Acta Medica Okayama

Volume 22, *Issue* 1 1968 *Article* 2

FEBRUARY 1968

An electron cytochemical demonstration and biochemical analysis of adenosine triphosphatase activity in cancer cell plasma membrane

Kimikazu Koshiba*

*Okayama University,

An electron cytochemical demonstration and biochemical analysis of adenosine triphosphatase activity in cancer cell plasma membrane*

Kimikazu Koshiba

Abstract

1. The studies of structure and function of the plasma membranes of cancer cells is extremely important for the elucidation of specificity of phenotypes of cancer cells. In order to bring this subject to light, plasma membranes, mitochondria, microsomes and nuclei have been isolated from the AH 130 ascites carcinoma cells and rat liver cells. The electron cytochemical observations and biochemical aays of M g^2+-Na+-K+-ATPase, ADPase, AMPase, and β-glycerophosphatase activities have been carried out before and after the fixation with glutaraldehyde. 2. M g^2+-ATPase and Mg^2+-N a +-K +-ATPase are present in the isolated plasma membranes, mitochondria and microsomes in both AH 130 cells and rat liver cells. ADPase and AMPase of the mitochondria and microsomes show far lower activities than those of the corresponding enzymes found in rat liver plasma membrane. ADPase and AMPase of AH 130 cell fraction exhibit activity much lower or zero. Generally, enzymatic activity of the AH 130 cell fraction is much lower than that of rat liver cell fraction. 3. Mg^2+-Na+-K+-ATPase is completely abolished by 5% glutaraldehyde fixation while it shows le effect on Mg^2+-ATPase in the plasma membrane. ADPase and AMPase activities of the mitochondria and microsomes are completely inhibited by glutaraldehyde fixation. AMPase of the plasma membrane of rat liver is completely abolished while ADPase activity is not affected in any way. 4. Only Mg^2+-ATPase can be demonstrated electron cytochemically. Cytochemical reaction products of Mg^2+-ATPase are located at the outer layer of the plasma membrane of the AH 130 cells and rat liver tiue. In the isolated membrane fractions it is located at the inner layer. 5. ρ-Chloromercuribenzoate has only a slight effect on Mg^2+-ATPase and Mg^2+-Na+-K+-ATPase activities of the rat liver membrane, while it inhibits these enzyme activities in the AH 130 cell membrane. NaF (1 mM) and NaN3 (1 mM) inactivate ADPase of the rat liver plasma memo brane. 6. In these experimental conditions, nonenzymatic hydrolysis of ATP by lead ions is not recognized. 7. It seems most reasonable to conclude that cytochemical electron microscopic demonstration of Mg^2+-ATPase after fixation with glutaraldehyde may serve as the absolute marker for the plasma membrane of ascites hepatoma and liver cells.

---

*PMID: 4305234 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 22, 11—36 (1968)

AN ELECTRON CYTOCHEMICAL DEMONSTRATION AND BIOCHEMICAL ANALYSIS OF ADENOSINE TRIPHOSPHATASE ACTIVITY IN CANCER CELL PLASMA MEMBRANE

Kimikazu KOSHIBA

Department of Biochemistry, Cancer Institute, Okayama University Medical School, Okayama, Japan (Director: Prof. T. Oda)

Received for publication, October 20, 1967

Characterization of the structure and function of plasma membrane of cancer cells is extremely important for the elucidation of specificity of phenotypes of cancer cells. In order to dissolve this problem, it is necessary to isolate purified plasma membrane. However, it may be said that valid markers for the plasma membrane have not been found yet. Thus, the isolation and purification of plasma membrane have not been fully appreciated. Adenosine triphosphatase is one of the most important enzymes that are widely distributed in the plasma membranes, mitochondria and other biological membrane systems. There are also the reports on biochemically-defined ATPase to be present in the isolated membrane of rat liver (1—5), erythrocytes (6, 7) and bacteria (8), which support the cytochemical evidence for the existence of membrane-bound ATPase in the intact cell. Cytochemical reaction products of this enzyme is visualized with the electron microscope and is usually demonstrated to be associated with only the plasma membranes of a variety of cell types (9—38).

In order to clarify whether or not the electron cytochemical reaction of ATPase activity plays a role of special markers in the isolated membranes, cytochemical observation and biochemical assay of Mg^{2+}-ATPase were conducted before and after fixation with glutaraldehyde of the plasma membranes, mitochondria, microsomes, and nuclei isolated from ascites hepatoma (AH 130 strain) cells and rat liver.

MATERIALS AND METHODS

Animals

Male Donryu rats about three or four months old were used. The animals had free access to water and oriental solid food prior to the experiments.

Isolation of cell membranes

AH 130 strain cell membrane: Cell membranes were isolated from AH 130 strain cells and rat liver cells by a modification (14) of Emmelot *et* al's method (1).

Male Donryu rats were injected intraperitoneally with 0.5 ml of AH 130 cells. The animals were killed 8 days after injection and ascites hepatoma cells were obtained. The cells were once filtered through four-layered nylon cloth. The filtrate was centrifuged at 1,100 rpm for 10 minutes in a Kubota centrifuge operated at 4°C, rotor No. 3. The pellet was suspended in 1 mM $NaHCO_3$ medium and centrifuged at 1,100 rpm for 10 minutes. The pellet was resuspended in a small quantity of the same medium and homogenized by moving a teflon pestle up and down in an ice cold surrounding. During the homogenization a drop of the homogenate was examined under a phase contrast microscope to check the degree of cell disruption. When the degree of the cell disruption reached about 80%, the homogenation was discontinued. The homogenate was suspended in twenty volumes of the same medium and centrifuged at 2,200 rpm for 10 minutes. The pellet was homogenized in five volumes of 1 mM $NaHCO_3$-1 mM $CaCl_2$ medium with a loose fitting teflon homogenizer and centrifuged at 3,000 rpm for 10 minutes. The supernatant was discarded and the pellet was homogenized with the same medium and centrifuged at 3,000 rpm for 10 minutes. This procedure was repeated several times until the supernatant became completely clear. The pellet was suspended in an equivalent volume of 0.25M sucrose and resuspended in two volumes of sucrose (d=1.34). Five ml of this suspension were transferred into a centrifugal tube of Spinco Model L preparative ultracentrifuge using rotor 30. Nine ml of 45 per cent sucrose (d=1.20) and nine ml of 42 per cent sucrose (d=1.18) and nine ml of 37 per cent sucrose (d=1.16) were gently layered over the suspension successively, then centrifuged at 30,000 rpm for one hour. A solid layer at the interphase between d=1.16 and d=1.18 was scooped. This centrifugal floatation was repeated and the same layer was collected. The fraction so harvested was suspended with twenty volumes of 1 mM $NaHCO_3$ medium and centrifuged at 10,000 rpm for 20 minutes. The pellet was designated as the cell membrane fraction.

Rat liver cell membrane: Rats were killed by decapitation, and liver tissues were perfused with cold 1mM $NaHCO_3$ (pH 7.4). The liver tissues were removed and weighted, chopped by scissors, and added with the same medium to give one gram of wet weight per 10 ml solution. The tissue was homogenized with a glass homogenizer by moving the pestle up and down in the ice cold surrounding. When the degree of cell disruption reached about 80 per cent, the homogenization was discontinued. The homogenate was suspended in twenty volumes of the same medium. This homogenate was filtered through four layered nylon cloth to remove tissue. The filtrate was centrifuged at 2,200 rpm for 10 minutes. The subsequent procedures were identical with those employed in the case of AH 130 cells.

