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Review

Megamitochondria formation - physiology and pathology

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Abstract

Mitochondria undergo structural changes simultaneously with their functional changes in both physiological and pathological conditions. These structural changes of mitochondria are classified into two categories: simple swelling and the formation of megamitochondria (MG). Data have been accumulated to indicate that free radicals play a crucial role in the mechanism of the MG formation induced by various experimental conditions which are apparently various. These include ethanol-, chloramphenicol- and hydrazine-induced MG formation. Involvement of free radicals in the mechanism of MG formation is showed by the fact that MG formation is successfully suppressed by free radical scavengers such as α-tocopherol, coenzyme Q_{10}, and 4-OH-TEMPO. Detailed mechanisms and pathophysiological meanings of MG formation still remain to be investigated. However, a body of evidence strongly suggests that enormous changes in physicochemical and biochemical properties of the mitochondrial membranes during MG formation take place and these changes are favorable for membrane fusion. A recent report showed that continous exposure of cells with MG to free radicals induces apoptosis, finding which suggests that MG formation is an adaptative process to unfavorable environments at the level of intracellular organelles. Mitochondria try to decrease intracellular reactive oxygen species (ROS) levels by decreasing the consume of oxygen via MG formation. If mitochondria succeed to suppress intracellular ROS levels, MG return to normal both structurally and functionally, and they restore the ability to actively synthesize ATP. If cells are additionally exposed to excess amounts of free radicals, MG become swollen, membrane potential of mitochondria ($\Delta\Psi m$) decreases, cytochrome c is released from mitochondria, leading to activation of caspases and apoptosis is induced.

Keywords: mitochondria - megamitochondria formation - free radicals - ethanol - chloramphenicol - hydrazine - apoptosis

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Introduction

Functions of mitochondria can be classified into two major categories [1, 2]: 1) General or common to mitochondria regardless of their sources of tissues: ATP synthesis and regulation of apoptotic processes; and 2) specific functions restricted to mitochondria in certain tissues: steroid synthesis in steroid producing organs such as adrenal cortex, ovary, testis and placenta; heat production in brown fat; and heme synthesis in erythroblasts in bone marrow.

Recent advance in molecular biology has disclosed a new important functional role of mitochondria. Data have been accumulated to indicate that mitochondria, probably regardless of their tissue sources, are deeply involved in the apoptotic processes. Among mitochondrial factors controlling the apoptotic processes are cytochrome c [3, 4], apoptosis-inducing factor (APAF) [5-8], Smac/DIABLO [second mitochondria-derived activators of caspases/direct IAP (inhibitors-of-apoptosis (IAP) proteins)]-binding protein with low pI [9, 10], Bcl-2 family [4, 11-13] and several procaspases including procaspase-2, -3 and -9 [14].

It has been believed for a long time that mitochondria are granular in their shapes scattered in the cytoplasm. Application of fluorescent dyes for visualization of mitochondria by confocal microscopy has established that the structure of mitochondria in cells is more dynamic than expected: when cells require a large amount of ATP mitochondria become granular separated from each other thus increasing their surface areas; when less amount of ATP is required they get together forming filamentous or string-like structures or branched structure resulting in decreases in their number per cell and their surface dimensions. When cells are exposed to various stimuli mitochondria may alter their shapes to adapt to new environments, and such structural changes of mitochondria may be classified into two categories: a simple swelling and the formation of megamitochondria (MG) [1, 2]. We can find a body of reports in the literature describing the MG formation both in human diseases and various experimental conditions. Typically, the presence of MG in the alcoholic liver disease has been adopted as a diagnostic tool in clinical medicine [15-19].

In this review, functional and morphological aspects of MG are overviewed based mainly on data published from our laboratory. The mechanisms of MG formation are discussed as well. Finally, pathophysiological meaning of the phenomenon is discussed with special reference to apoptosis.

I. Structural aspects of MG

1. Definition of MG

Detection of MG has been repeatedly reported in the literature in biopsied or autopsied specimens obtained from patients experiencing a variety of diseases and also in tissues or culture cells under various experimental conditions. In most cases, findings of MG have been accidental. When some mitochondria increase their sizes compared to those of the control they have been called “MG” or “extremely swollen mitochondria”. Thus, the criteria for MG have not been clear for a long time. Since mechanisms and pathophysiological meaning of MG are distinct from those of the simple swelling, as discussed later, the former must be strictly distinguished from the latter. Recently, we have proposed a simple criterion for defining MG (Fig. 1) [20]. Here, only morphological basis for MG is given in comparison to the swelling, and functional differences between them will be given later.

The question of to what extents mitochondria are enlarged by a simple swelling can be answered by the following simple experiments. When mitochondria isolated from beef heart (Figs. 1A-1C) or rat liver (Figs. 1D-1F) or hepatocytes isolated from rat liver (Fig. 1G-1F) are exposed to a hypotonic solution for 2 min for the former two cases and for 10 min for the latter case, the electron density of the matrix kept well in control mitochondria (Figs. 1A, 1D, 1G) is lost distinctly indicating that the inner mitochondrial membranes have lost their impermeability to sucrose and water has come into the matrix space resulting in the swelling of mitochondria (Figs. 1B, 1E, 1H). When they are further exposed to the

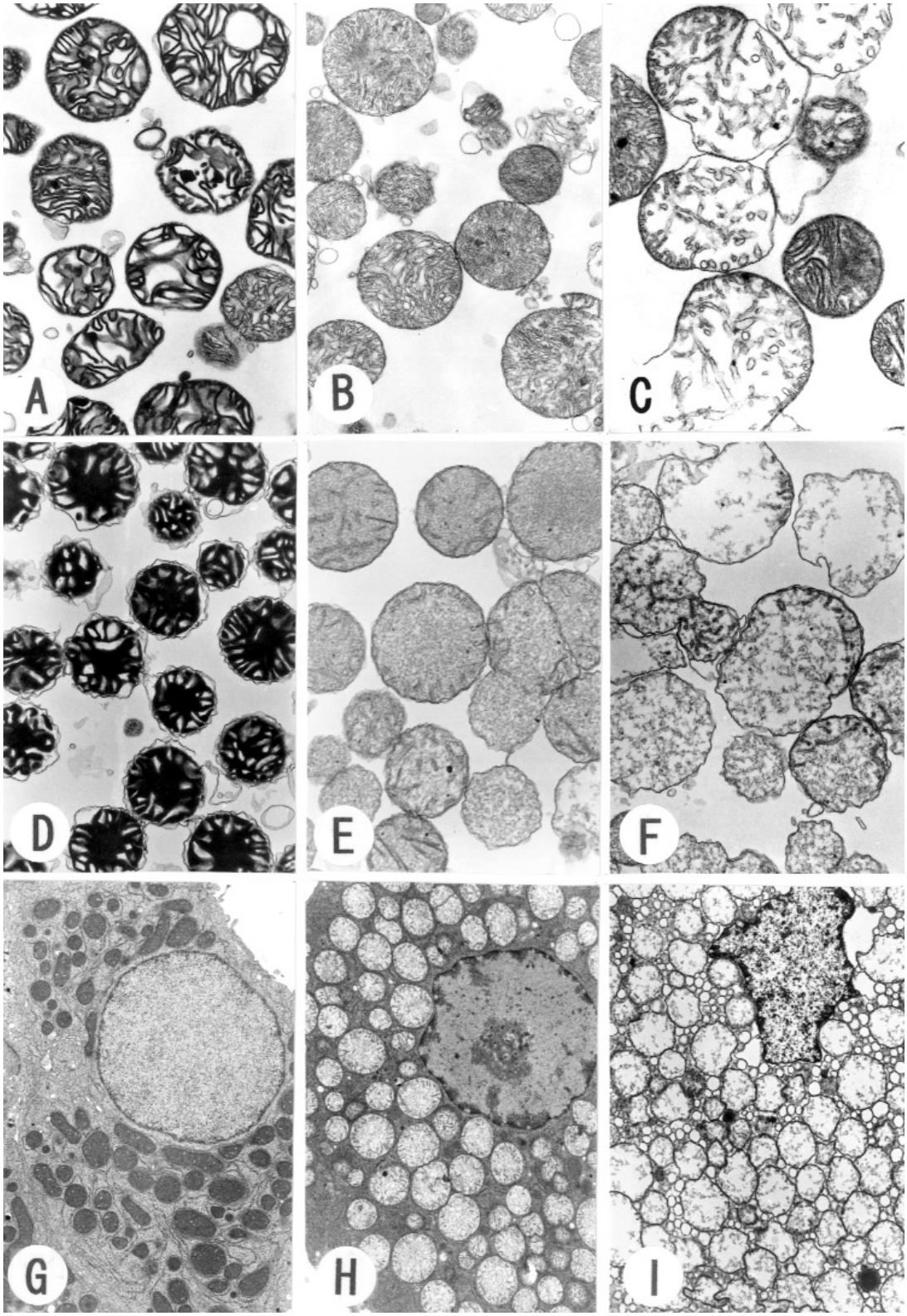


Fig. 1 Swelling of mitochondria in a hypotonic solution [modified from 1, 2, 20]. Beef heart (A-C) and rat liver (D-F) mitochondria, and isolated rat hepatocytes (G-I) were incubated with a medium containing 15 mM Na-acetate, 5 mM Tris-acetate, pH 7.4, at 25ºC for the former two kinds of mitochondria, and at 30ºC for the latter for various exposure times: control (A, D, G), 1 min (B, E), 5 min (C, F), 10 min (H), and 30 min (I). Magnification of electron micrographs: X10,000.

Table 1A MG formation in mammals under physiological conditions

Tissues (diseases)	Comments	Refs.
1. Sperm (mammalian)	*Fzo* encodes a transmembrane ATPase that becomes detectable on spermatid mitochondria in the late meiosis II, just prior to fusion, and disappears soon after the fusion is completed, corresponding *Fuzzy onions* in yeasts and *D. melanogaster*	21
2. Diaphragm (rat)	"Reticulum mitochondriale" in red fibers of adult rats; MG proved by serial-sectiong technique	22
3. Lymphocytes (mouse)	Two to three branched MG among 104 small mitochondria in the cell; proved by serial-sectioning technique	23
4. Ehrlich ascites tumor cells	MG proved by serial-sectioning technique	24
5. Liver (human)	MG related to aging; proved on one plane of section	25-29
6. Adrenal (human)	Fetal adrenal cortex; MG proved by serial-sectioning technique	30, 31

medium the outer mitochondrial membrane becomes ruptured losing its matrix proteins (Figs. 1C, 1F, 1I). It is clear from these results that mitochondria become at most 2-3 times larger in size by a simple swelling without losing their outer membrane. Thus, one may call MG when they exceed those extents in diameters compared to control ones.

2. Structure of MG

MG are detected both in physiological (Table 1A) and pathological conditions (Table 1B). The presence of MG in physiological conditions in mammals is restricted to certain tissues of the body. The case of skeletal muscles of the diaphragm is distinct from other cases because one huge, branched mitochondrion can be found in the myocyte [22] (Fig. 2A). Formation of such huge reticular mitochondrion is seen only in adult rats, and mitochondria scattered in the cell are detectable in rats one day after birth (Fig. 2B). Sperm mitochondria consist of four cylindrical ones, so-called "Nebenkern", running helically along the long axis of the sperm tail. Fzo1 appears temporarily during the developmental stage of the Nebenkern and disappears when it is completed (21). In yeast cells a variety of genes has been found to control the size and distribution of mitochondria in the cell (for review, 2, 196). Three genes have been detected to prevent the formation of MG: *mmm1* (197, 198) and *mdm12* (199) in yeast cells, and *mdm10* both in yeast cells (198) and *Podospora arserina* (200). Thus, mutants lacking these genes are endowed with MG. *Drp1* is the only one gene detected in human up to now (201). In Cos-7 cells transfected with mutant *Drp1* mitochondria appear as clusters of tubules suggesting the gene is important for the distributing mitochondrial tubules throughout the cell (201).

MG are associated with a variety of human diseases (Table 1B). It is evident from the table that liver and skeletal muscles are the two major tissues in which MG have been detected. Among these diseases listed in the table alcoholic liver injury (Fig. 3A) and mitochondrial myopathy (Fig. 3B) are distinct from others and the presence of MG has been used as a diagnostic tool in clin-

ical medicine because MG are frequently associated with these diseases. In most cases of biopsied or autopsied material except mitochondrial myopathy and alcoholic hepatic injury cases the frequency of MG is rather small. Furthermore, it is almost always the case to find a few MG among normal-sized mitochondria in a cell. Table 1C demonstrates a list of experimental conditions associated with MG formation in mammals. Similarly with MG formation in various diseases, the finding of MG associated with various experimental conditions is, in most cases, accidental.

Table 1B MG formation associated with human diseases

Tissues (diseases)	**Refs.**
1. Liver	
Alcoholic intoxication	16, 32-35, 38
Chronic familiar hyperphosphatemia	36
Gilbert's disease	37
Primary biliary chirrhosis	38
Chronic viral hepatitis	38-40
Halothane hepatitis	38, 41
Aspirine administration	42
Oral contraceptives	43, 44
Dubin-Johnson syndrome	39
Rotor's disease	45
Wilson's disease	46, 47
Weil's disease	48
Reye's syndrome	49, 50
Amyloidosis	38, 51
Systemic sclerosis	52
Mitochondrial myopathy	53
Primary amyloidosis	38
Extrahepatic biliary obstruction	38, 40
Diabetes	54
Type I hyperlipoproteinemia	54
Mucopolysaccharoidosis	55
2. Kidney	
Lipoid nephrosis	38
Membranous glomerulonephropathy	38, 56, 57
Acute glomerulonephritis	58, 59
Chronic pyelonephriis	59
Disseminated lupus erythematodes	59
Typ III glycogen disease	59
Gout	59
Eclampsia	59
Oncocytoma	60
Chronic lead intoxication	61
3. Bone marrow	
Myelogenous leukemia	62
Erythroleukemia	63
Iron, copper deficiency	64
Lymphoblastictic leukemia	65
4. Pancreas	
Kwashiokor disease	66
5. Parotic gland	
Warthin tumor	67, 68
6. Throid gland	
Oncocytoma	69
7. Cerebellum	
Mitochondrial myopathy	70
Musculo-oculodystrophy	71
8. Sweat gland	
Idiopathic cardiomyopathy	72
9. Heart	
Idiopathic cardiomyopathy	73, 74
Alcoholic cadiomyopathy	75-78
Eosinophilic heart disease	79
10. Skeletal muscle	
Mitochondrial myopahy	53, 80-92
Muscular dystrophy	93-101
Wednug-Hoffmann's disease	81, 102, 103
Periodic paralysis	81
Dermatomyositis	81, 104
Polymyositis	104, 105
Hypothyroidism	106
Diabetes	107
Alper's disease	108-110

Table 1C MG formation, experimentally induced in mammals.

