mitochondria isolated from rat fed on the polished rice.

Table 6 Relation between the Activation Energy for the Mitochondrial Swelling and the Fatty Acid Composition

	Activation Energy of Swelling	Fatty Acid (Area %)						
		$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:3\&4}$
Control*	16.2 Kcal.	21.73	3.37	25.0	13.75	23.9	—	12.23
Cold (5°C)*	19.3 Kcal.	32.10	2.96	29.75	17.4	16.0	—	1.79

* Mitochondria isclated from the liver of rat fed on "Oriental Kobo Kogyo Co.'s semisynthetic rat diet".

one group (5 rats) in a cold room (at 5°C) and another group (5 rats) in a warm room (at about 25°C). As the result it was found that the swelling rate of mitochondria in the group raised in the cold room was lowered and the activation energy required for swelling in this group was 19.7 Kcal as compared with 16.2 Kcal in the group raised in a warm room (Fig. 6). The compositions of fatty acids in these mitochondria are shown in Table 6.

A similar relation between the fatty acid composition and the swelling rate in rat liver mitochondria, observable in the course of 3'-Me-DAB feeding, was also found with the case of rats raised in cold room: the fatty acids of mitochondria which reduced swelling rate were largely composed of saturated fatty acid (C_{16}, C_{18}), but of less highly unsaturated fatty acids.

DISCUSSION

In the present study relationships between the fatty acid composition and the swelling rate in rat liver mitochondria were investigated. It is considered that the character and function of mitochondria have a close relation with the lipid component and structure in mitochondrial membrane. It has been observed that the increase of saturated fatty acids and the decrease of highly unsaturated fatty acids are accompanied by a fall in the swelling rate of mitochondria of the rat liver in 3'-Me-DAB feeding. As can be seen in Table 2, such highly unsaturated fatty acids as $C_{8:2}$, $C_{18:3}$, and $C_{20:3 \text{ or } 4}$ are present as the component fatty acids of glycerophosphatides which occupy about 80 per cent of the mitochondrial lipids.[8] Although the increase in saturated fatty acid in the liver was not so marked at the time of low rate swelling, the increase in saturated fatty acid was marked in the mitochondria. Therefore, it can be concluded that the degree of unsaturation of these glycerophosphatides is in a parallel relationship with that of the mitochondrial swelling. Moreover, it is considered that the flexibility of mitochondrial membrane is changed by the physical properties of the fatty acid residues which would exist as phospholipids

in the lipoprotein matrix. In addition, at the time of low rate swelling there could be observed a marked increase of acetone soluble lipid in rat liver, and in the fatty acid components oleic acid was markedly increased. This fact is suggestive of the formation of U-factor[30], indicating that the mitochondria are already in an uncoupling state due to oleic acid and consequently the swelling rate appears to be low. Actually, it has been already demonstrated by YAMAMOTO *et al.*[29] that the degree of the physiological swelling-shrinkage that is closely related to mitochondrial function is lowered as mentioned in the mitochondria in the foregoing, and that P/O ratio and respiratory control are also decreased.

Furthermore, metabolic insufficiency can often be observed in the mitochondria of those animals that are deficient in classical essential fatty acids such as linoleic, linolenic and arachidonic acids[23,25], and thus it is indicative of that these essential fatty acids are necessary for the proper function of mitochondria themselves. In other words, it is presumed that these essential fatty acids may be necessary for maintenance of proper structure or for the enzymic oxidation-reduction reactions associated with the active methylene hydrogen atoms which are held between the double bond.[23,24] However, it is not clear whether or not these changes in fatty acid composition of mitochondrial lipids occur following 3'-Me-DAB feeding, because the compositions of the animal tissue are greatly dependent on the feed, and also these are regulated to a certain extent by the feed the animals take in.[26,27] Nonetheless, in view of the facts that free fatty acid content is high in the cancer cells,[27] that the mitochondria of cancer cells are not so readily swollen and that stearic acid is found relatively abundantly in the lipids of liver of cancer bearing mouse or of cancer cells,[27] the phenomenon of saturation of mitochondrial lipids may be a sign of precancerous liver. In any event, it has been observed that in those mitochondria whose swelling tendency is considerably low, the amounts of saturated acids such as palmitic and stearic acids are always higher while those of unsaturated fatty acids are lower than those in the mitochondria that show a high rate swelling.

SUMMARY

1. For the purpose to clarify the relationship between the structural change and lipid composition of isolated rat liver mitochondria, lipid composition and swelling rate of mitochondria obtained from the rat of 3'-Me-DAB feeding and raised in cold room are measured, and the following results were obtained.

2. The mitochondria obtained from the liver of 3'-Me-DAB-fed rat and of rat raised in cold room show a low rate of swelling by addition of Na-oleate accompanied by the decrease in highly unsaturated fatty acids ($C_{18:3}$ and $C_{20:3\ or\ 4}$) and with the increase in saturated fatty acids (C_{16} and C_{18}).

3. Activation energy for the mitochondrial swelling is about 16.2 Kcal in the mitochondria obtained from normal rat liver, but requires 19.7 Kcal in the mitochondria that show a low rate of swelling. The fatty acid composition, especially in glycerophosphatides which occupy about 80 per cent of total lipids, is a structural component of mitochondrial membrane, undergoes the change from former to latter in the following fashion: $C_{16:0}$ 21.73→32.10, $C_{16:1}$ 3.37→2.96, $C_{18:0}$ 25.0→29.75, $C_{18:1}$ 13.75→17.40, $C_{18:2}$ 23.90→16.0 and $C_{20:3 \text{ or } 4}$ 12.23→1.79.

4. At the time of low rate swelling of mitochondria isolated from 3'-Me-DAB-fed rat liver, there could be observed a marked increase of the acetone soluble lipid (simple lipids) in the total liver lipids and in the fatty acid distribution of the acetone-soluble lipids, oleic acid was markedly increased (0.838→3.81%/dry liver), despite the fact that in the acetone-insoluble fractions or in the mitochondria there are no marked changes in the oleic acid contents (1.84→2.56% or 0.212→0.246%/dry liver).

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