Isolation of AH 130 cell nuclei: The nuclei were isolated from AH 130 cells

by a modified method of WIDNELL and TADA (39). The cells were suspended in 1 mM $NaHCO_3$ (pH 7.4) and centrifuged at 1,100 rpm for 10 minutes. The pellet was suspended in a small quantity of the same medium, and homogenized with a Teflon homogenizer by moving the pestle up to down in ice cold water. The homogenate was added with 1 M sucrose, 1 M $CaCl_2$, and 0.2M Tris-HCl buffer (pH 7.4) to make 0.25 M sucrose, 1.8 mM $CaCl_2$, and 10 mM Tris-HCl in final concentration. The homogenate was centrifuged at 1,100 rpm for 10 minutes. The pellet was homogenized in the 0.25 M sucrose-1.8 mM $CaCl_2$ medium with a loose fitting Teflon homogenizer. The homogenate was gently layered over the 0.34 M sucrose-1.8 mM $CaCl_2$ layer and centrifuged at 2,500 rpm for 10 minutes. The pellet was homogenized in the 2.2 M sucrose-1.8 mM $CaCl_2$ solution of about ten-fold volumes with loose fitting Teflon homogenizer and centrifuged at 28,900 rpm for one hour. Then supernatant was discarded. The pellet was suspended in the 0.25 M sucrose-1.8 mM $CaCl_2$ solution. The suspention was gently layered over the 0.34 M sucrose-1.8 mM $CaCl_2$ solution, and centrifuged at 2,500 rpm for 10 minutes. The pellet was homogenized in the 0.25 M sucrose-1.8 mM $CaCl_2$ and centrifuged at 1,100 rpm for 10 minutes. This pellet was the nuclear fraction.

Isolation of nuclei from rat liver: The liver tissues were weighted, chopped by scissors, and added with an ice cold 0.25 M sucrose-1.8 mM $CaCl_2$-10 mM Tris-HCl buffer (pH 7.4) to give one gram of wet weight per 10 ml solution. The homogenization was carried out with a glass homogenizer in cold water. The homogenate was once filtered through four-layer nylon cloth to removed connective tissue. This homogenate was centrifuged at 1,100 rpm for 10 minutes. The subsequent procedures were the same as in the case of AH 130 cells.

Isolation of AH 130 strain cell mitochondria: The cells were filtered through four-layer nylon cloth. The filtrate was centrifuged at 2,200 rpm for 10 minutes. The pellet was diluted with 5 volumes of an ice cold 0.25 M sucrose-0.1 mM EDTA-2 mM-Tris-HCl buffer (pH 7.4) and centrifuged at 2,200 rpm for 10 minutes. The proteinase in the pellet was added to give one milligram per gram of wet cells and kept at 0°C for 15 minutes. This pellet was homogenized with Teflon homogenizer, the homogenate was diluted in twenty times with the same medium, and centrifuged at 2,500 rpm for 10 minutes. The supernatant was centrifuged at 6,000 rpm for 10 minutes. The pellet was homogenized in the same medium, and centrifuged at 6,000 rpm for 10 minutes. The pellet was homogenized in the same medium. The homogenate was gently layered over 0.34 M sucrose and centrifuged at 2,500 rpm for 10 minutes. The supernatant was centrifuged at 6,000 rpm for 10 minutes. The pellet was homogenized in 0.25 M sucrose and centrifuged at 5,000 rpm for 10 minutes. This pellet was taken as mitochondria fraction.

Isolation of rat liver mitochondria: The liver tissues were weighted, chopped by scissors and added with an ice-cold 0.25 M sucrose-0.1 mM EDTA-2 mM Tris-HCl buffer (pH 7.4) to give one gram of wet weight per 10 ml solution. The homogenization was carried out with a glass homogenizer. The homogenate was

centrifuged at 2,500 rpm for 10 minutes. The pellet was suspended in the same medium. The homogenate was gently layered over 0.34 M sucrose and centrifuged at 2,500 rpm for 10 minutes. The following procedures were the same in the case of AH 130 cells.

Isolation of microsomes from AH 130 cells and rat liver: After the mitochondrial fraction was removed as described above, its supernatant was centrifuged for 10 minutes at 10,000 rpm. The microsomes were isolated by centrifugation of the supernatant for one hour at 30,000 rpm in a medium identical with that used for the isolation of plasma membranes or mitochondria. All operations were carried out at 0°C or 4°C.

Assay of enzyme

The enzyme assay was carried out as follows: Mg^{2+}-ATPase medium consisted of 5.0 mM $MgCl_2$, 25 mM Tris-HCl buffer (pH 7.6), 5 mM substrate (sodium salt of ATP, or ADP, or AMP, or β-glycerophosphate). The reaction was conducted with the addition of 0.05—0.10 ml of membrane fractions, and these fractions were omitted in the control reaction. Mg^{2+}-Na^+-K^+-ATPase medium was added with 100 mM NaCl and 10 mM KCl in the same medium. The final volume was adjusted to 1.0 ml with deionized water. The final concentration of protein of each fraction in the reaction medium ranged from 0.1 to 0.5 mg protein per ml of medium. After preincubation in water-bath for 5 minutes at 37°C, the reaction was started by the addition of 0.1 ml substrate. The incubation was continued for 15 minutes at 37°C. 0.1ml of NaN_3 (1mM), NaF (1 mM), or *p*-chloromercuribenzoate (2×10^{-4}M) was added to the substrate medium for the study of the inhibitory effects upon enzymatic activity. The reaction was stopped by the addition of 1.0 ml of 16% cold perchloric acid, and aliquots of 1.0 ml were taken out for the determination of inorganic phosphate. The inorganic phosphate was determined by the method of TAKAHASHI (40), a modification of the method of Berenblum-Chain. Proteins in each fraction were determined by the method of BIURET (41) or LOWRY (42).

Electron cytochemical demonstration of adenosine triphosphatase activity: For determining the site of ATPase activity, the membrane fractions were fixed for 30 minutes at 0°C with 5 per cent glutaraldehyde in 0.25 M sucrose buffered with 0.1M cacodylate buffer (pH 7.4). The cell and tissue were fixed for one hour with the same medium. They were rinsed overnight in 0.25 M sucrose buffered with 0.1 M cacodylate. For the demonstration of ATPase activity in these samples, Wachstain-Meisel medium modified by ODA (9) was used as the incubation medium. The medium consists of 5 mM adenosine triphosphate Na salt, 50 mM histidine buffer (pH 7.4), 5 mM $MgCl_2$, 2 mM Pb $(NO_3)_2$, 5 mM KCl, 0.1 M NaCl, and 0.1 M sucrose.

The fixed plasma membranes, nuclei, mitochondria and microsomes were incubated for 15 minutes at 30°C in a freshly prepared medium. The cell or tissue was incubated for 30 minutes at 30°C in the same medium. After reaction the samples were washed with the following medium: 0.1 M sucrose, 50 mM histidine buffer (pH 7.4), 5 mM KCl, 0.1 M NaCl; and centrifuged.

The tissue and cells were briefly rinsed in the same medium. In order to check the substrate specificity of the ATPase reaction, ATP was replaced with ADP, AMP or β-glycerophosphate.

Electron microscopy

Dehydration, embedding, section staining and microscopy: Samples of the precipitated material were immersed in freshly prepared ice-cold fixative solution containing 1% osmium tetroxide and 0.14 M veronal-acetate buffer (pH 7.4) for one hour or overnight. The samples were then dehydrated through a series of ethanol solution of graded concentration, embedded in Epon 812 and sectioned with the glass knife using a Porter-Blum microtome MT-2. The sections were mounted on copper grids and stained with uranyle acetate and lead citrate. Specimens were examined in a Hitachi HU-11-D electron microscope.