A. Tissues

Experimental conditions	Sources of tissues (animals)	Refs.
1. Hormonal disturbance		
hypophysectomy	adrenal cortex (rat)	111-113
aminoglutethimide	adrenal cortex (rat)	114
thyroxin	liver (rat)	115,116
anabolic steroids	liver (sheep)	117
cortisone	Liver (rat)	118
calcitonin	bone marrow (rat, monkey, mini-pig)	119
2. Ethanol intoxication	liver (rat, monkey, mini-pig)	120-127
3. Malnutrition		
starvation, protein deficiency	liver (mouse,rat)	128-133
riboflavin deficiency	liver (mouse,rat),kidney (mouse)	134-139
choline deficiency	liver (rat)	140
vitamin B_1deficiency	heart (rat)	64,141,142
essential fatty acid deficiency	liver (mouse)	143
iron, copper deficiency	heart, bone marrow, liver (rat)	64,144
4. High protein diet	liver (rat)	145
5.Drug		
chloramphenicol	liveer (moude), adrenal cortex (rat)	146-148
ethidium bromide	liver (mouse)	149-150
α,α'-dipyridyl	kidney, liver (mouse)	151
nialamide	liver (mouse)	152
cuprizone	liver (mouse)	153-156
diethyldithiocarbamate(DDC)	liver (mouse)	157
ionicotinic acide derivatives	liver (mouse)	158
ammonia derivatives	liver (mouse, rat)	159
hydrazine	liver (mouse,rat)	160
popylthiouracil	liver (rat)	161
2-DAB	liver (rat)	162
puromycin	cerberum (mouse)	163
glycerin	kidney (rat)	164
reserpin	heart (mouse, dog)	165, 166
ouinidine	heart (dog)	167
DNP	heart (dog)	167
crotoxin (rattle-snake venom)	skeletal muscle (rat)	168
6. Hypoxia, ischemia, oxygen toxicity	heart, liver (dog, rat) skeletal muscle (rat)	167-176
7. Amyloidosis	liver (monkey)	51
8. Virus		
encephalomyocarditis virus	liver (mouse)	177
9. X-ray irradiation	heart (rabbit)	178
10. Exercise	heart (dog)	179

B. C,ulture cells

Chemicals	Cells	Conditions#	Refs.
1. Hydrazine	primary culture rat hepatocytes, RL-34 (JCRB 0247), COS-1 (JCRB 9082) IAR-20 (JCRB 0610)	2 mM, 22-24 h	180-183
2. H_2O_2	primary culture rat hepatocytes, RL-34, COS-1, IAR-20	0.2 mM, 22 h	180,181
3. Chloramphenicol	primary culture rat hepatocytes, RL-34, COS-1, IAR-20	200-300 μg/ml, 22 h	181-184
	HeLa	20-40 μg/ml	186
4. Ethidium bromide	RL-34, COS-1, IAR-20	200-300 μg/ml, 22 h	181-184
	L cells (mouse fibroblasts)	1-2 μg/ml, 3-4 h	185
		20-40 μg/ml,	186
	Hela cells	1-2 μg/ml, 24 h	189
	human bone marrow (lymphocytes, platelets)	10 μg/ml, 3.5-18 h	188
5. Erythromycin	primary culture rat hepatocytes, RL-34, COS-1, IAR-20	200 μg/ml, 22 h	181
6. Indomethacin	primary culture rat hepatocytes, RL-34, COS-1, IAR-20	0.3-0.5 mM, 22 h	181
7. Methylglyoxal-bis-guanylhydrazine	primary culture rat hepatocytes, RL-34, COS-1, IAR-20	50 μg/ml, 22 h	181
	mouse leukemia L120	1-10 μg/ml, 24 h	190
8. Ethanol	rat cardiac myocytes	12.5-500 mM, 5 min	191,192
	RL-34	1-3%, 6 h	193
9. Anti-Fas antibody	Jurket T cells	100 μg/ml, 6 h	194
10. Ischemia	rat cardiac myocytes	2 h	195

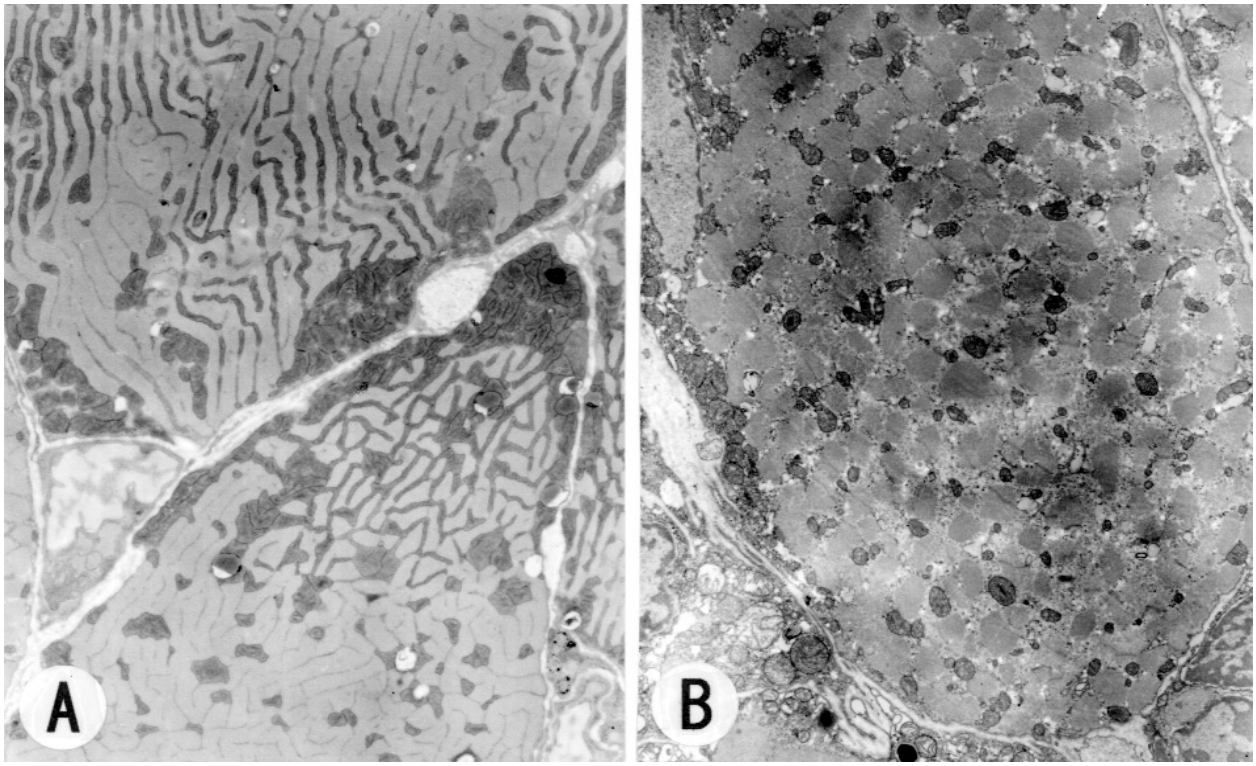


Fig. 2 Ultrastructural appearances of diaphragm mitochondria. A. Reticular networks of mitochondria in an adult rat aged 8 weeks. B. Granular mitochondria separated from each other in a rat aged one day after birth. Magnification of electron micrographs: x5,000.

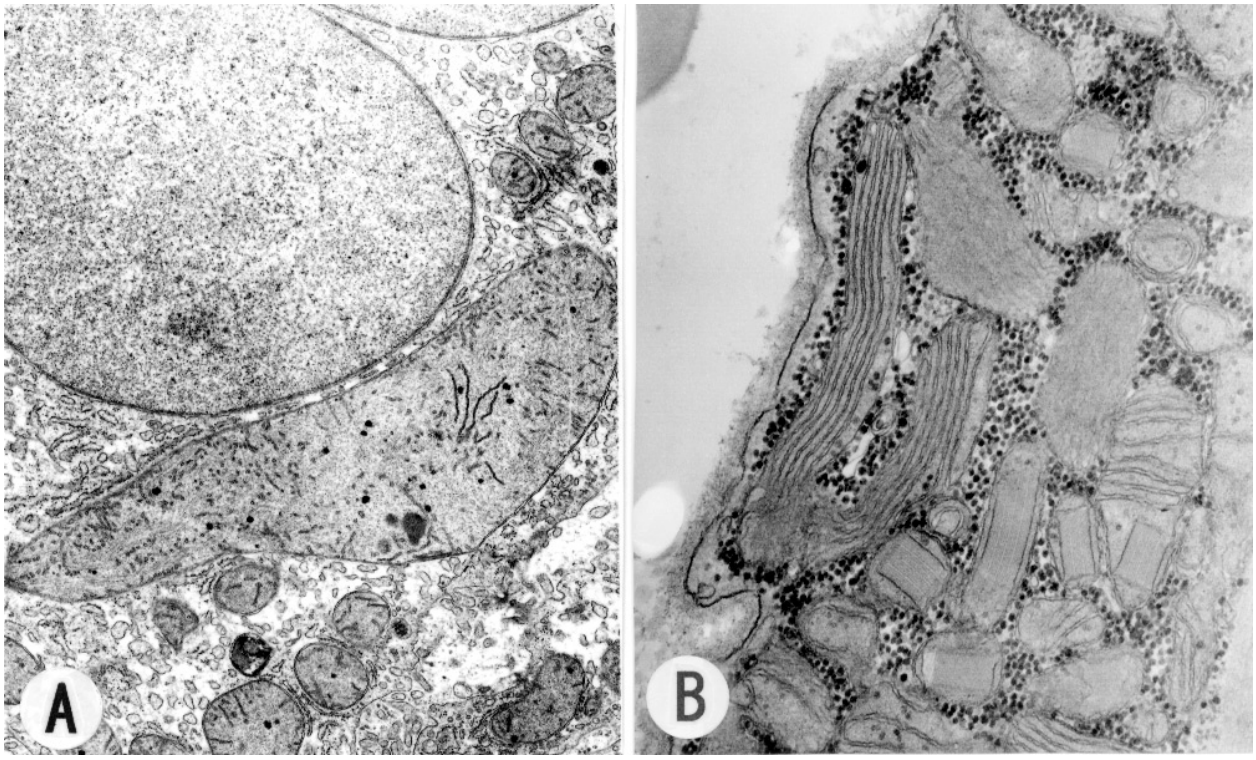


Fig. 3 MG associated with human diseases. A. Alcoholic hepatic disease (x11,500). B. Mitochondrial myopathy (x23,000).

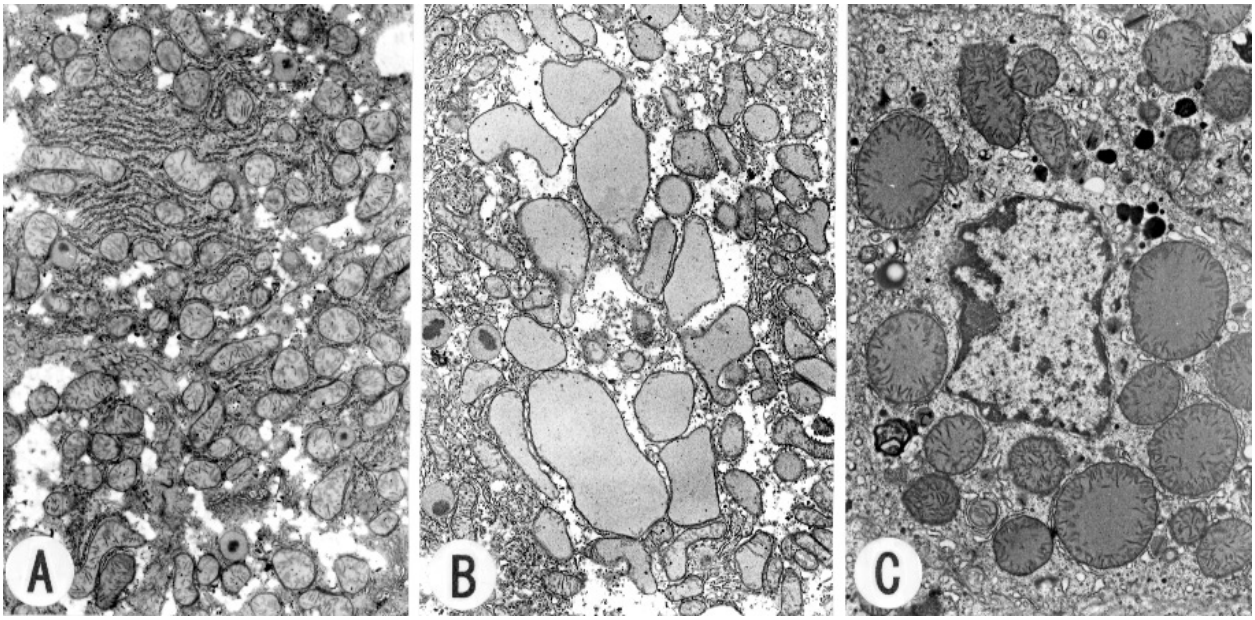


Fig. 4 Ethanol- and 1-octadecanol-induced MG in the rat liver. A. Control. B. Animals were given 32% (v/v) ethanol in a drinking water for two months. C. Animals were fed with a powdered diet containing 20% 1-octadecanol for 2 months. Magnification of electron micrographs: x7,800.

Ethanol-induced MG in experimental animals have two distinct morphological characteristics [202]: only a few MG in each cell are found in the liver of rats fed with ethanol for one month, and the number of MG distinctly increases when the duration of etahnol treatment is prolonged for up to 2-3 months (Fig. 4B). Both MG and the surrounding mitochondria, even smaller in their sizes as compared to those of the control animals (Fig. 4A), are almost devoided of cristae. Ethanol-induced MG formation with the characteristics described above, is reproducible with propanol and butanol treatments [202, 203]. When the number of carbon chains in alkyl alcohols is increased, MG which are distinct from ethanol-induced MG are formed [202]. For instance, with 1-octadecanol treatment, almost every mitochondrion becomes enlarged to various degrees resulting in a decreased number of mitochondria per cell (Fig. 4C).

Survey of the chemicals capable to induce MG including cuprizone has led us speculate that ammonia derivatives with electron-releasing substituting groups might induce MG [159]. Based on this assumption, we tested hydrazine (NH_2-NH_2), one of the chemicals with the simplest chemical structure among ammonia derivatives with electron-releasing substituting groups, if it was capable of inducing MG in the mouse or rat livers. We found that hydrazine successfully induced MG in all hepatocytes of mice or rats treated for 2-3 days with the former and 7-8 days with the latter (Fig. 5) [157, 158].

II. Functional aspects of MG

1. Isolation procedures for MG

It is essential to analyze functional aspects of MG in order to elucidate the mechanisms of MG formation under various pathological conditions. The MG fraction obtained by a routine isolation procedure for normal mitochondria always consists of a mixed population of mitochondria enlarged to various degrees and normal-sized ones. Thus, a serious question may be raised: does the MG fraction obtained by a routine isolation procedure for normal mitochondria really reflect the function of MG? Data obtained from our lab-

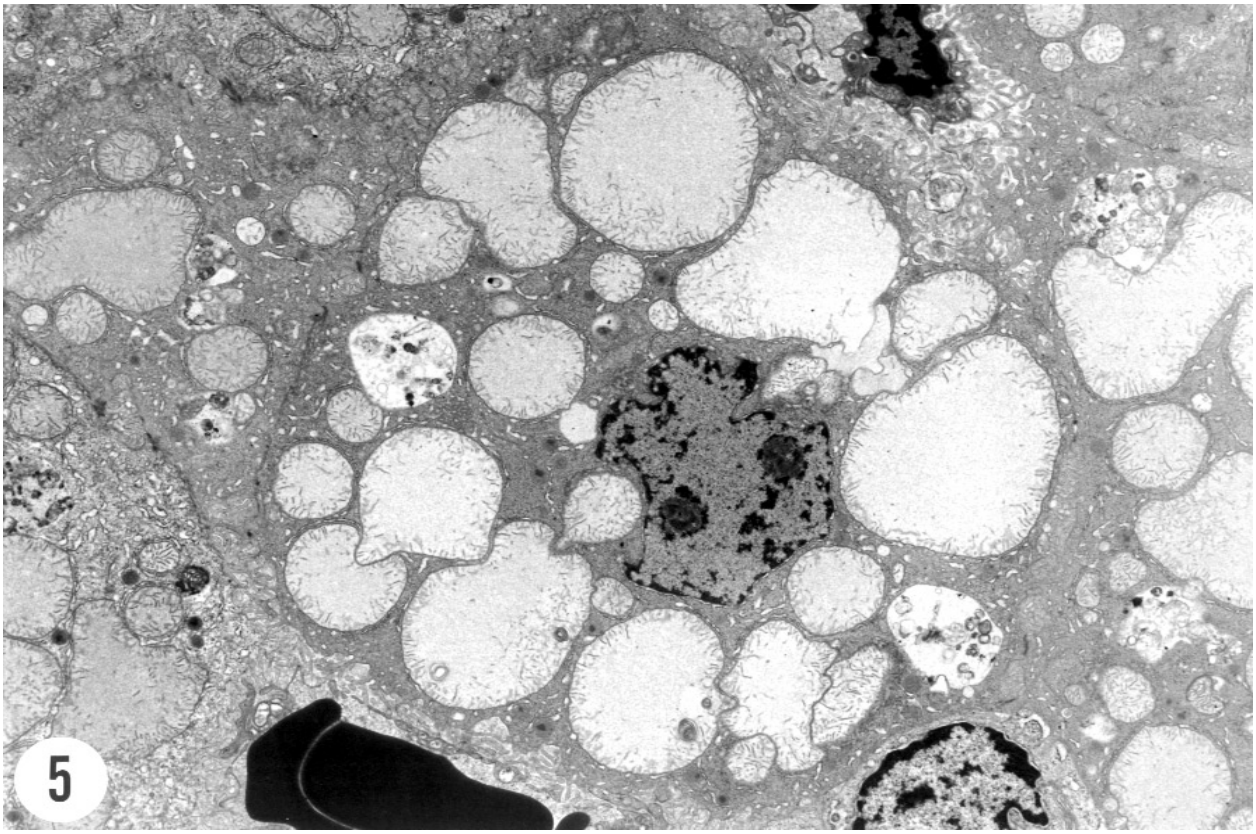


Fig. 5 Hydrazine-induced MG in the rat liver. Animals were fed a 1% hydrazine-diet for 8 days (x6,000).