RESULTS

Mg^{2+}-Na^+-K^+-ATPase activity in AH 130 cells and rat liver tissue

Under the experimental conditions employed in this instance, the Mg^{2+}-Na^+-K^+-ATPase reaction was confined to both plasma membranes. The intensity of reaction products in the AH 130- and liver cells was about the same. The reaction products were distributed on the plasma membrane in the form of discrete dots. In the AH 130 cells, the enzymatic reaction products were seen on the microvilli and infolding plasma membrane (Figs. 1, 2). The same in the liver tissue were on bile canaliculi and plasma membrane (Figs. 3, 4). These electron dense granules were located at the outer layer of the plasma membrane, but such reaction products were not detected in nuclei, mitochondria and microsomes. In the case of the AH 130 cell, the enzymatic reaction products could not be recognized when ADP, AMP and β-glycerophosphate were employed as the substrate.

Mg^{2+}-Na^+-K^+-ATPase activity of AH 130- and rat liver plasma membrane fractions

The plasma membrane isolated from AH 130 cells and rat liver cells were observed as a triple layered structure of about 80 Å in width. The membrane fraction isolated from rat liver had still adhered to each other by way of desmosome and tight junction (Figs. 5, 8). But these features were not recognized in the plasma membrane isolated from AH 130 cells (Fig. 6). Generally, the cellular membrane fractions exhibited a variety of structural organization according to their functional specialization in the cell. The electron cytochemical reaction products in the isolated cell membrane were located at the inner layer of the membrane (Figs. 7, 9). On the other hand, in the nuclei, mitochondria and microsomes fixed

with glutaraldehyde the reaction products were not observed (Figs. 10—19).

Analysis of nonenzymatic hydrolysis of adenosine triphosphate salt by lead ions

Recently, the nonenzymatic hydrolysis of ATP salt by lead ions or buffer has been reported (58, 59). In order to elucidate this problem, nonenzymatic hydrolysis of ATP salt by lead ions in the histidine buffer instead of Tris-malate buffer was studied by biochemical analysis. As shown in Table 1, under this experimental condition nonenzymatic hydrolysis of ATP salt by the Pb ion is not recognized. Thus, in this experimental condition, the reaction elicited by the electron cytochemical and biochemical methods reflects real enzymatic activities. It seems that the nonspecific reaction by the nonenzymatic hydrolysis of ATP salt is not reflected.

Table 1. Effect of Pb $(NO_3)_2$ on various nucleoside phosphates

	Inorganic phosphate liberated mμ moles/mg protein/min	
	+Pb $(NO_3)_2$	−Pb $(NO_3)_2$
Histidine buffer		
ATP	5	5
Tris-HCl buffer		
ATP	9	8
ADP	9	9
AMP	0	1
β-gly-P	1	1

The test system for electron cytochemical method contained···5 mM Na_2ATP, 2 mM Pb $(NO_3)_2$, 5 mM $MgCl_2$, 5 mM KCl, 100 mM NaCl, 100 mM sucrose, and 50 mM histidine buffer (pH 7.4), incubation 15 min, temperature 30°C. For biochemical method···5 mM ATP, ADP, AMP and β-glycerophosphate Na salt, 5 mM $MgCl_2$, 2mM Pb $(NO_3)_2$, 100mM NaCl, 10 mM KCl, and 25mM Tris-HCl buffer (pH 7.6), incubation 15 min, temperature 37°C

Specific activities of the Mg^{2+}-ATPase, Mg^{2+}-Na^+-K^+-ATPase, ADPase, AMPase, and β-glycerophosphatase of the fresh membrane fractions

The specific activities of freshly prepared membrane fractions are listed in Tables 2—5. ATPase is a better substrate than ADP and AMP, while β-glycerophosphate does not serve as substrate. The Mg^{2+}-ATPase and Mg^{2+}-Na^+-K^+-ATPase activities of the plasma membranes and the Mg^{2+}-ATPase activity of mitochondria were much higher than the corresponding activities of microsomes. The ADPase activity of rat liver plasma membrane was much higher than the activity of other membrane fractions.

Table 2 Effect of glutaraldehyde fixation on ATPase activity in cell components isolated from rat ascites hepatoma (AH 130 strain) cell

AH 130 cell components	Mg^{2+}-ATPase								Mg^{2+}-Na^+-K^+-ATPase							
	Before glutaraldehyde fixation				After glutaraldehyde fixation				Before glutaraldehyde fixation				After glutaraldehyde fixation			
	ATP	ADP	AMP	β-gly-P	ATP	ADP	AMP	β-gly-P	ATP	ADP	AMP	β-gly-P	ATP	AMP	ADP	β-gyl-P
Nuclei	31	1	5	4	4	~0	2	3	21	18	6	3	2	~0	2	3
Mitochondria	131	59	19	11	13	3	2	~0	170	41	20	7	22	~0	3	3
Microsome	85	48	20	6	~0	~0	~0	~0	90	37	34	9	7	1	4	4
Plasma membrane	127	26	10	3	117	10	1	1	247	28	11	3	132	10	5	2

The values indicate specific activity of phosphatase, expressed as mμ moles of inorganic phosphate released per mg of protein min. at 37°C
β-gly-P ····· β-glycerophosphate

Table 3 Effect of glutaraldehyde fixation on ATPase activity in cell components isolated from rat liver cells

Rat liver cell components	Mg^{2+}-ATPase								Mg^{2+}-Na^+-K^+-ATPase							
	Before glutaraldehyde fixation				After glutaraldehyde fixation				Before glutaraldehyde fixation				After glutaraldehyde fixation			
	ATP	ADP	AMP	β-gly-P	ATP	ADP	AMP	β-gly-P	ATP	ADP	AMP	β-gly-P	ATP	ADP	AMP	β-gly-P
Nuclei	5	~0	1	~0	~0	~0	~0	~0	6	1	2	~0	2	~0	2	~0
Mitochondria	400	50	14	5	~0	~0	1	1	398	49	19	4	~0	~0	2	2
Microsome	115	47	44	1	~0	~0	~0	~0	91	54	39	3	5	1	~0	~0
Plasma membrane	324	110	196	~0	244	103	~0	~0	378	74	125	~0	224	65	0	~0

The values indicate specific activity of phosphatase, expressed as mμ moles of inorganic phosphate released per mg of protein per min. at 37°C
β-gly-P ····· β-glycerophosphate

Table 4 Inhibitory effects of NaF, NaN_3 and P. C. M. B. on ATPase and ADPase activities in the plasma membrane isolated rat ascites hepatoma (AH130) cells

	Inorganic phosphate liberated (mμ moles/mg protein/min)			
	None	$NaF^{10^{-3}}$	$NaN_3{}^{10^{-3}}$	p. c. m. b. 2×10^{-4}
Mg^{2+}-Na^+-K^+-ATPase	158	195	118	59
Mg^{2+}-Na^+-K^+-ADPase	29	77	19	25
Mg^{2+}-ATPase	95	49	37	17
Mg^{2+}-ADPase	43	59	24	27

Table 5 Inhibitory effects of NaF, NaN_3 and P. C. M. B. on ATPase and ADPase activities in the plasma membrane isolated from rat liver cells

	Inorganic phosphate liberated (mμ moles/mg protein/min)			
	None	$NaF^{10^{-3}}$	$NaN_3^{10^{-3}}$	p. c. m. b. 2×10^{-4}
$Mg^{2+}Na^+$-K^+-ATPase	436	421	382	363
Mg^{2+}-Na^+-K^+-ADPase	226	141	94	220
Mg^{2+}-ATPase	408	455	235	292
Mg^{2+}-ADPase	255	141	78	255

The nucleotidase activity of liver plasma membranes, using AMP as substrate, proved to be high, but it was significantly lower in the plasma membrane preparation of AH 130 cells. The plasma membrane isolated from AH 130 cells showed much lower Mg^{2+}-ATPase and Mg^{2+}-Na^+-K^+-ATPase activities than those of the plasma membrane from rat liver. In general, a specific activity of the AH 130 cell membrane fractions was much lower than that of the liver fraction. As shown in Tables 2—5, the liberation of inorganic phosphate was somewhat activated by Na^+ and K^+ in the plasma membrane. However, the activating effect of Na^+ and K^+ was not clear at mitochondria and microsome fractions. The activity of the isolated nuclear fraction was scarcely recognized. It seems that most of the activity of the AH 130 cell nuclear fraction is an artifact resulting from the contamination of other membrane fragments.

Effect of glutaraldehyde fixation

Tables 2 and 3 show the effect of fixation with 5% glutaraldehyde solution. Activities of the Mg^{2+}-ATPase, Mg^{2+}-Na^+-K^+-ATPase, ADPase, and AMPase could be domonstrated in the mitochondrial, microsomal and nuclear fractions after glutaraldehyde fixation. Mg^{2+}-ATPase activity of plasma membrane fractions from both AH 130 cells and liver cells was not inhibited by fixation in the present condition. On the contrary, the activity stimulated by Na^+ and K^+ was completely abolished by fixation. The results of these observations indicate that the localization of Mg^{2+}-Na^+-K^+-ATPase activity cannot be demonstrated electron cytochemically. The AMPase activity of liver plasma membranes was completely inhibited, but the ADPase activity was not.

Inhibitory effect of NaF, NaN_3 and p-chloromerucuric benzoate

As shown in Tables 4 and 5 *p*-chloromerucuric benzoate (1.0×10^{-4}M) did not a significantly affect (0～10% inhibition) the Mg^{2+}-ATPase and Mg^{2+}-Na^+-K^+-ATPase activities of the liver membrane, but inhibited those

activities in AH 130 cell membrane (30%～60%). NaN_3 (10^{-3}M) inhibited the Mg^{2+}-ATPase of both membrane fractions, but had no effect on the Mg^{2+}-Na^+-K^+-ATPase nor NaF (10^{-3}M) had any effect on the Mg^{2+}-ATPase and Mg^{2+}-Na^+-K^+-ATPase activities. On the other hand, *p*-chloromercuric benzoate had shown no effect on the ADPase of the both membrane fractions. NaF and NaN_3 inhibited the ADPase of rat liver membrane fraction by 50%, but no such effect was observed in the AH 130 cell membrane.

DISCUSSION

The plasma membrane is currently receiving much attention in morphological and biochemical studies by many workers. Especially, the physical and chemical changes that occur in the plasma membrane of the cell, the active transport, envelopmental formation of RNA virus, cell surface antigens, and action of various agents such as enzymes, hormones and drugs are the subjects of controversy. On the other hand, the molecular structure of the membrane in the interpretation of electron micrographs and X-ray diffraction patterns is nothing but an imperfect and speculative model. The solution of this problem has now been restrained by lack of the methodology involved with fractionation, purification and fixation of the membrane.

The methods of NEVILLE (2) or its modification by EMMELOT *et al.* (1) are the procedures employed in the isolation of plasma membranes. By these methods the yield of membrane is low, and causes drastic alteration of the cellular structure On the other hand, the isolation can be accomplished with an isotonic medium instead of the hypotonic medium, reported by TAKEUCHI (48), Coleman *et al.* (5), and Wallach *et al.* (45—49) Red cell membrane, mitochondrial and microsomal membranes are easier to prepare than the plasma membrane. However, microsomal membrane is continuous with the plasma membrane or nuclear membrane at several points, and the mutual contamination is inevitable. The contamination by other cellular fragments is higher in plasma membrane fraction obtained from cancer cell than that from liver cell. Many methods of the membrane isolation of cancer cell have been reported. WALLACH and KAMAT (45—49) used Ehrlich ascites cells as material and fractionated by density gradient centrifugation in sucrose polymer. The electron microscope descriptions of their final plasma membrane fraction are not available. In the case of ascites cells, they do not have desmosomes or structures resembling the bile space. So, it is in fact difficult to distiguish plasma membrane frag-

ments from intracellular smooth membrane fragments. In this experiment, the separation of the plasma membrane element from AH 130 cell was more difficult than in the case of the liver.

It is well known that Mg^{2+}-ATPase, Mg^{2+}-Na^{+}-K^{+}-ATPase and AMPase are intrinsic constituents of the liver plasma membrane. On the other hand, an electron cytochemical demonstration of the activities of these enzymes in plasma membrane has been reported by many workers. At first, GLICK and FISHER (50, 51), introduced the staining method for the demonstration of ATPase activity. Since then, WACHSTEIN and MEISEL (52) used lead method for histochemical demonstration of ATPase, and many histochemical demonstration of ATPase activity in tissue sections have been reported by light microscopy(53–56) or electron microscopy. However, this method gives varying results due to the variation in the type of fixation, the time of fixation, the components of incubation medium and its concentration. NOVIKOFF *et al.*(57)(1958) have shown that lead does inhibit ATPase activity. Recently, ROSENTHAL and MOSES (58, 59), have reported that Pb ions cause the nonenzymatic hydrolysis of adenosine triphosphate, inhibiting ATPase activity. But NOVIKOFF (60) has found that the staining reactions obtained in the Wachstein-Meisel and similar media reflect real enzymatic activities for various considerations. On the other hand, MOSES *et al.*(61) state that the localization of lead salt deposits to plasma membranes does not alone constitute sufficient evidence for the localization of enzyme activity. In the histidine buffer used in this experiment, the nonenzymatic hydrolysis of adenosine triphosphate was not recognized, but it seems that a precise examination is necessary for the analysis of specimens of lower enzymatic activity.

In order to demonstrate the localization of enzymatic activity in situ with electron microscopy, it is necessary to avoid the migration of soluble and diffusible enzymes. SABATINI *et al.* (20) introduced the preservation method of cellular ultrastructure and enzymatic activity by aldehyde fixation The prefixation of glutaraldehyde has been used by many workers in the recent years The preservation of morphology proves to be sufficiently good with the use of glutaraldehyde. But enzyme activity cannot always be determined satisfactorily. The biochemical analysis suggests that the ATP splitting enzymes are widely localized in the plasma membranes, mitochondria, microsomes, muscle fibers, and others. After the fixation with glutaraldehyde, however, ATPase activity is mainly demonstrated in the plasma membrane and cannot be demonstrated in other membrane fractions. It has been found that the increase of Mg^{2+}-ATPase activity due to the addition of Na^{+} and K^{+} ions was abolished by

glutaraldehyde fixation. Such evidences indicate that only Mg^{2+}-ATPase is demonstrable by electron cytochemical method.

In the present work, data indicate that N^+ and K^+ activation of Mg^{2+}-ATPase activity cannot be demonstrated histochemically, although it is not clear whether or not Mg^{2+}-ATPase and Mg^{2+}-Na^+-K^+-ATPase are different enzymes. It seems, however, that many observations reflect the structural differences in enzymic proteins in the plasma membrane(64-66).

The mitochondria and microsomes are rich in Mg^{2+}-ATPase activity, but the increase in activity due to the additon of Na^+ and K^+ ions is not recognized, and ATPase activity is inhibited by glutaraldehyde. The ADPase and AMPase activities of these fractions are low, being inhibited by the fixation. Thus the electron cytochemical localization of ATPase, ADPase and AMPase activities of these fractions cannot be demonstrated after glutaraldehyde fixation.