oratory may partly answer this question [204]. The liver homogenate obtained from rats fed with a hydrazine-diet for 8-9 days or from those fed with ethanol in drinking water for two months were divided into two parts: one part of the homogenate was used for the isolation of mitochondria by the method routinely used in our laboratory. The homogenate was centrifuged for 10 min at 700g. The supernatant was centrifuged for 10 min at 7,000g, and the pellet was washed three times and used as the mitochondrial fraction [designated as (700-7,000)$gR^{2'''}$]. Another part of the homogenate was used to obtain the light and heavy mitochondrial fractions. The homogenate was centrifuged for 10 min at 500g. The supernatant was centrifuged for 10 min at 2,000g. The pellet was washed three times yielding a heavy mitochondrial fraction [designated as (500-2,000)gR^2]. After obtaining the heavy mitochondrial fraction, the supernatant was centrifuged for 10 min at 7,000g. The pellet was washed three times yielding a light mitochondrial fraction [designated as (2,000-7,000)gR^2]. As shown in Fig. 6, the difference in size of mitochondria between the heavy and light mitochondria fractions obtained from the control animals is not remarkable while the one obtained from the experimental animals is distinct. The rate of the oxygen consumption and phosphorylating abilities of mitochondria isolated from hydrazine- and ethanol-treated rat livers were decreased compared to those of mitochondria isolated from the corresponding paired control animals (Table 2) [183, 204]. It should be noted that there is essentially no difference between the (500-2,000)gR^2 and (2,000-7,000)gR^2 fractions obtained from experimental animals in the rate of oxygen consumption, the contents of cytochromes, some enzyme activities, the rate of generation of reactive oxygen species (ROS), and the membrane potential of mitochondria ($\Delta\Psi m$) (Fig. 7, Table 2) [183, 204]. These data strongly suggest that a routine isolation pro-

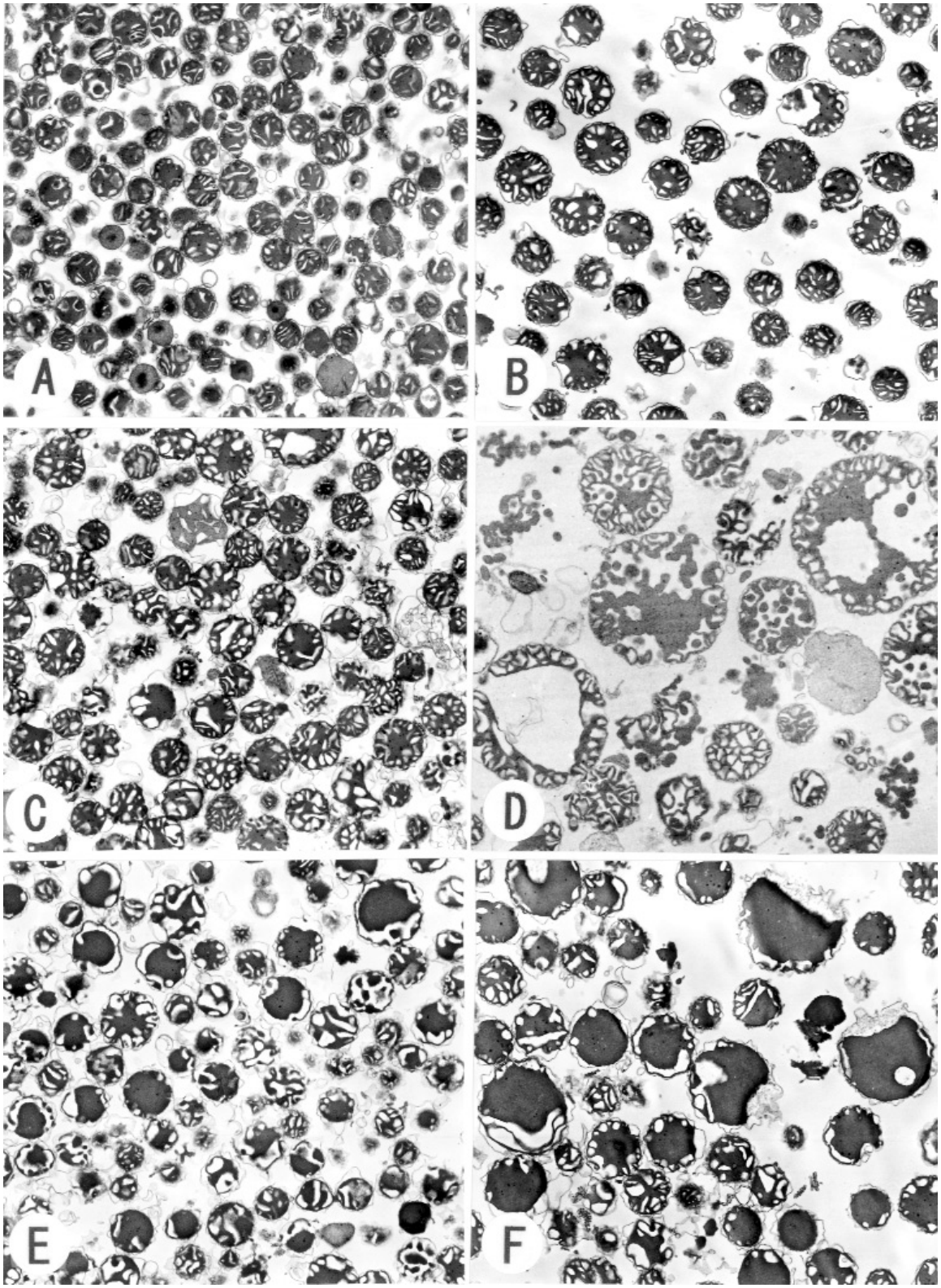


Fig. 6 Ultrastructural appearances of the heavy and light mitochondrial fractions obtained from hydrazine- and ethanol-treated rat livers [modified from 204]. The light and heavy mitochondrial fractions obtained from control animals (A and B, respectively), those obtained from animals treated with a 1% hydrazine-diet for 8 days (C and D, respectively), and those obtained from animals fed with 32% ethanol in drinking water for one month (E and F, respectively) were fixed for electron microscopy. Magnification of electron micrographs: x10,000.

Table 2A Oxygen consumption of mitochondria isolated from hydrazine- and ethanol-treated rat livers after various centrifugation procedures*(cited from 204).

Sources of mitochondria	State 3 (natoms O/mg protein per min)	State 4	RCI	ADP/O	State 3 (natoms O/mg protein per min)	State 4	RCI	ADP/O
Paired control								
(700-7000)gR^2	130.6 ± 4.6	30.4 ± 0.7	4.31 ± 0.23	1.89 ± 0.06	99.9 ± 3.5	20.0 ± 7.2	5.00 ± 0.46	2.82 ± 0.02
(500-2000)gR^2	145.2 ± 3.2	29.0 ± 0.3	4.96 ± 0.26	1.84 ± 0.04	100 6 ± 4.8	21.9 ± 1.8	4.59 ± 0.32	2.72 ± 0.03
(2000-7000)gR^2	134.9 ± 2.5	35.3 ± 0.5	3.82 ± 0.19	1.88 ± 0.02	108.7 ± 2.6	21.9 ± 4.2	4.96 ± 0.43	2.69 ± 0.72
Hydrazine								
(700-7000)gR^2	109.3 ± 3.8^b	32.3 ± 0.2	3.39 ± 0.30	1.66 ± 0.01	88.5 ± 2.8	17.5 ± 0.9	5.06 ± 0.30	2.41 ± 0.06^b
(5000-2000)gR^2	103.1 ± 2.6	31.1 ± 0.6	3.32 ± 0.19	1.65 ± 0.05	91.5 ± 4.9	20.1 ± 1.8	4.55 ± 0.38	2.39 ± 0.08^d
(2000-7000)gR^2	112.3 ± 4.8	33.6 ± 0.5	3.34 ± 0.39	1.67 ± 0.03	85.6 ± 4.0^d	19.6 ± 2.1	4.58 ± 0.29	2.31 ± 0.05
Paired control								
(700-7000)gR^2	121.0 ± 4.6	24.2 ± 1.4	5.06 ± 0.37	1.66 ± 0.04	78.8 ± 3.5	10.8 ± 1.1	7.63 ± 0.96	2.68 ± 0.04
(500-2000)gR^2	118.9 ± 3.3	25.2 ± 0.8	4.71 ± 0.22	1.72 ± 0.05	82.9 ± 2.8	14.0 ± 1.3	5.90 ± 0.20	2.60 ± 0.02
(2000-7000)gR^2	122.4 ± 2.9	24.2 ± 1.2	5.05 ± 0.21	1.64 ± 0.02	84.6 ± 4.0	14.9 ± 2.0	5.68 ± 0.18	2.57 ± 0.03
Ethanol								
(700-7000)gR^2	46.1 ± 3.0^a	11.1 ± 0.8^a	4.19 ± 0.11^d	1.76 ± 0.03	48.9 ± 2.2^a	11.2 ± 0.9	4.42 0.24^b	2.53 ± 0.03
(500-2000)gR^2	50.3 ± 4.8$_a$	12.6 ± 0.5^a	3.98 ± 0.32	1.74 ± 0.04	52.2 ± 1.6^a	11.8 ± 1.0	4.44 ± 0.42^b	2.55 ± 0.05
(2000-7000)gR^2	57.4 ± 2.9^a	14.4 ± 0.6^b	4.00 ± 0.12	1.68 ± 0.02	53.0 ± 3.3^a	11.4 ± 0.5	4.63 ± 0.35^c	2.46 ± 0.01

*Animals were placed on a 1% hydrazine-diet for 8 days or fed with 32% ethanol in drinking water for 2 months. See the text for isolation of various mitochondrial fractions. Data are the averages and the standard error (mean ± SE) of three different experimens. Data on experimental groups are statistically different from those of the corresponding control at: a ($p<0.001$); b($0.001<p<0.01$); c($0.01<p<0.02$); d($0.02<p<0.05$). RCI, respiratory control index; ADP: adenine diphosphate.

cedure for normal mitochondria can be applied to the isolation of MG. The data described above, demonstrating that heavy and light mitochondrial fractions are functionally the same might not be unexpected since mitochondria, apparently separated from each other on one plane of section, are often connected to each other on another plane of section when serial sectioning technique for electron microscopy is employed. Namely, large mitochondria and small ones are connected to each other in the cell (Fig. 8) [204]. Thus, it might be reasonable to assume that the function of a mitochondrial fraction obtained by a routine isolation procedure represents the MG one although some of them might be fragmented during the homogenization procedures and also lost in the residue after the centrifugation of the homogenate at 500g or 700g.

Table 2B Contents of cytochromes in hydrazine- and ethanol-treated rat liver mitochondria obtained by various isolation procedures*(cited from 204).

Sources of mitochondria	Cytochromes (nmol/mg protein)				
	a +a_3	**b**	**c_1**	**c**	**c +c_1**
Paired control					
(700-7000)gR_2'''	0.145 ± 0.009	0.135 ± 0.007	.0106 ± 0.001	0.174 ± 0.012	0.280 ± 0.013
(500-2000)gR_2'''	0.128 ± 0.006	0.142 ± 0.005	0.134 ± 0.004	0.216 ± 0.006	0.350 ± 0.009
(2000-7000)gR_2'''	0.043 ± 0.003	0.072 ± 0.002	0.108 ± 0.003	0.198 ± 0.003	0.308 ± 0.008
Ethanol					
(700-7000)gR_2'''	0.070 ± 0.005[b]	0.088 ± 0.006[a]	0.065 ± 0.006[a]	0.196 ± 0.009	0.271 ± 0.008
(500-2000)gR_2'''	0.020 ± 0.001[a]	0.100 ± 0.001	0.110 ± 0.004	0.219 ± 0.008	0.329 ± 0.009
(2000-7000)gR_2'''	0.022 ± 0.001[b]	0.067 ± 0.002	0.109 ± 0.002	0.196 ± 0.007	0.305 ± 0.004
Paired control					
(700-7000)gR_2'''	0.287 ± 0.018	0.194 ± 0.010	0.115 ± 0.006	0.210 ± 0.007	0.325 ± 0.012
8500-20009gr_2'''	0.278 ± 0.011	0.190 ± 0.008	0.110 ± 0.010	0.212 ± 0.005	0.322 ± 0.005
(2000-7000)gR_2'''	0.266 ± 0.009	0.182 ± 0.008	0.114 ± 0.004	0.213 ± 0.005	0.327 ± 0.011
Hydrazine					
(700-7000)gR_2'''	0.238 ± 0.009	0.180 ± 0.010	0.097 ± 0.004[d]	0.179 ± 0.003[a]	0.276 ± 0.008[b]
(500-2000)gR_2'''	0.240 ± 0.005[d]	0.176 ± 0.011	0.090 ± 0.006	0.176 ± 0.004[a]	0.268 ± 0.006[b]
(2000-7000)gR_2'''	0.211 ± 0.004[c]	0.156 ± 0.009[d]	0.089 ± 0.006	0.171 ± 0.002[a]	0.260 ± 0.003[b]

*Animals were placed on a 1% hydrazine-diet for 8 days or given 32% ethanol in drinking water for 2 months. Variou mitochondrial fractions used were the sam as those described in Table 2A. Data are the averages and standard error (mean ± SE) of three different experiments. Data on experimental groups are statistically different from those of the corresponding control at: a($p<0.001$); b($0.001< p<0.01$); c($0.01<p<0.02$), d($0.02<p<0.05$).

2. Function of MG

Effects of chronic ethanol intoxication on the respiratory rate of hepatic mitochondria have been extensively studied by many investigators (206-210). It is generally agreed that ethanol causes a decrease in state 3 respiration when NADH-linked substrates are used (211). Decreases in the rate of oxygen consumption and lowered phosphorylating ability of MG have also been reported by others in the case of neuromuscular disorders [70, 212-214]. It is also generally agreed that the contents of cytochrome $a+a_3$ and b, especially that of the former, are remarkably decreased in the hepatic mitochondria of rats after chronic ethanol consumption [210, 215, 216].

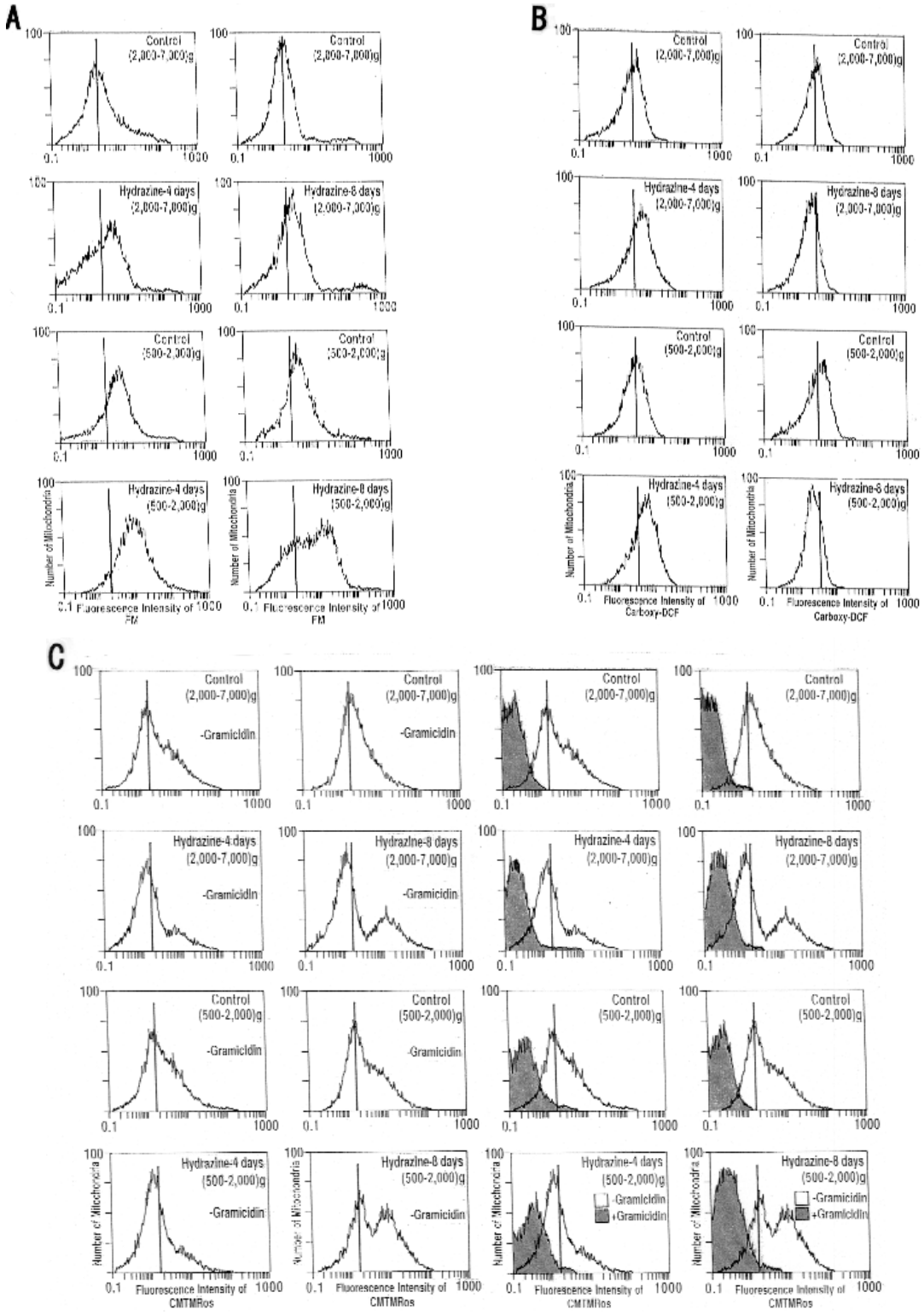


Fig. 7 Flow cytometric analysis on the heavy and light mitochondrial fractions obtained from the liver of rats fed a 1% hydrazine-diet for 4 days and 8 days [modified from 182, 204]. The heavy and light mitochondrial fractions obtained from control and experimental groups were stained with a fluorescent dye Green FM to detect changes in the volume of mitochondria (A), with carboxy-H_2-DCFDA to detect changes in the rate of the generation of ROS from mitochondria (B), and with CMTMRos to detect changes in $\Delta\Psi m$ (C). Note similar trends in heavy and light mitochondrial fractions (both in the control and experimental animals).