It is reported that AMPase is present in plasma membrane (1)(3)(26)(67)(70)(71) and periplasm space of bacteria (69). AMPase activity of plasma membrane is higher than that in other fractions. But this activity is completely inhibited by glutaraldehyde. On the other hand, ADPase activity of the plasma membrane has been demonstrated in bile canaliculi or microsomes of the liver by the use of biochemical(1, 3, 72), histochemical(73) and electron microscopic methods(7, 63). EMMELOT *et al.*(3) state that the ADPase activity measured at pH 7.2 in the liver plasma membrane is zero or very small, and this lower activity may be due to either the loss or the inactivation of the enzyme during isolation of the membrane. The high activity found in this experiment *in vitro* may be due to the difference in the methods of isolating the membrane.

HORI *et al.* (73) reported the existence in bile canaliculi of more than two enzymes such as ATPase, formalin-resistant ATPase-like enzyme, ADPase and apyrase in their histochemical study of ATPase in cytoplasm. ADPase activity in the liver plasma membrane fraction was considered to be due to the existence in bile canaliculi of enzymes which are related to the dephosphorylation of both ATP and ADP. In fact, ADPase activity of isolated liver plasma membranes was not inhibited by glutaraldehyde and PCMB, but this activity was inhibited by NaF and NaN_3. All these characteristics well accord with those of the results reported by HORI *et al.*(73). On the other hand, ADPase activity of the AH 130 plasma membrane showed a very low activity. Hence, it may be considered that this activity is largely due to bile canaliculi structure. According to the biochemical and electron cytochemical data reported herein, it is clear that Mg^{2+}-ATPase activity is detectable only in the plasma membrane when

fixed with glutaraldehyde. For the characterization of the isolated membrane, the absolute marker is necessary. Reported membrane markers are chemical, morphological and immunological markers. EMMELOT *et al* (77) (17) reported that sialic acid is a chemical component of rat liver plasma membrane as demonstrated by the biochemical and electron cytochemical methods. However, it is not well known whether sialic acid exists only in the cytoplasmic membrane and its cytochemical reaction is inhibited by fixation. BENEDETTI *et al.* (78) report that hexagonal pattern of the membrane can be demonstrated after negative staining at 37°C of isolated plasma membranes from rat liver. But SEKI *et al.* (79) state that the hexagonal pattern recognized in the liver plasma membrans can scarcely be seen in AH 130 cell. Likewise, the histological features such as desmosomes, bile spaces and globular knob are not recognized in AH 130 cell. MARINETTI *et al.* (80) claim that a fluorcesent stilbene isothiocyanate disulfonic acid serves as a specific chemical marker for the plasma membranes of an intact cell. However, a specific chemical marker in the cell fraction is invariably found in the mitochondrial fraction.

The characterization of structure and function of the plasma membrane has been reported by several authors. It appears, however, most reasonable to conclude from the results of our investigation that an electron cytochemical reaction of Mg^{2+}-ATPase activity may serve as an effective means of special marker for the plasma membranes of cancer and liver cells.

SUMMARY

1. The studies of structure and function of the plasma membranes of cancer cells is extremely important for the elucidation of specificity of phenotypes of cancer cells. In order to bring this subject to light, plasma membranes, mitochondria, microsomes and nuclei have been isolated from the AH 130 ascites carcinoma cells and rat liver cells. The electron cytochemical observations and biochemical assays of Mg^{2+}-Na^{+}-K^{+}-ATPase, ADPase, AMPase, and β-glycerophosphatase activities have been carried out before and after the fixation with glutaraldehyde.

2. Mg^{2+}-ATPase and Mg^{2+}-Na^{+}-K^{+}-ATPase are present in the isolated plasma membranes, mitochondria and microsomes in both AH 130 cells and rat liver cells. ADPase and AMPase of the mitochondria and microsomes show far lower activities than those of the corresponding enzymes found in rat liver plasma membrane. ADPase and AMPase of AH 130 cell fraction exhibit activity much lower or zero. Generally, enzymatic

activity of the AH 130 cell fraction is much lower than that of rat liver cell fraction.

3. Mg^{2+}-Na^{+}-K^{+}-ATPase is completely abolished by 5% glutaraldehyde fixation while it shows less effect on Mg^{2+}-ATPase in the plasma membrane. ADPase and AMPase activities of the mitochondria and microsomes are completely inhibited by glutaraldehyde fixation. AMPase of the plasma membrane of rat liver is completely abolished while ADPase activity is not affected in any way.

4. Only Mg^{2+}-ATPase can be demonstrated electron cytochemically. Cytochemical reaction products of Mg^{2+}-ATPase are located at the outer layer of the plasma membrane of the AH 130 cells and rat liver tissue. In the isolated membrane fractions it is located at the inner layer.

5. *p*-Chloromercuribenzoate has only a slight effect on Mg^{2+}-ATPase and Mg^{2+}-Na^{+}-K^{+}-ATPase activities of the rat liver membrane while it inhibits these enzyme activities in the AH 130 cell membrane. NaF (1 mM) and NaN_3 (1 mM) inactivate ADPase of the rat liver plasma membrane.

6. In these experimental conditions, nonenzymatic hydrolysis of ATP by lead ions is not recognized.

7. It seems most reasonable to conclude that cytochemical electron microscopic demonstration of Mg^{2+}-ATPase after fixation with glutaraldehyde may serve as the absolute marker for the plasma membrane of ascites hepatoma and liver cells.

ACKNOWLEDGEMENTS

The author wishes to express deep thanks to Professor T. ODA for many helpful discussions and suggestions during this work.

The author also thanks Dr. S. OMURA for helpful advice on the preparation of plasma membranes, and Dr. G. YAMAMOTO for helpful advice on the assay of adenosine triphosphatase activity.

REFERENCES

1. EMMELOT, P., BOS, C. J., BENEDETTI, E. L. and RUMKER, P. H.: Studies on plasma membranes. 1. Chemical composition and enzyme content of plasma membranes isolated from rat liver. *Biochem. Biophys. Acta* 90, 126, 1964
2. NEVILLE, D. M.: The isolation of a cell membrane fraction from rat liver. *Biophys. Biochem. Cytol.* 8, 413, 1960
3. EMMELOT, P. and BOS, C. J.: Studies on plasma membranes. 3. Mg^{2+}-ATPase, (Na^{+}-K^{+}-Mg^{2+})-ATPase and 5′-nucleosidase activity of plasma membranes isolated from rat liver. *Biochem. Biophys. Acta* 120, 369, 1966
4. LIEHERMAN, I., LANSING, A. I. and LYNCH, W. E.: Nucleoside triphosphate pyrophos-

phohydrolase of the plasma membrane of the liver cell. *J. Biol. Chem.* 242, 736, 1967
5. COLEMAN, R., MICHELL, R. H., FINEAN, J. B. and HAWTHORNE, J. N.: A purified plasma membrane fraction isolated from rat liver under isotonic conditions. *Biochem. Biophys. Acta* 135, 573, 1967
6. POST, R. L., MERRITT, C. R., KINSOLVING, C. R. and ALBRIGHT, C. D.: Membrane adenosine triphosphatase as a participation in the active transport of sodium and potassium in the human. *J. Biol. Chem.* 235, 1796, 1960
7. PULLMAN, E. M., PENEFSKY, H. S., DATTA, A. and RACKER, E.: Partial resolution of the enzymes catalyzing oxidative phosphorylation. 1. Purification and properties of soluble dinitrophenol-stimulated adenosine triphosphatase. *J. Biol. Chem.* 235, 3322, 1960
8. ABRAMS, A., MCNAMARA, D. and LOHNSON, B.: ATPase in isolated bacterial cell membranes. *J. Biol. Chem.* 235, 3659, 1960
9. ODA, T. and SEKI, S.: Molecular structure and biochemical function of the microvilli membrane of intestinal epithelial cells with special emphasis on the elementary particles. *J. Electron Microscopy* 14, 210, 1965
10. ODA, T.: Cytochemical electron microscopic demonstration of adenosine triphosphatase in unfixed mitochondria. *J. Electron Microscopy* 14, 343, 1965
11. HELLMAN, I. T. and HELLIRSTROM, C.: Quantitative studies on isolated pancreatic islets of mammals.: Adenosine triphosphatase activity in normal and obese-hyperglycemice. *J. Histochem. Cytochem.* 12, 491, 1964
12. EPSTEIN, M. A. and HOLT, S. J.: The localization by electron microscopy of HeLa cell surface enzymes splitting adenosine triphosphate.: *J. Cell Biol.* 19, 325, 1963
13. GOLDFISHER, S., ESSNER, E. and NOVIKOFF, A. A.: The localization of phosphatase activities at the level of ultrastructure. *J. Histochem. Cytochem.* 12, 72, 1964
14. OMURA, J., IWATA, S., TAKE, S., YAMAMOTO, G., SEKI, S., KOSHIBA, K. and ODA, T.: Isolation and biochemical as well as electron microscopic observation of the plasma membrane of cancer cells. *Acta Med. Okayama*, to be published.
15. NOVIKOFF, A. B., ESSNER, E. and GOLDFISHER, S.: The interpretation of ultrastructure. *Sym. Intern. Soc. Cell. Biol.* p. 145, Academic Press, New York, 1962
16. OTERO-VILARDEBO, L. R., LANE, N. and GOODMAN, G. C.: Localization of phosphatase activities in cilonic goblet and absorptive cells. *J. Cell Biol.* 21, 486, 1964
17. ROSTGAARD, J. and BARRNETT, R. J.: Fine structure localization of nucleoside phosphatase in relation to smooth muscle cells and unmyelinated nerves in the small intestinal of the rat. J. Ultrastruct. Res. 11, 193, 1964
18. SPATTER, H. W., NOVIKOFF, A. B. and MASEK, B.: Adenosine triphosphatase activity in the cell membranes of kidney tubule cells. *J. Biophys. Biochem. Cytol.* 4, 765, 1958
19. EPSTEIN, M. A. and HOLT, S. J.: Electron microscope observations on the surface adenosine triphosphatase-like enzymes of HeLa cells infected with herpes virus. *J. Cell Biol.* 19, 337, 1963
20. SABATINI, D. D., MILLER, F. and BARRNET, R. J.: Aldehyde fixation for morphological and enzyme histochemical studies with the electron microscope. *J. Histochem. Cytochem.* 12, 57, 1964
21. NORTH, R. J.: The localization by electron microscopy of nucleoside phosphatase activity in guinea pig phagocytic cells. *J. Ultrastruct. Res.* 16, 83, 1966
22. HCFF, H. F. and GRAF, J.: An electron microscopic study of phosphatase activity in the endothelial cells of rabbit aorta. *J. Histochem. Cytochem.* 14, 719, 1966
23. ASHWORTH, C. T., LUIBEL, F. J. and STEWART, S. C.: The fine structural localization of adenosine triphosphatase in the small intestine, kidney, and liver of the rat. *J. Cell Biol.* 17, 1, 1963
24. TORACK, R. M.: Adenosine triphosphatase activity in rat brain following differential

fixation with formaldehyde, glutaraldehyde, and hydroxyadipaldehyde. *J. Histochem. Cytochem.* 13, 191, 1964

25. LAZARUS, S. S. and BARDEN, H.: Histochemistry and electron microscopy of mitoc ondrial adenosine triphosphatase. *J. Histochem. Cytochem.* 9, 285, 1961
26. SABATINI, D. D., BANSCH, K. and BARRNETT, R. J.: Cytochemistry and electron microscopy. *J. Cell Biol.* 17, 19, 1963
27. NOVIKOFF, A. B. and MASEK, B.: Adenosine-triphosphatase and 5'-nucleotidase activities in the plasma membrane of liver cells as revealed by electron microscopy. *J. Biopys. Cytol.* 4, 711, 1958
28. KHPLAN, S. E. and NOVIKOFF, A. B.: The localization of adenosine triphosphatase activity in rat kidney: electron microscopic examination of reaction product in formalin-calcium fixed frozen section. *J. Histochem. Cytochem.* 7, 295, 1959
29. NOVIKOFF, A. B. and BIEMPICA, L.: Cytochemical and electron microscopic examination of Morris 5123 and Reuber H-35 hepatomas after several years of transplantation. Proceeding of the U. S.-Japan Joint Conference on Biochemical and Biological Eval tation of Malignancy in Experimental Hepatomas. Kyoto, 1965
30. WHIT, J. G. and KRIVIT, W.: Fine structural localization of adenosine triphosphatase in human plates and other blood cells. *Blood* 27, 554, 1965
31. VETHAMANY, V. G. and LAZARUS, S. S.: Ultrastructural localization of Mg^{2+}-de, en ent dinitrophenol-stimulated adenosine triphosphatase in human blood platelets. *J. Histochem. Cytochem.* 15, 267, 1966
32. WACHSTEIN, M. and BESEN, M.: Electron microscopic study in several mammalian species of the reaction product enzymatically liberated from adenosine triphosphate in the kidney. *Lab. Invest.* 13, 476, 1964
33. PERSIJN, J. P., DAEMS, W. T., DEMAN, J. C. and MEIJER, A. C.: The demonstration of adenosine triphosphatase activity with the electron microscope. *Histochmie* 2, 372, 1691
34. NAGANO, T.: Localization of adenosine triphosphatase activity in the rat sperm tail as revealed by electron microscopy. *J. Cell Biol.* 25, 101, 1962
35. LAZARUS, S. S. and BARDEN, H.: Histochemistry and electron microscopy of mitochondrial adenosine triphosphatase. *J. Histochem. Cytochem.* 10, 285, 1962
36. WHACHSTEIN, M and FERNENDEZ, C.: Electron microscopic localization of n cleoside triphosphatase in endoplasmic reticulum of liver and pancreas. *J. Histochem. Cytochem.* 14, 203, 1966
37. MAS, P. and NAKAO, K.: Variation of localization of AMPase and ATPase activities at the plasma membrane of human prastatic epithelial cells: An electron microscopic study. *J. Histochem. Cytochem.* 14, 203, 1966
38. ODA, T., IWATA, S., OMURA, S., SEKI, S., YAMAMOTO, G., HATASE, O., HAYASHI, H. and WAKABAYASHI, A.: Isolation and characterization of plasma membrane from AH 130 strain cancer cells and liver cells of rat. Proceedings of the 9th International Cancer Congress, Abstracts of papers, 154, 1966
39. WIDNELL, C. C. and TADA, J. R.: A procedure for the isolation of enzymically active rat-liver nuclei. *Biochem. J.* 92, 313, 1964
40. TAKAHASHI, T.: The method of the determination of true inorganic phosphorus, creatine phosphate in mammalian tissues and studies on the phosphoamidase, creatine phosphokinase activities of the boar spermatozoa. *J. Japanese Biochemical Society* 26, 690, 1955 (in Japanese)
41. GORNALL, A. L. BARDAWILL, C. J. and DAVID, M. M.: The determination of protein by the biuret reaction. *J. Biol. Chem.* 177, 751, 1949
42. LOWRY, O. H., ROSENBROUGH, N. J., FORR, A. L. and RANDELL, R. T.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265, 1951