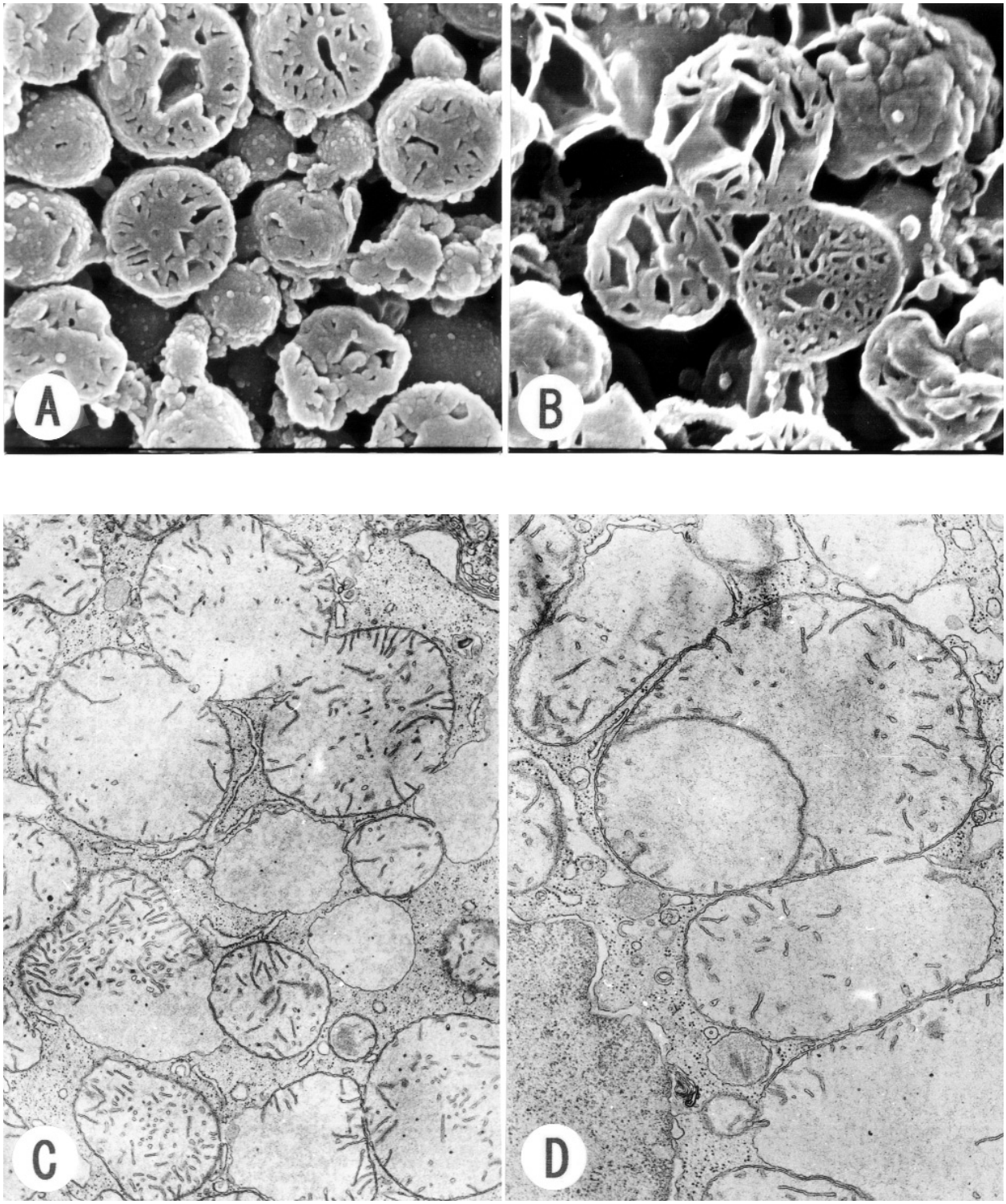


Fig. 8 Ultrastructural aspects of hydrazine-treated rat liver mitochondria [modified from 204]. A and B: Scanning electron micrographs of mitochondria isolated from the livers of the control (A) and those of animals fed a 1% hydrazine-diet for 8 days (B). C and D: Transmission electron micrographs of the liver of the control (C) and treated animals used for Fig. B. Magnification of electron micrographs: A and B, x30,000; C and D, x20,000.

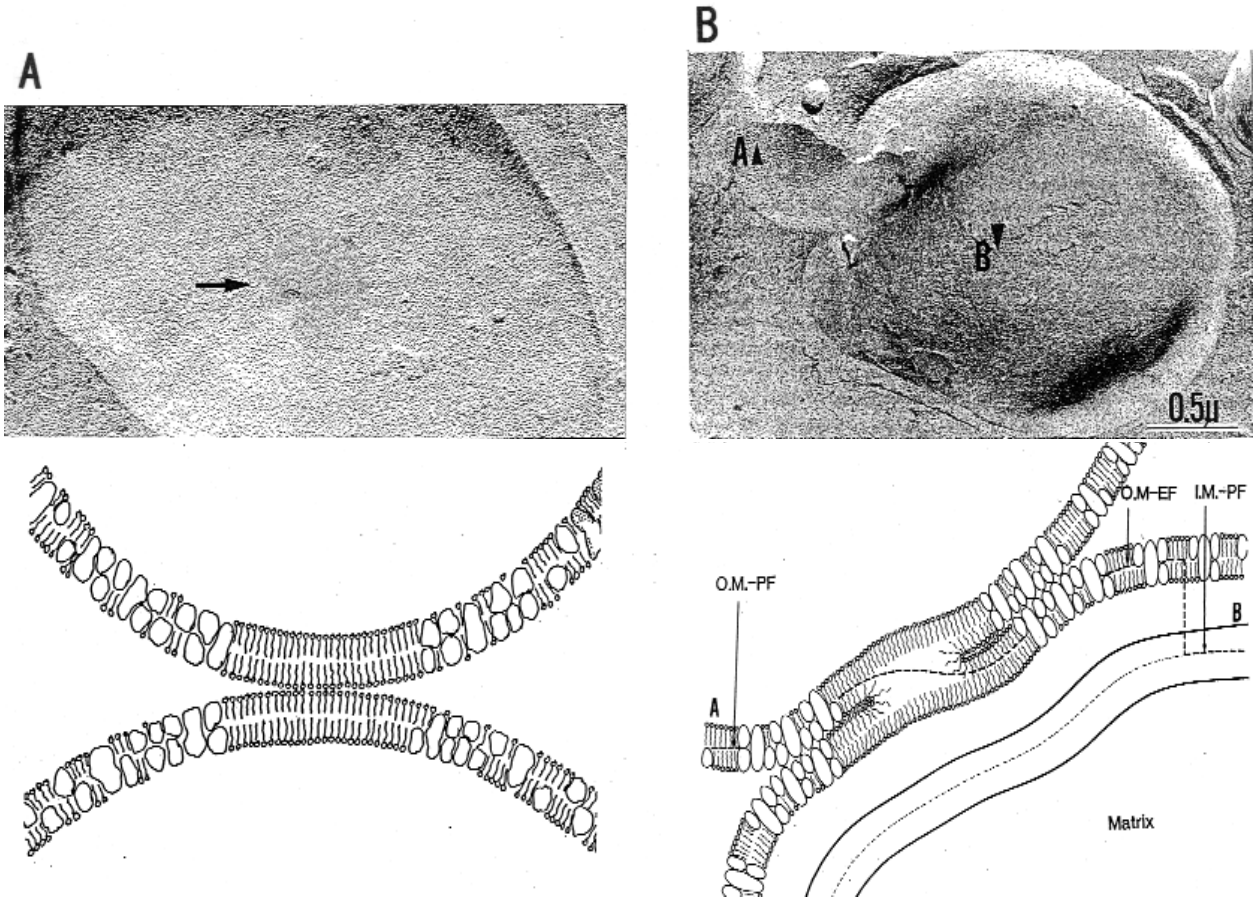


Fig. 9 Freeze-fracture electron micrographs demonstrating particle-free regions in the outer mitochondrial membrane (A) and the fusion of adjacent mitochondrial outer mitochondrial membrane (B) in a hydrazine-treated mouse liver [cited from 160 (A), 159 (B)] A. The electron micrograph was obtained from a mouse fed a 1% hydrazine-diet for 3 days. Schematic presentation (bottom). B. Cluster formation is observed on the protoplasmic face of the outer membrane of one mitochondrion (arrowhead A). The fracture face is continuous on the exoplasmic face of the outer membrane of adjacent mitochondria, followed by the protoplasmic face of the inner membrane (arrowhead B). Cluster formations are again seen on the fracture face of the inner membrane. Schematic presentation (bottom). IM, inner membrane; OM, outer membrane; PF, protoplasmic face; EF, exoplasmic face. Magnification of electron micrographs: A, 34,000; B, 54,600.

III. Mechanisms of the MG formation

1. Membrane fusion as a mechanism for the MG formation

There are several possible mechanisms for the MG formation [157]: swelling or hypertrophy of individual mitochondrion, fusion of adjacent mitochondria and the suppression of the division of mitochondria. Number of mitochondria per cell may not be altered except for the case of fusion. The first possibility is excluded since sizes of MG significantly exceed those caused by a simple swelling. Hypertrophy of mitochondria is another possible mechanism of the formation of MG. However, there is only one report in the literature describing the induction of MG in rat livers by feeding animals with a high-protein diet [217]. Disturbance in the dividing process of mitochondria may result in the formation of MG. Chloramphenicol-induced formation of MG has been accepted as a typical example, since it is known to specifically inhibit protein synthesis in mitochondria. Experimental morphological evidence supporting involvement of the membrane fusion in the processes of the MG formation are:

1) Freeze-fracture studies on the outer membranes of mitochondria isolated from the liver of

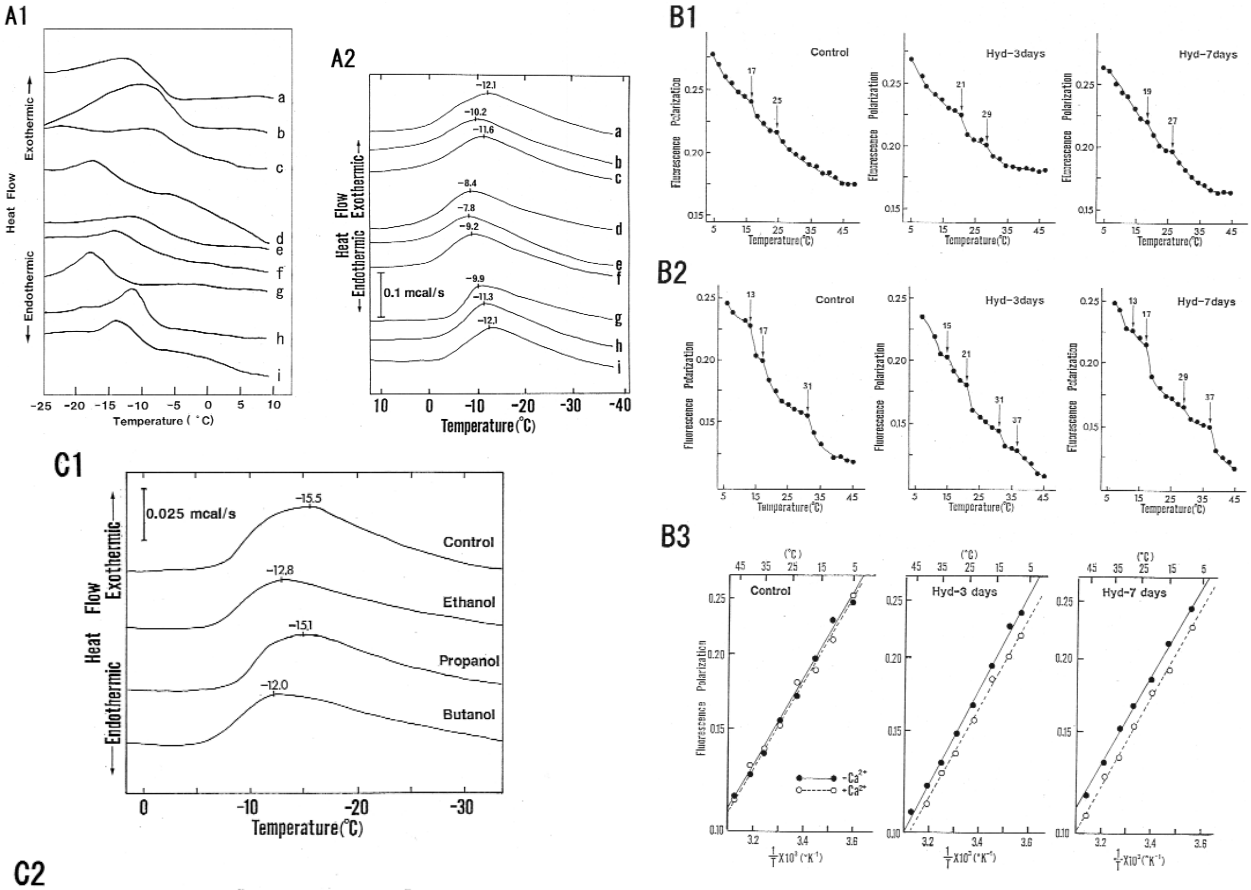


Fig. 10 Changes in physico-chemical properties of mitochondria during the MG formation induced by hydrazine or ethanol. **A1.** Calorimetric cooling curves of hydrazine-fed rat liver mitochondria and mitochondrial membranes obtained from 1% hydrazine-fed rats. Whole mitochondria: a, control; b, hydrazine-3 days; c, hydrazine-7 days. The outer membrane fraction: d, control; e, hydrazine-3 days; f, hydrazine-7 days. The inner membrane fraction: g, control; h, hydrazine-3 days; i, hydrazine-7 days [cited from 222]. **A2.** Calorimetric cooling curves of lipids extracted from mitochondria and submitochondrial membranes obtained from 1% hydrazine-fed rat liver. Whole mitochondria: a, control; b, hydrazine-3 days; c, hydrazine-7 days. The outer membrane fraction: d, control; e, hydrazine-3 days; f, hydrazine-7 days. The inner membrane fraction: g, control; h, hydrazine-3 days; i, hydrazine-7 days [cited from 224]. **B.** Temperature dependence of the fluorescence polarization of DPH in hydrazine-treated rat liver mitochondria and mitochondrial extracted lipids. Liver mitochondria were obtained from control rats, those fed with hydrazine 3 days (hyd-3 days) and those fed with hydrazine for 7 days (Hyd-7 days) [cited from 224]. **B1:** Phase transition curves of liver liver mitochondria of hydrazine-fed rats obtained with the fluorescence polarization of DPH. **B2:** Phase transition curves of lipids extracted from liver mitochondria of hydrazine-fed animals obtained with the fluorescence polarization of DPH. **B3:** Effects of Ca^{2+} on temperature dependence of the fluorescence polarization of DPH in lipids extracted from hydrazine-fed rat liver mitochondria. Experiments were carried out in the absence and presence of 50 mM Ca^{2+}. Values of the fluorescence polarization of DPH are plotted in semilogarithnic scale against $1/T$ x 10^3 (K^{-1}). **C.** Physicochemical properties of ethanol-treated rat liver mitochondria [cited from 223] **C1:** Calorimetric cooling curves of mitochondria were obtained from the liver of rats fed with ethanol, propanol or butanol for 3 months. **C2:** Changes in thermotropic lipid phase transition temperatures of mitochondria and submitochondrial fractions obtained from the liver of rats treated with ethanol for 3 months. *Ti*: initial transition temperature; *Tm*: maximum transition temperasture; *Tf*: final transition temperature.