43. TAKEUCHI, M. and TERAYAMA, H.: Preparation and chemical composition of rat liver cell membranes. *Exp. Cell Res.* 40, 32, 1964
44. KAMAT, V. B. WALLACH, D. F. H.: Separation and partial purification of plasma membrane fragment from Ehrlich carcinoma microsomes. *Science* 148, 1343, 1965
45. WALLACH, D. F. H. and KAMAT, V. B.: Preparation of plasms membrane fragment from mouse ascites-tumor cells. *Enzymology*, Vol. 3
46. WALLACH, D. F. H. and ULLERY, D.: Studies on the surface plasmic membrane of Ehrlich ascites carcinoma cells. 1. The hydrolysis of ATPase and related nucleotidase by microsomal membranes. *Biochem. Biophys. Acta* 64, 526, 1962
47. WALLACH, D. F. H. and ULLERY, D.: Studies on the surface and cytoplasmic membrane of Ehrlich ascites carcinoma cells. 1. Alkali-cation activated adenosine triphosphate hydrolysis in microsomal membrane fraction. *Biochem. Biophys. Acta* 88, 620, 1964
48. WALLACH, D. F. H. and HAGER, E. B.: Plasma and cytoplasmic membrane fragment from Ehrlich ascites carcinoma. *Proc. N. A. S.* 52, 721, 1964
49. WALLACH, D. F. H. and HAGER, E. B.: Association of cell surface antigens with microsomal membrane fractions derived from Ehrlich ascites carcinoma cells. *Nature* 196, 1004, 1963
50. GLICK, D.: Histochemical localization of adenosine triphosphatase. *Science* 103, 599, 1964
51. GLIDK, D. and FISCHER, E. E.: The histochemical localization of adenosine triphosphatase in plant and animal tissue. *Science* 102, 429, 1945
52. WACHSTEIN, M. and MEISEL, E.: Histochemistry of hepatic phosphatase at a physiological pH. with special reference to the demonstration of bile canaliculi. *Ameri. J. Clin. Path.* 27, 13, 1957
53. FREIMAN, D. G. and KAPLAN, N.: Studies on the histochemical differentiation of enzymes hydrolysing adenosine triphosphatase. *J. Histochem. Cytochem.* 8, 159, 1960
54. PADYKULA, H. and HERMAN, E.: Factors affecting the activity of adenosine triphosphatase and other phosphatase as measured by histochemical techniques. *J. Histochem. Cytochem.* 3, 161, 1955
55. PADYKULA, H. and HERMAN, E.: The specificity of the histochemical method for adenosine triphosphatase. *J. Histochem. Cytochem.* 3, 170, 1955
56. PEARSE, A. G. E.: Histochemistry. Little, Brown and Co, Boston., 1960
57. NOVIKOFF, A. B., HAUSMAN, D. H. and PODBER, E.: The localization of adenosine triphosphatase in liver : *In situ* steining and cell fractionation studies. *J. Histochem. Cytochem.* 6, 61, 1958
58. ROSENTHAL, A. S., MOSES, H. L., BEAVER, D. L., and SCHUFFMAN, S. S: Nucleoside phosphatase by lead ion. *J. Histochem, Cytochem.* 14, 698, 1965
59. MOSES, H. L., ROSENTHAL, A. S., BEAVER, D. L., and SCHUFFMAN, S. S.: Lead ion and phosphatase histochemistry. 2. Effect of adenosine triphosphatase hydrolysis by lead ion on the histochemical localization of adenosine triphosphatase activity. *J. Histochem. Cytochem.* 14, 702, 1966
60. NOVIKOFF, A. B.: Enzyme localizations with Wachstein-Meisel procedures: real or artifact. *J. Histochem. Cytochem.* 15, 353, 1967
61. MOSES, H. L. and ROSENTHOL, A. S.: On the significance of lead-catalyzed hydrolysis of nucleoside phosphates in histochemical systems. *J. Histochem. Cytochem.* 15, 353, 1967
62. BOSCH, R.: On the histochemical demonstration of sodium activated adenosine triphosphatase. *J. Histochem. Cytochem.* 15, 114, 1967
63. TORMEY, J. M.: Significance of the histochemical demonstration of ATPase in epithelia noted for active transport. *Nature* 210, 820, 1966
64. JARNEFELT, J.: Conversion of the Na^+ and K^+ independent part of the brain microsomal ATPase to a form requiring added Na^+ and K^+.: *Biochem. Biophys. Res. Commun.*

17, 330, 1964

65. Skou, J. C. and Heberg, C.: The effect of suphydryl-blocking reagent and urea on the ($Na^+ + K^+$)-activated enzyme system. *Biochem. Biophys. Acta* 110, 359, 1965
66. Ernst, S. A.' Goertenillier, C. C. and Ellis, R. A.: The effect of salt regimens on the development on (Na^+-K^+) dependent ATPase activity during the growth of salt glands of duckings. *Biochem. Biophys. Acta* 135, 682, 1967
67. Essher, E., Novikoff, A. B. and Masek, B.: Adenosine triphosphatase and 5′-nucleotidase activity in the plasma membrane of liver cells as revealed by electron microscopy. *J. Biophysic. Biochem. Cytol.* 4, 711, 1958
68. Neu, H. C.: The 5′-nucleotidase of escherichia coli. 1. Purification and properties. *J. Biol. Chem.* 242, 3896, 1967
69. Neu, H. C.: The 5′-nucleotidase of Escherichia coli: 2. Surface localization and purification of the E. Coli 5′-nucleotidase inhibitor. *J. Biol. Chem.* 242, 3905, 1967
70. Woodin, A. M. and Wieneke, A. A.: Composition and properties of a cell membrane fraction from the polymorphonuclear leucocyte. *Biochem. J.* 99, 493, 1966
71. Coleman, R. and Finean, J. B.: Some properties of plasma membrane isolated from guinea-pig tissue. *Biochem. J.* 97, 39 p, 1965
72. Novikoff, A. B. and Heus, M.: A microsomal nucleoside diphosphatase. *J. Biol. Chem.* 238, 710, 1963
73. Hori, S. H. and Chang, J. P.: Histochemical study of adenosin triphosphatase in cytoplasm. 1. Histochemical study of adenosine triphosphatase in cytoplasma. *J. Histochem. Cytochem.* 11, 71, 1963
74. Emmelot, P. and Bos, C. J.: Differential effect of neuraminidase sensitive sialic acid in the K^+dependent phosphohydrolysis of *p*-nitrophenolphosphate by isolated rat-liver plasma membranes. *Biochem. Biophys. Acta.* 121, 375, 1966
75. Emmelot, P. and Bos, C. J.: Differential effect of neuraminidase on the Mg^{2+}-K^+-Na^+-ATPase and 5′-nucleotidase of isolated plasma membrane. *Biochem. Biophys. Acta* 99, 578, 1965
76. Emmelot, P. and Bos, C. J.: On the participation of neuraminidase sensitive sialic acid in the K^+-dependent phosphohydrolysis of p-nitrophenol phosphate by isolated rat-liver plasma membranes. *Biochem. Biophys. Acta* 115, 244, 1966
77. Benedetti, E. L. and Emmelot, P.: On the fine structure of cellular membrances. Sixth International Congress for Electron Microscopy, Kyoto, 1966
78. Benedatti, E. L. and Emmelot, P.: Electron microscopic observation on negatively stained plasma membranes isolated from rat liver. *J. Cell. Biol.* 26, 299, 1965
79. Seki, S., Omura, S. and Oda, T,: The surface structure of ascites hepatoma cancer cell, to be published.
80. Marinetti, G. V. and Gray, G. M.: A fluorescent chemical marker for the liver cell plasma membrane. *Biochem. Biophys. Acta* 135, 580, 1967