Table 3A Analysis of lipids on hydrazine-treated rat liver mitochondrial and submitochondrial fractions[a]

	Whole mitochondria			Outer membrane			Inner membrane		
	Control	3 days	7 days	Control	3 days	7 days	Control	3 days	7 days
Cholesterol	3.9 ± 0.3	5.2 ± 0.3[g]	3.3 ±0.2	33.2 ± 3.1	43.0 ± 2.9[g]	37.1 ± 2.5	1.4 ± 0.2	1.5 ± 0.1[g]	1.4 ± 0.1
Total phospholipids[c] (%)	244.5 ±13.5	405.4 ±15.5[d]	231.8 ±9.3	435.0 ±21.7	584.8 ±25.7[d]	517.4 ±10.0[e]	310.0 ±36.2	424.5 ±26.8[c]	315.0 ±10.4
PC	36.7 ± 0.9	29.8 ± 0.9[e]	30.4 ±0.7[e]	44.3 ± 0.7	32.4 ± 0.6[d]	37.7 ± 0.5[d]	32.1 ± 0.6	33.0 ± 0.8	30.7 ± 0.6
PE	28.7 ± 0.7	36.9 ± 1.2[e]	33.4 ±0.9[f]	28.9 ± 0.7	33.2 ± 0.6[e]	32.8 ± 0.7[e]	33.5 ± 0.8	40.6 ± 0.7[d]	35.7 ± 0.4[c]
PI	6.2 ± 0.3	6.3 ± 0.3	7.0 ±0.5	6.8 ± 0.4	8.0 ± 0.4	7.2 ± 0.6	2.3 ± 0.1	2.6 ± 0.1	2.2 ± 0.1
PS	3.2 ± 0.1	7.4 ± 0.3[d]	5.5 ±0.5[e]	1.7 ± 0.1	4.8 ± 0.3[d]	3.4 ± 0.3[e]	1.0 ± 0.1	4.0 ± 0.1[d]	2.8 ± 0.2[d]
CL	14.1 ± 0.3	14.2 ± 0.8	10.9 ±0.8[f]	5.6 ± 0.7	9.1 ± 0.4[e]	5.8 ± 0.4	18.3 ± 0.6	10.9 ± 0.5[d]	15.4 ± 0.9
SM	2.2 ± 0.2	2.4 ± 0.1	2.3 ±0.2	5.0 ± 0.2	4.7 ± 0.3	5.0 ± 0.2	3.0 ± 0.1	3.0 ± 0.1	3.1 ± 0.2
LPC	2.7 ± 0.2	3.7 ± 0.4[f]	2.3 ±0.2	2.1 ± 0.3	3.5 ± 0.3[f]	3.6 ± 0.1[e]	2.7 ± 0.1	3.7 ± 0.3[f]	3.1 ± 0.2
PE/PC	0.97± 0.04	1.24± 0.03[d]	1.09±0.01[d]	0.66± 0.02	1.03 ± 0.03[d]	0.87± 0.03[d]	1.04± 0.02	1.23± 0.04[e]	1.20± 0.06

[a] Mitochondria and submitochondrial fractions were obtained from rats fed a 1% hydrazine diet for 3 days and 7 days. Phospholipid species analyzed were phosphatidylcholine (PC), Phosphatidyethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), sphingomyelin (SM), and lysophosphatidylcholine (LPC). Data on the experimental group are statistically different from those of the control group at d(p,0.001), e(0.001<p<0.01), f(0.01<p<0.02), and g(0.02<p<0.05). [b]μg/mg protein. [c]nmol/Pi/mg protein.

Table 3B. Fatty acid compositions of hydrazine-treated rat liver mitochodrial membranes[a] (cited from 222)

Fatty acids (%)	**Whole mitochondria**			**Outer membrane**			**Inner membrane**		
	Control	**3 days**	**7 days**	**Control**	**3 days**	**7 days**	**Control**	**3 days**	**7 days**
16:0	20.8 ±0.7	11.7 ±1.4[c]	14.7 ±0.4[b]	29.4 ±1.0	18.0 ±0.5[b]	25.4 ±1.6	24.0 ±0.9	21.1 ±1.0	18.8 ±1.0[c]
16:1	1.5 ±0.1	1.1 ±0.1	1.3 ±0.1	3.5 ±0.3	1.6 ±0.2[c]	2.7 ±0.4	4.7 ±0.8	5.6 ±0.3	4.8 ±0.5
18:0	22.3 ±1.0	18.1 ±0.7[d]	24.3 ±0.7	28.7 ±1.9	22.1 ±1.3	22.7 ±1.5[e]	30.8 ±1.1	20.6 ±1.3[b]	32.8 ±0.9
18:1	8.0 ±0.4	10.5 ±1.5[e]	6.0 ±0.4[c]	14.7 ±2.1	23.7 ±0.5[b]	18.9 ±1.5	12.4 ±0.8	13.8 ±0.5	12.0 ±0.6
18:2	16.7 ±0.4	31.8 ±0.7[b]	22.6 ±1.4	6.4 ±0.5	17.9 ±0.4[b]	19.0 ±1.5[b]	16.5 ±1.4	27.1 ±1.5[c]	19.5 ±0.7
18:3	1.8±0.3	0.9 ±0.1[d]	1.4 ±0.1	1.2 ±0.6	0.3 ±0.1[d]	1.0 ±0.2	1.2 ±0.1	0.8 ±0.1[d]	1.2 ±0.1
20:4	21.6 ±0.4	22.1 ±0.7	22.9 ±0.9	17.4 ±0.9	15.0 ±1.1	16.6 ±0.8	9.8 ±0.9	10.9 ±1.0	10.5 ±0.6
22.6	6.4 ±0.3	6.6 ±0.6	7.4 ±0.5	1.2 ±0.1	1.0 ±0.1	1.2 ±0.1	0.9 ±0.1	0.7 ±0.1	0.5 ±0.1[e]
U/S[f]	1.33±0.06	2.36±0.15[b]	1.57±0.04[d]	0.73±0.05	1.51±0.06[c]	1.10±0.06[c]	0.83±0.04	1.38±0.03[b]	0.93±0.05

[a]Mitochondria and submitochondrial fractions were obtained from rats fed a 1% hydrazine diet for 3 days and 7 days. Data are expressed as means ± SE. Data on experimental group are statistically different from the control group at subscripts b(p<0.001), c(0.001<p<0.01), d(0.01<p<0.02), and e(0.02<p<0.05). [f]The ratio of the total amount of unsaturated fatty acids (U) to that of saturated fatty acids (S).

Table 3C Fatty acid compositions of phospholipid classes from hydrazine-treated rat liver mitochondria[a] (cited from 224)

Fatty Acid (%)	PC			PE			PI	
	C	3H	7H	C	3H	7H	C	3H
16:0	25.6 ± 1.6	19.4 ± 2.0*	19.7 ± 0.9**	24.7 ± 1.2	17.2 ± 0.7**	16.2 ± 0.4**	25.7 ± 1.7	20.5 ± 1.2*
16:1	0.7 ± 0.2	0.2 ± 0.1*	0.5 ± 0.1	0.7 ± 0.1	0.4 ± 0.2	1.4 ± 0.3*	0.4 ± 0.2	0.5 ± 0.1
18:0	24.8 ± 0.7	27.0 ± 1.6	31.7 ± 1.4**	32.4 ± 0.6	39.4 ± 1.9*	45.3 ± 1.3**	32.7 ± 2.9	32.8 ± 0.4
18:1	7.4 ± 0.3	5.2 ± 0.4**	4.4 ± 0.2**	5.7 ± 0.2	4.2 ± 0.2**	2.8 ± 0.3**	5.3 ± 0.4	3.9 ± 0.5
18:2	15.0 ± 1.9	17.5 ± 3.6	12.0 ± 0.7	5.6 ± 0.6	4.5 ± 0.7*	2.2 ± 0.2**	12.1 ± 1.3	13.9 ± 0.2
20.4	27.0 ± 0.9	30.5 ± 2.3	31.5 ± 0.7**	31.2 ± 0.8	33.8 ±. 0.9	32.7 ± 1.4	21.2 ± 2.3	29.6 ± 1.0
U/S[b]	1.04 ± 0.09	1.22 ± 0.16	0.93 ± 0.06	0.78 ± 0.05	0.75 ± 0.05	0.65 ± 0.34*	0.75 ± -.05	0.88 ± 0.05

Fatty Acid (%)	PI	PS			CL		
	7H	C	3H	7H	C	3H	7H
16:0	24.5 ± 1.9	24.3 ± 0.6	18.1 ± 0.3**	21.1 ± 2.6	8.2 ± 0.2	8.2 ± 0.3	7.9 ± 0.2
16:1	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	1.2 ± 0.4	1.4 ± 0.6	0.8 ± 0.2
18:0	31.0 ± 1.0	27.1 ± 2.2	20.8 ± 0.7*	19.6 ± 1.2**	7.2 ± 0.3	7.2 ± 0.2	6.9 ± 0.1
18:1	12.4 ± 0.6**	7.4 ± 0.7	10.9 ± 0.5**	9.0 ± 0.3**	16.5 ± 0.7	17.1 ± 0.6	15.9 ± 0.7
18:2	22.6 ± 0.7**	20.7 ± 1.4	42.0 ± 0.7**	41.3 ± 2.3**	62.9 ± 0.9	61.9 ± 0.5	63.9 ± 0.7
20.4	11.3 ± 0.8**	20.1 ± 2.0	8.0 ± 0.8**	8.7 ± 0.7**	4.3 ± 0.1	4.9 ± 0.3	4.5 ± 0.3
U/S[b]	0.85 ± 0.05	0.96 ± 0.11	1.62 ± 0.05**	1.50 ± 0.15**	4.20 ± 0.44	4.72 ± 0.51	5.46 ± 0.32

[a] Mitochondria were obtained from rats fed a 1% hydrazine diet for 3 days (3H) and 7 days (7H). Phospholipid species analyzed were phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin (CL). Data on the experimental group are statistically different from those of the control group (C) at *($0.01 < P < 0.05$) and **($P < 0.01$). [b] The ratio of the total amount of unsaturated fatty acids (U) to that of saturated fatty acids (S).

rats or mice fed with a hydrazine-diet; the lateral separation between the particles and smooth continuum in the fracture face of the outer membrane and cluster formations in the protoplasmic fracture face of the outer membrane (Fig. 9) [159, 160]. According to the current hypothesis on the membrane fusion, intramembrane proteins are pushed aside (cluster formation) when two apposed membrane fuse (218-220);

2) Mitochondria of the liver of rats fed a cuprizone-diet are often connected to each other by narrow stalks (150);

3) A serial-sectioning technique for electron microscopy revealed (in cuprizone-fed mouse

Table 3D Contents of divalent cations in serum and liver fractions of hydrazine-treated rats[a] (cited from 222)

Metal	Samples	Control	Hydrazine	
			3 days	7 days
Ca^{2+}	Serum	2.66 ± 0.17	2.89 ± 0.12	2.58 ± 0.18
	Homogenates	2.56 ± 0.44	2.54 ± 0.51	2.50 ±.0.38
	Mitochondria	14.0 ± 1.8	22.8 ± .2.3[c]	15.2 ± 0.9
Mg^{2+}	Serum	1.57 ± 0.23	1.29 ± 0.42	1.30 ± 0.55
	Homogenates	39.5 ± 5.9	22.1 ± 2.7[c]	34.3 ± 1.6
	Mitochondria	46.9 ± 9.2	38.6 ± 8.5	38.9 ± 2.5
Cu^{2+}	Serum	23.2 ± 0.79	16.5 ± 0.29[b]	18.8 ± 0.45[b]
	Homogenates	0.206 ± 0.036	0.215 ± 0.032	0.316 ± 0.012
	Mitochondria	0.406 ± 0.020	0.529 ± 0.048	0.481 ± 0.015[c]

[a] Rats were fed a 1% hydrazine diet for 3 days and 7 days. Data are expressed as nmol/mg protein for homogenates and mitochondria, nmol/ml for serum (means ± SE; superscripts *b*: $0.001 < p < 0.01$, *c*: $0.02 < ,p < 0.05$).

Table 3E Exothermic phase transition temperatures and enthalpy values for liver mitochondria and submitochondrial fractions obtained from rats fed a 1% hydrazine-diet for 3 days and 7 days[a] (cited from 222)

Fraction	Duration of Hydrazine feeding (days)	*Ti*[f] (°C)	*T*m[g] (°C)	*T*f[h] (°C)	*T*f - *T*i (°C)	Δ*H*
Whole mitochondria	0	-6.4 ± 0.2	-12.8 ± 0.1	-23.8 ± 1.4	17.3 ± 1.5	-0.1526 ± 0.0100
	3	-4.5 ± 0.3[c]	-9.5 ± 0.1[b]	-26.1 ± 0.7	21.6 ± 0.9	-0.1154 ± 0.0067[e]
	7	-3.1 ±.0.1[b]	-8.9 ± 0.3[c]	-19.1 ± 0.1	16.0 ± 0.1	-0.0751 ± 0.0114[b]
Inner membrane fraction	0	-14.3 ± 0.3	-17.7 ± 0.1	-26.7 ± 0.1	12.5 ± 0.4	-0.0583 ± 0.0057
	3	-8.0 ± 0.2	-10.9 ± 0.3[b]	-18.4 ± 0.4	10.5 ± 0.3[d]	-0.0631 ± 0.0011
	7	-9.4 ± 0.4[b]	-12.9 ±0.3[b]	-21.9 ± 0.4[b]	12.8 ± 0.8	-0.0497 ± 0.0052
Outer membrane fraction	0	-12.6 ± 0.7	-17.0 ± 0.6	022.7 ± 0.1	10.1 ± 0.4	-0.0365 ± 0.0011
	3	-5.6 ± 0.1[b]	-12.6 ± 1.0[e]	-22.0 ± 0.4	17.0 ± 0.2[b]	-0.0689 ± 0.0067[e]
	7	-7.7 ± 1.4[e]	-13.5 ± 0.3[d]	-19.8 ± 0.6[e]	12.5 ± 0.2	-0.0343 ± 0.0002

[a]Data are the averages of five different experiments (means ± SE). Data on the experimental group are different from those of the control group at superscripts *b*($p < 0.001$), *c*($0.001 < p < 0.01$), *d*($0.01 < p < 0.02$), and *e*($0.02 < p < 0.05$). The enthalpy value (ΔH) is expressed as cal/g wet wt of each sample. [f]The initial transition temperature. [g]The maximum transition temperature. [h]The final transition temperature.

Table 3F Exothermic phase transition temperatures and enthalpy values for lipids extracted from hydrazine-treated rat liver mitochondria[a] (cited from 224)

Duration of hydrazine treatment (days)	T_i[b] (°C)	T_m[c] (°C)	T_f[d] (°C)	$T_f - T_i$ (°C)	ΔH (cal/g wet wt)
0	2.4 ± 0.7	-13.3 ± 0.6	-31.2 ± 0.6	17.9 ± 0.3	4.1
3	3.8 ± 0.6	-8.8 ± 0.8*	-29.4 ± 0.1	20.5 ± 0.1	5.5
7	5. ± 0.1	-11.1 ± 0.1**	-29.6 ± 0.7	18.5 ±.0.6	4.9

[a]Mitochondria were obtained from rats fed a 1% hydrazine diet for 3 days and 7days averages of five different experiments (means ± SE). Data on the experimental group from those of the control group at *($0.01 < p < 0.02$) and **($0.02 < p < 0.05$). [b]The initial transition temperature. [c]The maximum transition temperature. [d]The final transition temperature.

Table 3G Summary of the changes in DSC thermograms in hydrazine-treated rat liver mitochondria (cited from 224).

	The maximum transition temperature (°C)		ΔH (cal/g wet wt)	
Fraction	**Control**	**3H[a]**	**Control**	**3H[a]**
Extracted lipids[b]	-14.7	-9.5	3.955	5.653
Extracted lipids + Ca^{2+}[c]	-12.7	-8.1	4.618	4.921
Extracted phospholipids	-13.4; -20.4	-10.7; -22.3	3.065	3.552
Extracted phospholipids+ cholesterol	-11.8	- 6.9	3.200	4.815
Mixture of phospholipids[d]	-4.0; -17.0	-1.3; 13.7	0.278	0.600
Mixture of phospholipids[d] +Ca^{2+}	0	3.0	0.305	0.461
PS-PC mixture[e]	-4.6; -15.9	-0.8; -17.0	3.392	5.213
PS-PC mixture[e] + Ca^{2+}	-4.1; -15.7	-0.7; -17.7	3.697	4.166
PE-PC mixture[f]	-7.6; -16.6	-4.9	6.421.	7.261
PE-PC mixture[f] + Ca^{2+}	-5.8	-1.7	6.678	4.823

[a]Rats fed a 1% hydrazine diet for 3 days. [b]Total lipids extracted from mitochondria. [c]Phospholipids after exclusion of cholesterol from lipids. [d]Mixture of artificial phospholipids with compositions similar to those of mitochondria of control animals and to those of 3H-mitocondria. [e]Mixture of PS and PC with PS/PC ratios similar to those of mitochondria of the control animals and to those of 3H-mitochondria. [f]Mixture of PE and PC with PW/PC ratios similar to those of mitochondria of the control animals and to those of 3H-mitochondria.

Table 3H Exothermic phase transition temperatures and enthalpy values for liver mitochondria and submitochondrial fractions from ethanol-, propanol- and butanol-treated rats[a] (cited from 223).

Fraction	Alcohol[f]	Ti[g] (°C)	Tm[h] (°C)	Tf[i] (°C)	Tf –Ti (°C)	ΔH
Whole mitochondria	C	4.9 ± 0.7	-1.6 ± 0.3	-27.8 ± 0.1	22.9 ± 0.7	-0.092 ± 0.021
	E	-4.0 ± 0.5	-12.6 ± 0.2[c]	-18.3 ± 0.4[b]	14.4 ± 0.1[c]	-0.047 ± 0.007[d]
	P	-6.5 ± 0.3	15.5 ± 2.4	-27.5 ± 1.8	21.1 ± 1.5	-0/078 ± 0.013
	B	-4.3 ± 0.3	11.6 ± 0.1[b]	-18.6 ± 1.6[e]	14.3 ± 1.3[e]	-0.036 ± 0.007[c]
Inner membrane fraction	C	-16.2 ± 0.1	-20.2 ± 0.4	-28.2 ± 0.3	12.0 ± 02	-0.050 ± 0.004
	E	-15.5 ± 0.1	-21.7 ± 0.5	-26.1 ± 0.2[e]	11.5 ± 0.8	-0.058 ± 0.010
	P	-14.8 ± 0.1[d]	-19.4 ± 0.1	-28.5 ± 0.1	13.7 ± 0.2[e]	-0.062 ± 0.003
	B	-17.1 ± 0.1[c]	-25.6 ± 0.2[c]	-31.9 ± 0.1[c]	14.8 ± 0.1[c]	-0.060 ± 0.015
Outer membrane fraction	C	-10.9 ± 0.1	-19.5 ± 0.4	-28.2 ± 0.2	17.3 ± 0.1	-0.066 ± 0.006
	E	-11.8 ± 0.1	-16.3 ± 0.1[d]	-23.6 ± 0.3[c]	11.8 ± 0.4[c]	-0.058 ± 0.008
	P	-10.6 ± 0.1	-17.1 ± 0.1[e]	-26.2 ± 0.3[e]	15.6 ± 0.4[e]	-0.048 ± 0.004[e]
	B	- 7.9 ± 0.2[c]	-16.5 ± 0.3[e]	-21.3 ± 0.3[c]	13.4 ± 0.5[c]	-0.049 ± 0.003[e]

[a]Animals were given 32% ethanol, 32% propanol or 6.9% butanol in drinking water for 3 months. Data are averages and standard error (S.E.) for 5 different experiments.Values are significantly different from those of the control at *b*($p<0.001$), *c*($0.001<p<0.01$), *d*($0.01<p<0.02$), and *e*($0.02<p<0.05$). The enthalpy value (*ΔH*) is expressed as cal/g wet wt of each sample. [f]C, control; E, ethanol; P, propanol; B, butanol. [g]Initial transition temperature. [h]Maximum transition temperature. [i]Final transition temperature.

liver) that mitochondria separated from each other on one plane are connected to each other on different planes of section indicating that mitochondria are connected to each other in the process of MG formation [191], and

4) A distinct decrease in the number of mitochondria per cell in hydrazine-treated rat liver has been observed [181].

We have presented a body of evidence to demonstrate that distinct physico-chemical and biochemical changes in the mitochondrial membranes take place during MG formation induced by ethanol, chloramphenicol and hydrazine, all favorable for the membrane fusion [159, 209, 221-225]. Biochemical an physico-chemical changes occur in mitochondrial membranes of liver cells after 2-3 days of treatment with hydrazine, when MG are not yet induced [222-230]. These changes include: increases in the relative amount of phosphatidylethanolamine and acidic phospholipids subclasses; increases in the ratio of unsaturated to saturated fatty acids in phospholipid domains of mitochondrial membranes; increases in the content of Ca^{2+} in mitochondria; and increases in the membrane fluidity of mitochondrial membranes (Fig. 10, Table 3). Similar results were also observed in ethanol-, propanol- and butanol-induced MG formation [223]. Thus, we may deduce from these data that the membrane fusion plays a key role in the mechanism of the MG formation, although a possible role of the suppression of the dividing pro-

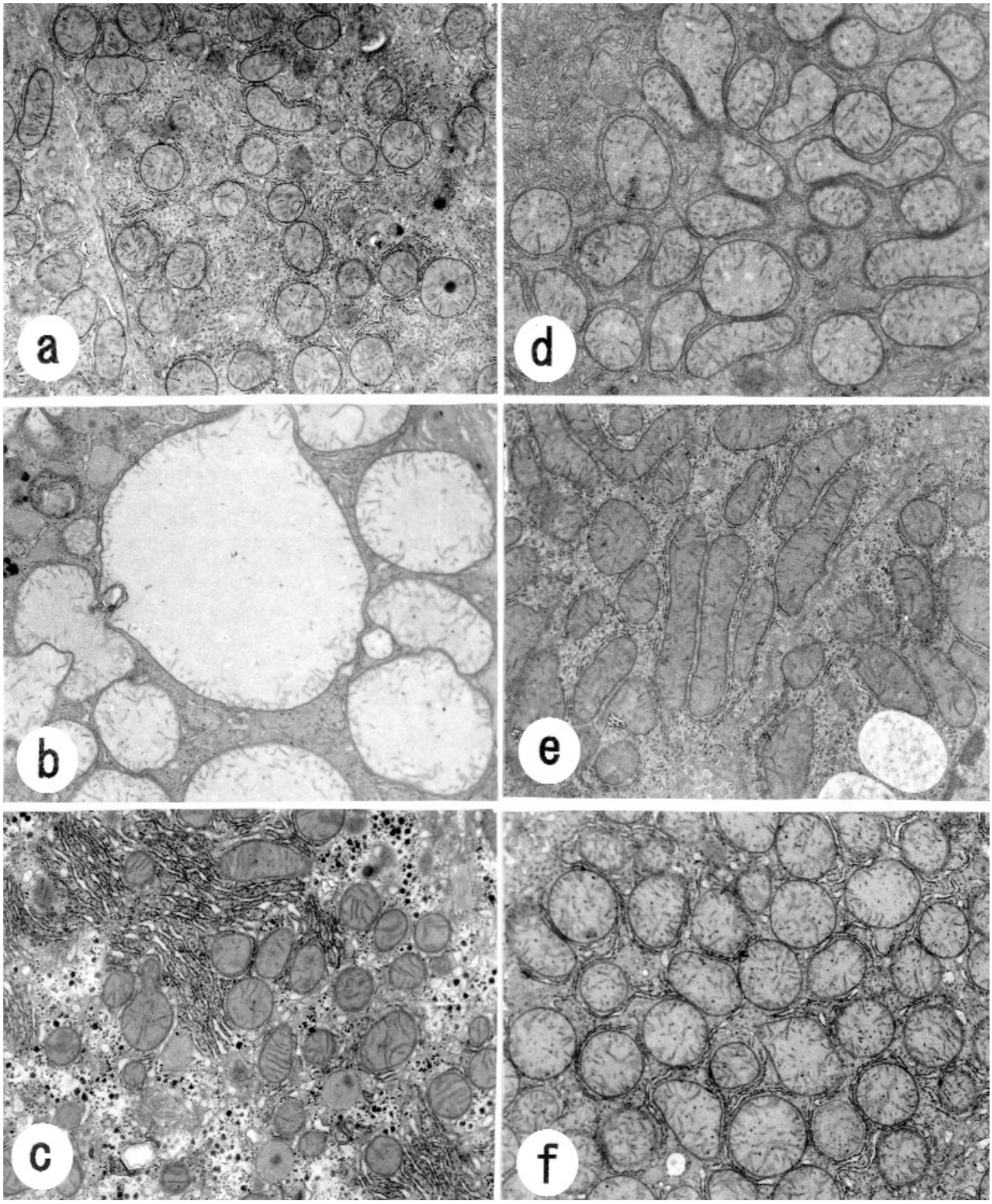


Fig. 11A. Suppression of hydrazine- and ethanol-induced MG formation and enhanced lipid peroxidation by scavengers of free radicals. **(b-f).** Electron micrographs of the liver of rats fed a 1% hydrazine-diet for 8 days in the absence (b) and presence of a combined treatment with 4-OH-TEMPO (c), CoQ_{10} (d), α-tocopherol (e) or allopurinol (f). Control: **(a).** 4-OH-TEMPO and allopurinol were given to the animals by subcutaneous injection (5 mg/100 g of body weight), α-tocopherol (70 mg/100 g of body weight) and CoQ_{10} (10 mg/100 g of body weight) by ip daily during the course of the experiments Magnification of electron micrographs: x12,000.

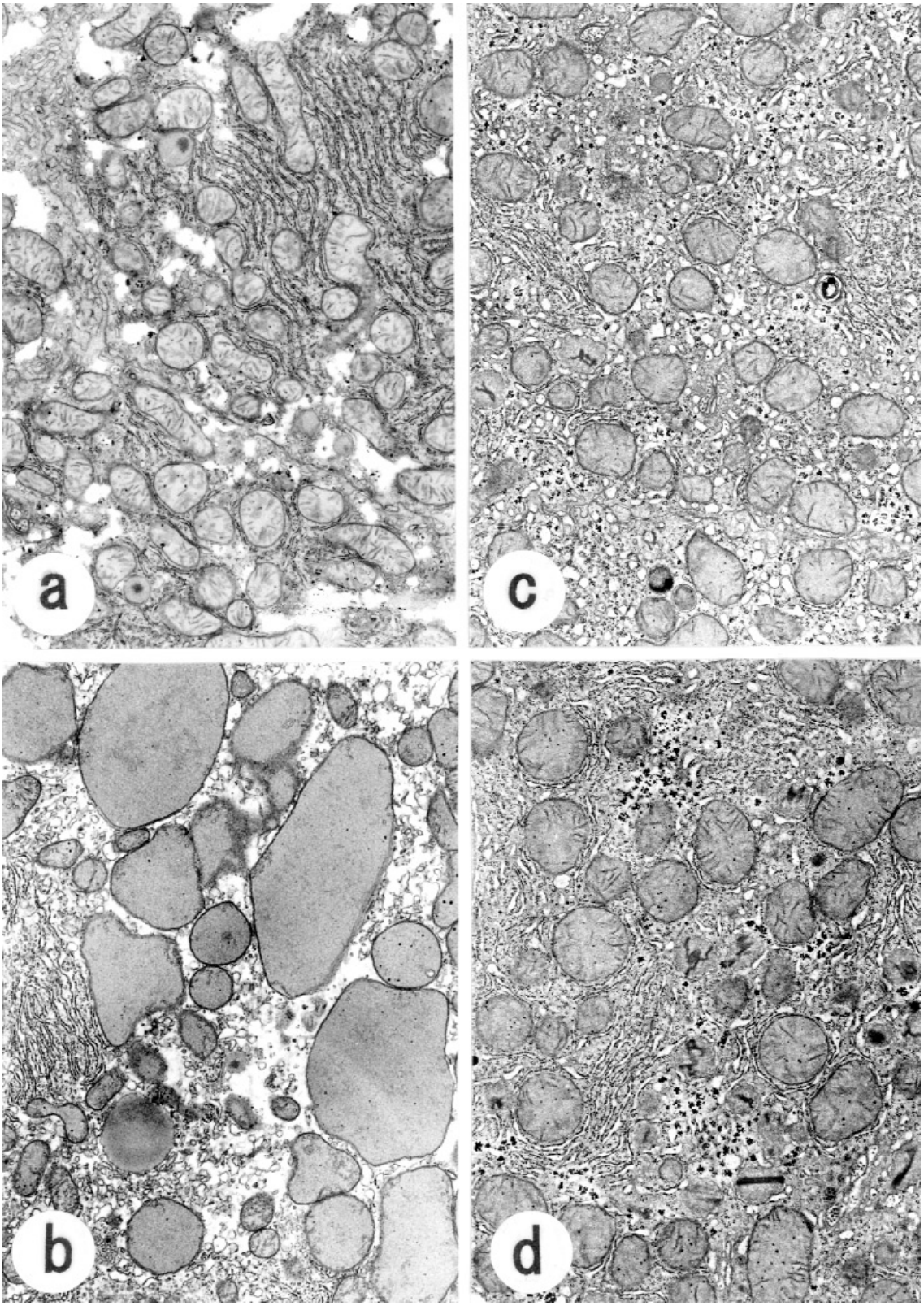


Fig. 11B. Electron micrographs of the liver cells in rats given 32% ethanol in a drinking water four weeks in the absence (b) and presence of a combined treatment with 4-oH-TEMPO (c) or allopurinol (d). Control: (a). 4-OH-TEMPO and allopurinol were given every other day. Magnification of electron micrographs: x10.000.

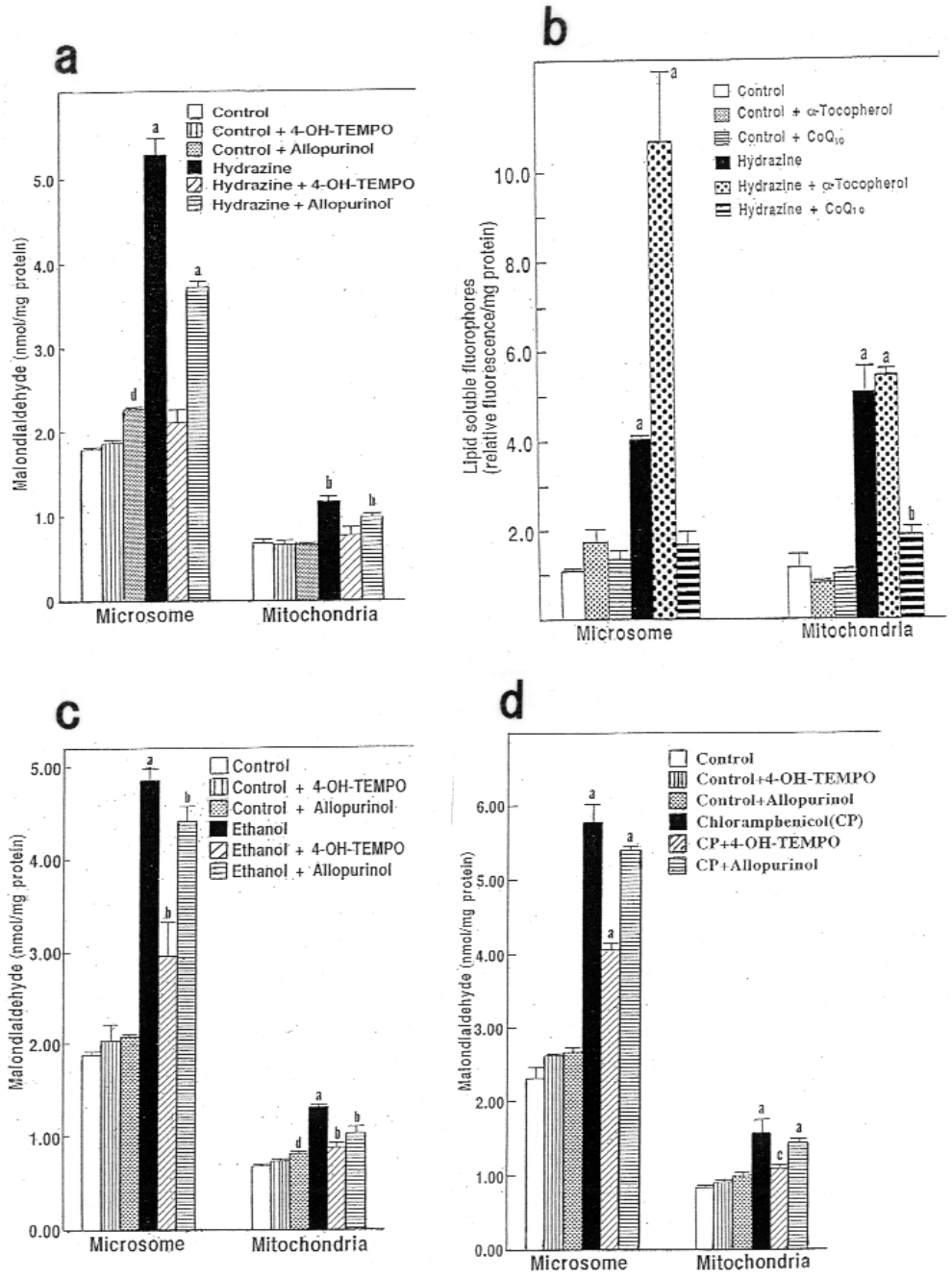


Fig. 11 C. Effects of scavengers of free radicals on enhanced lipid peroxidation in mitochondria and microsomes obtained from the livers of rats treated with hydrazine (a, b), ethanol (c) or mice treated with chloramphenicol (CP) (d) in the presence and absence of scavengers of free radicals. Treatment of animals with hydrazine or ethanol are the same as those described in the legend to fig. 11A.

cess of mitochondria cannot be excluded. Then, a question may arise: what is the mechanism by which the mitochondrial membranes undergo such enormous biochemical and physicochemical changes favorable for the membrane fusion?

2. Free radicals and MG formation

The first trial to suppress the MG formation was done by coenzyme Q_{10} (CoQ_{10}) using hydrazine-induced MG (226, 230). The original idea was

Table 4 Effects of 4-OH-TEMPO and allopurinol on changes in coupling efficiency of mitochondria of ethanol-treated rat livers[a] (cited from 225)

Animals	Succinate			
	state 3 (natoms O/mg	state 4 prot/min)	RCI	ADP/O ratio
Control	132.5 ± 6.7	27.1 ± 1.6	4.99 ± 0.57	2.04 ±0.03
Control + 4 -OH-TEMPO	115.5 ± 0.1	24.0 ± 1.2	4.84 ± 0.24	1.84 ± 0.05
Control + Allopurinol	114.9 ± 2.6	23.0. ± 2.0	5.09 ± 0.35	1.84 ± 0.05
Ethanol	68.9 ± 1.7[c]	14.6 ± 0.3[c]	4.93 ± 0.18	1.60 ± 0.03[b]
Ethanol + 4 -OH –TEMPO	72.4 ± 5.3[c]	15.4 ± 0.5[c]	4.68 ± 0.23	1.86 ± 0.05
Ethanol + Allopurinol	78.2 ± 5.9[c]	15.3 ± 2.5[e]	5.38 ± 0.60	1.99 ± 0.04

[a]Rats were given 32% ethanol in a drinking water for four weeks. 4-OH-TEMPO or allopurinol was given to the animals every other day by subcutaneous injection during the course of the experiment (5 mg/100 g of body wt). Data are the averages and standard error (mean ± SE) of three different experiments. Values in experimentalgroups are statistically different from those of the control at *b*($p<0.001$), *c*($0.001<p<0.01$), *d*($0.01<p<0.02$), *e*($0.02<p<0.05$).

based on the assumption that when cells are exposed to hydrazine mitochondria may be forced to consume more oxygen to deal with extra electrons released from hydrazine. As a result, relative hypoxia in the cell may be induced. Hypoxia and anoxia are known to induce MG in heart and skeletal muscles (169-176). Thus, relative hypoxia possibly induced by hydrazine may cause the formation of MG. If this is the case, externally added CoQ_{10} could suppress the hydrazine-induced MG by receiving extra electrons released from hydrazine. Actually, CoQ_{10} successfully prevented the hydrazine-induced formation of MG in rat livers [226, 230]. Another possible explanation for the effectiveness of CoQ_{10} in the prevention of hydrazine-induced MG formation is its scavenging action on free radicals. It has turned our that lipid peroxidation was significantly and invariably enhanced during the MG formation induced by hydrazine, ethanol and chloramphenicol [225-230]. Scavengers for free radicals such as TEMO and α-tocopherol were found to be effective in suppressing the enhanced lipid peroxidation and preventing the MG formation (Fig. 11) [225-230]. Also, allopurinol, a xanthine oxidase inhibitor, was found partly effective in suppressing the MG formation [227]. Furthermore, 4-hydroxy-2,2,6,6-tetramethyl-piperidin-1-oxyl (4-OH-TEMPO) has been shown to improve decreased coupling efficiencies of mitochondria caused by ethanol intoxication (Table 4) [225]. Enhanced mitochondrial lipid peroxidation in liver of rats or mice treated with inducers of MG was suppressed by free radicals scavengers.

Recently, we have established an *in vitro* model to study the mechanism of the MG formation using cell cultures from various sources and inducers of free radicals [180]. The MG formation induced by chloramphenicol in RL-34 cells was successfully suppressed by cycloheximide or 4-OH-TEMPO [184]. However, the question of how free radicals modify the mitochondrial membranes favoring for the membrane fusion still remains to be addressed.

IV. Pathophysiological meaning of the megamitochondria formation

A clue to the answer to the question of the pathophysiological meaning of MG formation came from the studies on culture cells. When cells of various cell lines are cultured with a variety of chemicals that are known to directly or indirectly

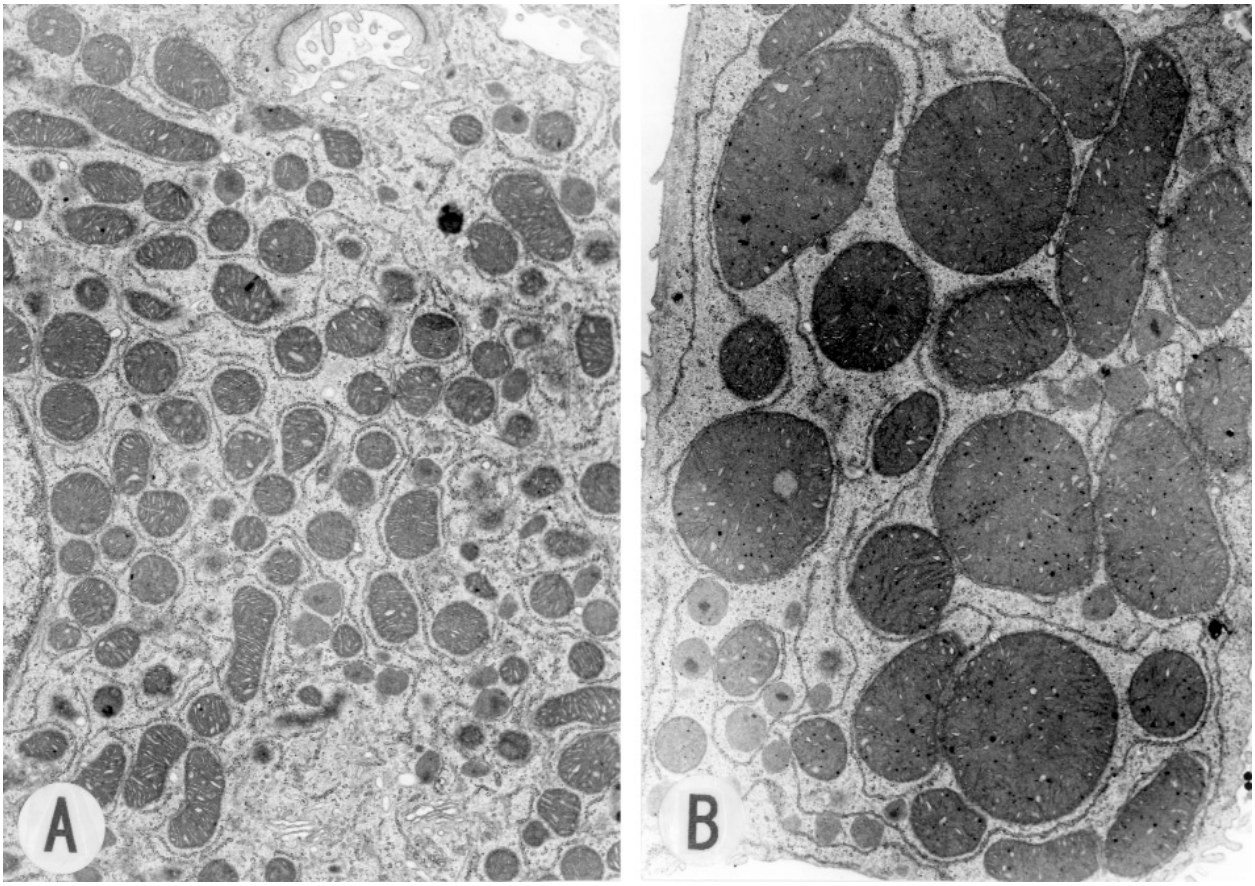


Fig. 12 Electron micrographs of hydrazine-induced formation of MG in isolated rat hepatocytes [cited from 181]. Hepatocytes were cultured for 22 h in the absence (A) or presence (B) of 2 mM hydrazine. Magnification of electron micrographs: x10,000.

generate free radicals, MG are invariably induced. Filamentous mitochondria stained with a fluorescent dye CMXRos in control cells become granular [181]. Electron microscopic examination of these enlarged granular mitochondria revealed by confocal microscopy shows that they are actually MG. For example, mitochondria in isolated rat hepatocytes treated with hydrazine for 22 h become extremely enlarged with distinct decreases in their number per cell (Fig. 12). When the cultivation time of the cells with inducers of free radicals is prolonged for 72 h, nuclei of the cells become condensed to various degrees which are characteristic of apoptotic cells (Fig. 13) (181, 184). In the case of chloramphenicol, 49% of RL-34 cells treated with the chemical for 72 h become apoptotic (184). Apoptotic changes of the cells are evidenced by the ladder formation, PI staining of the nucleus, changes in the membrane potential of mitochondria ($\Delta\Psi m$) and flow cytometric analysis on cell cycle (Fig. 14). (181). All these data have shown that a certain population of cells become apoptotic in the presence of MG. Then, a question arises: What is the correlation between the phenomenon of the MG formation and succeeding apoptotic changes of the cells? To answer this question, a series of events which takes place during and after the formation of MG has been extensively studied both *in vitro,* using culture cells, and *in vivo,* using animals, paying special attention to several key events which might be intimately related to the initiation of apoptosis: structural changes of MG, changes in $\Delta\Psi m$, those in the content of cytochromes of mitochondria,

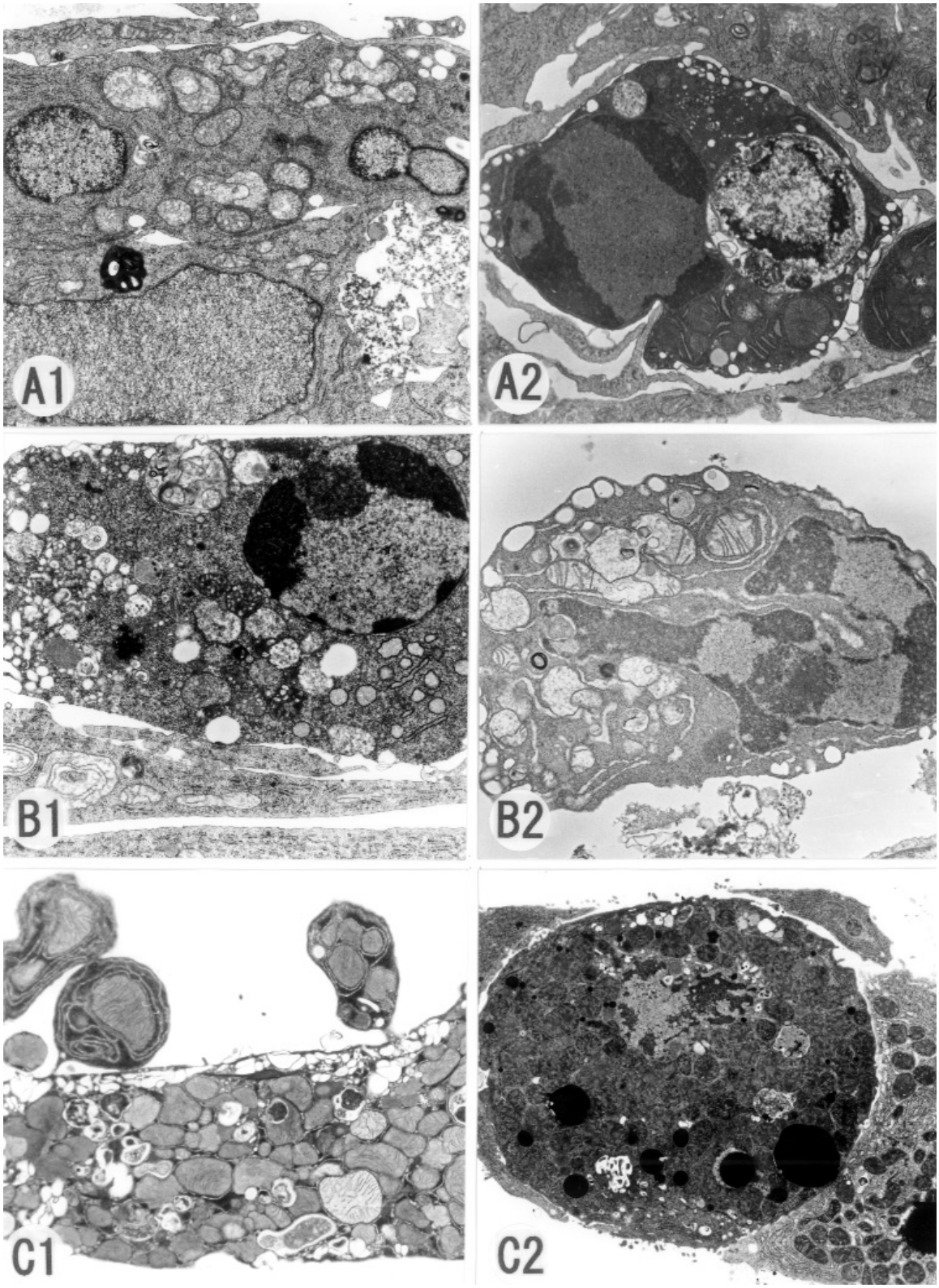


Fig. 13 Electron micrographs demonstrating apoptotic changes of culture cells treated for 72 h with inducers of free radicals [modified from 2, 181] A1, A2: chloramphenicol (CP) -treated RL-34 cells; B1, B2: CP-treated IAR-20 cells; C1, C2: hydrazine-treated rat hepatocytes. Concentrations of chemicals: CP, 300 μg/ml; hydrazine, 2 mM. Magnifications of electron micrographs: A, B: x10,000; C, X5,500.

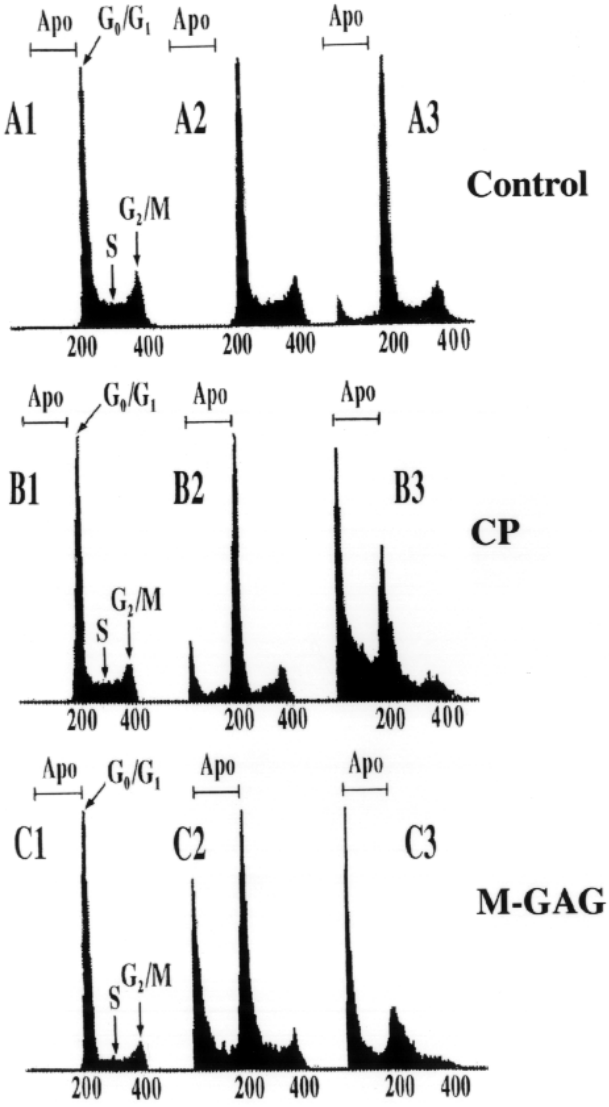


Fig. 14 Cell cycle analysis on CP- and M-GAG-treated RL-34 cells [cited from 181]. Cells were cultured in the absence (A1-A3) or presence of CP (300 μg/ml) (B1-B3) or M-GAG (50 μg/ml) (C1-C3). Cultivation time: 24 h (A1, B1, C1); 48 h (A2, B2, C2); 72 h (A3, B3, C3).

especially that of cytochrome c, which activates caspases, and those in the intracellular level of ROS and the rate of the generation of ROS from MG. The series of events occurring both *in vivo* and *in vitro* in the cells treated with various kinds of free radical-generating chemicals for various lengths of time are summarized as follows: (1) When MG are newly formed, their matrix density is similar to that of normal mitochondria, whereas they become pale with time, indicating that they become secondarily swollen. For example, when RL-34 cells are cultured for 22 h in the presence of chloramphenicol, MG with a similar density to mitochondria of the control cells are detected. When they are further exposed to the chemical for additional 12-24 h, MG with a pale matrix are detected, indicating that they become secondarily swollen (Fig. 15A, a-c). MG with a condensed matrix and those with a pale matrix are obtained in the liver of mice fed a chloramphenicol-diet for 9-10 days and 14-15 days, respectively (Fig. 15A, d-f). Similarly, MG with a condensed matrix are also obtained in the liver of rats treated with a hydrazine-diet for 4-5 days, whereas those with a pale matrix are obtained when animals are treated with the toxic diet for 8-9 days (Fig. 15A, g-i) [182, 183]; (2) The formation of MG is preceded by remarkable increases in the intracellular levels of ROS. Intracellular levels of ROS are remarkably elevated in the cells endowed with MG with a condensed matrix, and they become further increased in the cells with MG with a pale matrix. The rate of the generation of ROS from MG with a condensed matrix is increased, whereas it is decreased in MG with a pale matrix as compared to that of the control mitochondria (Fig. 15B, a, c) [182-184]. (3) $\Delta\Psi m$ of MG with a condensed matrix remains in the same level as that of the control mitochondria or sometimes slightly decreased, whereas that of MG with a pale matrix is distinctly decreased compared to that of the control mitochondria (Fig. 15B, b, d) (182-184); (4) The content of cytochrome c in MG with a condensed matrix remains at a similar level to that of the control mitochondria whereas the cytochrome $a+a_3$ content of MG with a pale matrix becomes distinctly decreased (Table 5).

In physiological conditions, enzymatic and non-enzymatic defense systems against free radicals work effectively within the cell, so that cells are devoid of free radical-mediated various injuries. However, when the level of free radicals exceeds a certain level, mitochondria become enlarged to various degrees lowering the rate of the oxygen consumption and phosphorylating abilities. Phosphorylating abilities and oxygen consumption of MG induced by various experi-

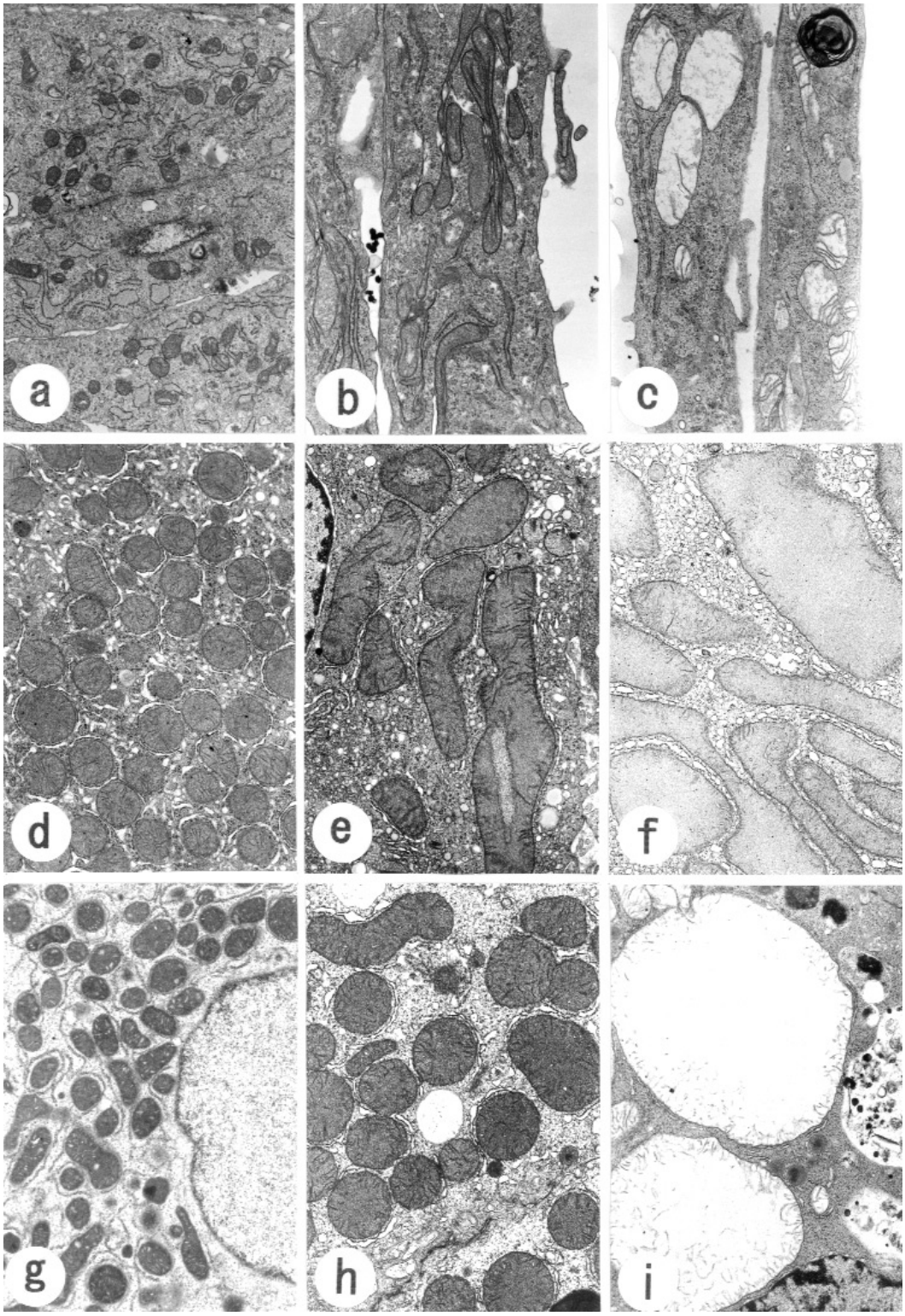
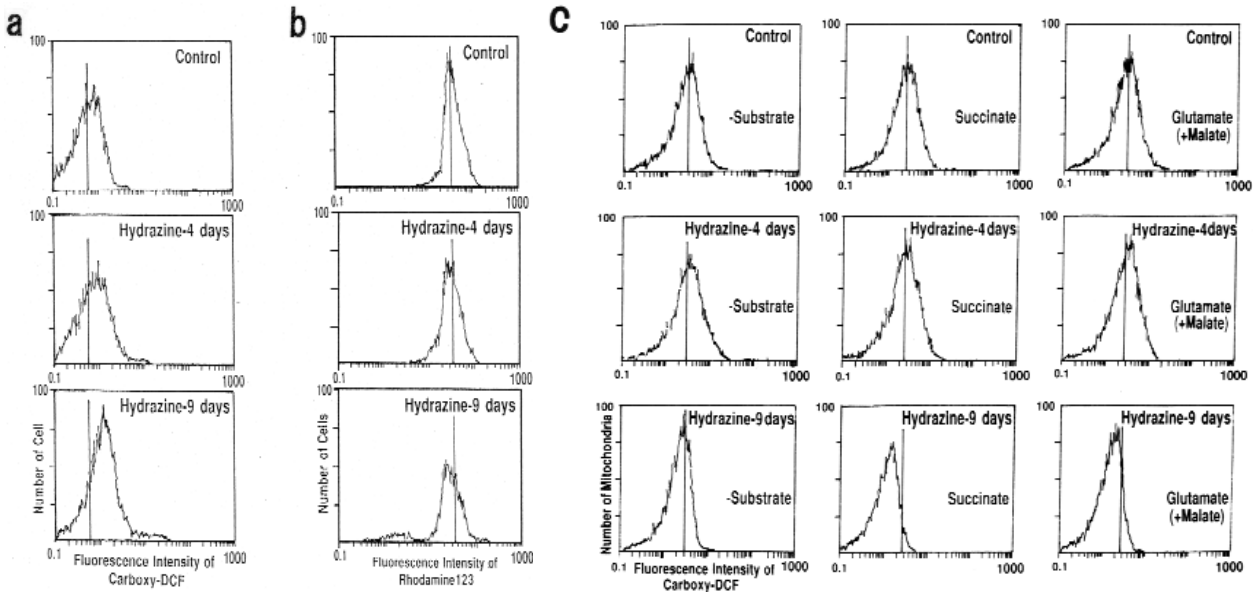


Fig. 15A Morphological and functional differences between MG with a condensed matrix and those with a pale matrix due to swelling. Electron micrographs demonstrating MG with a condensed matrix and those with a pale matrix [modified from 182, 183]. RL-34 cells were culture for 22 h (b) or 48 h (c) in the presence of CP (300 μg/ml). Control: (a). The liver was obtained from mice fed with 2% chloramphenicol-diet for 9-10 days (e) or 14-15 days (f). The liver was obtained from rats fed with a 2% hydrazine-diet for 4 days (h) or 9 days (i). Control: (g). Magnifications of electron micrographs: X10,000. Comparison of the rate of the generation of ROS and $\Delta\Psi m$ between MG with a condensed matrix and those with a pale matrix [cited from 184].



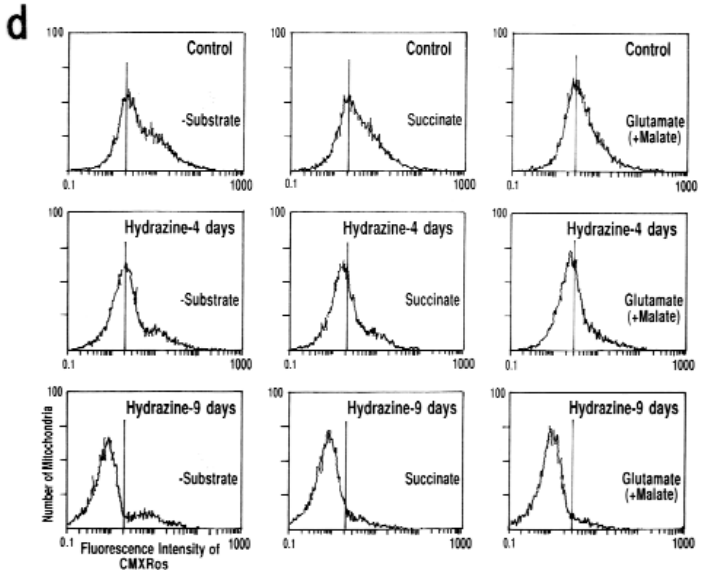
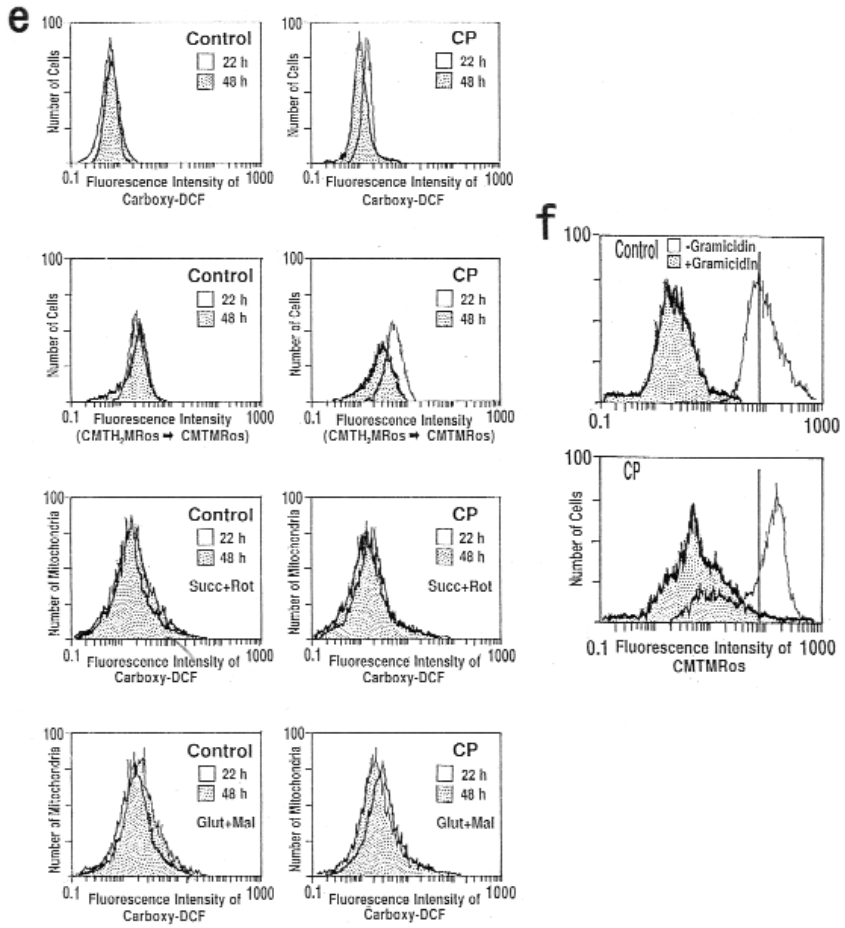


Fig. 15B Hepatocytes and mitochondria were isolated from the liver of rats fed a hydrazine-diet for 4 days and 9 days, respectively, and intracellular levels of ROS (a) and of ΔΨm (b), the rate of the generation of ROS from mitochondria (c) and ΔΨm (d) were measured. Carboxy-H_2-DCFDA was used to measure ROS. CMTMRos and rhodamine 123 were used to measure the intracellular levels of ΔΨm and ΔΨm, respectively. RL-34 cells were cultured for 22 h and 48 h, respectively, in the presence and absence of CP (300 μg/ml). The overall intracellular level of ROS was measured using carboxy-H_2-DCFDA (e). The extracellular generation of ROS was measured using $CMTH_2MRos$. Mitochondria were isolated from RL-34 cells cultured for 22 h and 48 h, respectively in the absence and presence of CP (300 μg/ml) (e). The ratew of the generation of ROS was measured using carboxy-H_2-DCFDA. Oxidizable substrates used were succinate or glutamate (+malate) (e). Intracellular overall ΔΨm of hepatocytes cultured for 48 h in the presence and absence of CP (300 μg/ml) was measure using CMTMRos in the presence and absence of gramicidin (50 μg/ml).

mental conditions or obtained from human biopsied materials are often lower to various degrees compared to those of the control, as mentioned above. Mitochondrial respiration accounts for about 90% of cellular uptake and 1-2% of the oxygen consumed is converted to ROS [231, 232]. MG may generate less amounts of ROS because of lower rates of respiration. Cells then stop growing due to a shortage of ATP supply from MG. It has been shown previously that the

Fig. 15B cont'd



intracellular level of ATP in hepatocytes and the ability of MG to synthesize ATP are actually remarkably decreased in rats treated chronically with ethanol (233). If free radicals are removed from the cell, MG may return to normal functionally and structurally. However, if cells are further exposed to excess amounts of free radicals, swelling of MG proceeds, resulting in further

Table 5 Changes in the contents of cytochromes in hydrazine-treated rat liver mitochondria[a] [cited from 182]

	Cytochromes (nmol/mg/protein)				
Condition	**a + a3**	**b**	**c1**	**c**	**c + c1**
Control	0.287 ± 0.018	0.194 ± 0.010	0.115 ± 0.006	0.210 ± 0.007	0.325 ± 0.012
Hyd 4 –5 days	0.254 ± 0.020	0.188 ± 0.016	0.100 ± 0.002	0.205 ± 0.008	0.305 ± 0.008
Hyd 8 –9 days	0.238 ± 0.009[c]	0.180 ± 0.006	0.095 ± 0.004[d]	0.179 ± 0.003[a]	0.274 ± 0.008[b]

[a]Mitochondria were isolated from the liver of rats given a 0.5% hydrazine-diet for 4-5 days and 8-9 days, respectively. Values are expressed as nmol/mg protein. Each value is the expressed as mean ± SE of five different determinations in animals. Data on experimental animals are statistically different from those of the control animals at *b*($p<0.001$), *c*($0.001<p<0.01$), *d*($0.01<p<0.02$), *e*($0.02<p<0.05$)

decreases in ΔΨm which in turn causes remarkable decreases in the intracellular level of ATP. Cytochrome c and AIF released from swollen MG may activate caspases, and cause nuclear condensation and DNA fragmentation, which characterize apoptotic changes of the cell. Free radicals do not always induce the formation of MG: if the intracellular levels of free radicals exceed those which cells can accommodate themselves to, cells may become necrotic with forming MG. It is well known that MG are formed in various tissues of cold-blooded animals during hibernation [234]. Mitochondria in the cell of various tissues of such animals are required to generate a certain amount of ATP, which is sufficient for the maintenance of the basal metabolism to keep animals alive during hibernation. Therefore, there may be no need for mitochondria in the cell of hibernating animals to respire actively resulting in the generation of large amounts of ROS. If this is the case, the MG formation could be regarded as an adaptive process to unfavorable environments at a level of subcellular organelles.

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