Explanation of Figures

Key to Abbreviations

N: Nucleus, M: Mitochondria, G: Golgi apparatus, ER: Endoplasmic reticulum, Mi: Microvilli, D: Desmosome

Fig. 1 An electron micrograph of ascites hepatoma (AH 130) cell. Fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. Microvilli, Golgi apparatus, mitochondria, endoplasmic reticulum, lysosome, nuclei and polysome are shown in the cytoplasm. × 25,000

Fig. 2 An electron cytochemical demonstration of magnesium ion-activated adenosine triphosphatase of AH 130 cells. Fixation: 5% glutaraldehyde, Staining: uranylacetate and lead citrate, Postfixation: 1% OsO_4, Reaction product is seen in the plasma membrane including microvilli membrane. Organellae in the cytoplasma free of reaction product. × 15,000

Fig. 3 An electron micrograph of normal rat liver. Fixation: 5% glutaraldehyde, Post-fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. × 40,000

Fig. 4 An electron cytochemical demonstration of magnesium ion-activated adenosine triphoshatase of normal rat liver. Fixation: 5% glutaraldehyde, Post-fixation: OsO_4, Staining: uranyl acetate and lead citrate. Reaction product is seen in the plasma membrane and membrane of bile canaliculus microvilli. Mitochondria and endoplasmic reticulum are free of reaction product. × 15,000

Fig. 5 An electron micrograph of a crude nuclei fraction isolated from normal rat liver cells. Fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. Nuclei, plasma membrane and connective tissue are seen. The plasma membrane is shown still adhering to each other by way of desmosomes or tight junction. × 15,000

Fig. 6 An electron micrograph of isolated AH 130 plasma membrane. Fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. × 56,000

Fig. 7 An electron cytochemical demonstration of magnesium ion-activated adenosine triphosphatase of isolated AH 130 plasma membrane. Fixation: 5% glutaraldehyde. Post-fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. Reaction product is seen in the inner side of the plasma membrane. × 80,000

Fig. 8 An electron micrograph of isolated rat liver plasma membrane. Fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. × 25,000

Fig. 9 An electron cytochemical demonstration of magnesium ion-activated adenosine triphosphatase of isolated rat liver plasma membrane. Fixation: 5% glutaraldehyde, Post-fixation: 1% OsO_4, Staining: Uranyl acetate and lead citrate. × 112,000

Fig. 10 An electron micrograph of mitochondrial fraction of AH 130 cell. Fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. × 20,000

Fig. 11 An electron cytochemical demonstration of magnesium ion-activated adenosin triphosphatase of isolated mitochondria of AH 130 cell. No reaction product is observed. Fixation: 5% glutaraldehyde, Post-fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. × 20,000

Fig. 12 An electron micrograph of mitochondrial fraction of rat liver cell. Fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. × 33,000

Fig. 13 An electron cytochemical demonstration of magnesium ion-activated adenosine triphosphatase of isolated mitochondria of rat liver cells. Fixation: 5% glutaraldehyde, Post-fixation: 1% OsO_4, Reaction product is not seen in the inner or outer membrane. × 33,000

Fig. 14 An electron micrograph of microsomal fraction of AH 130 cell. Fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. × 42,000

Fig. 15 An electron cytochemical demonstration of magnesium ion-activated adenosine triphosphatase of isolated microsomes of AH 130 cell. Fixation: 5% glutaraldehyde, Post-fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. Ribosome is seen, but reaction product is not. × 108,000

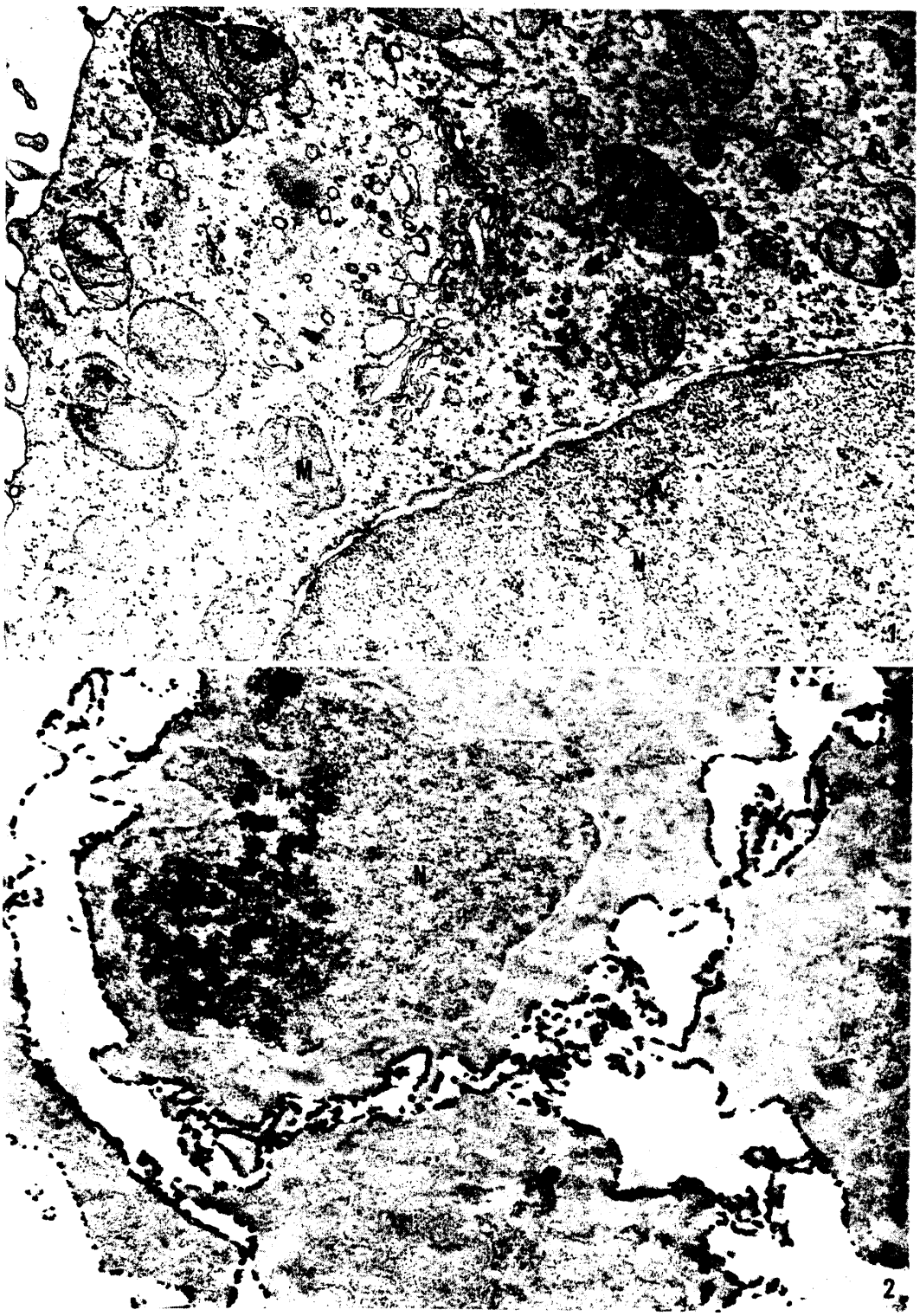
Fig. 16 An electron micrograph of microsomal fraction of rat liver cell. Fixation: 1% OsO_4, Staining: uranyl acetae and lead citrate. × 90,000

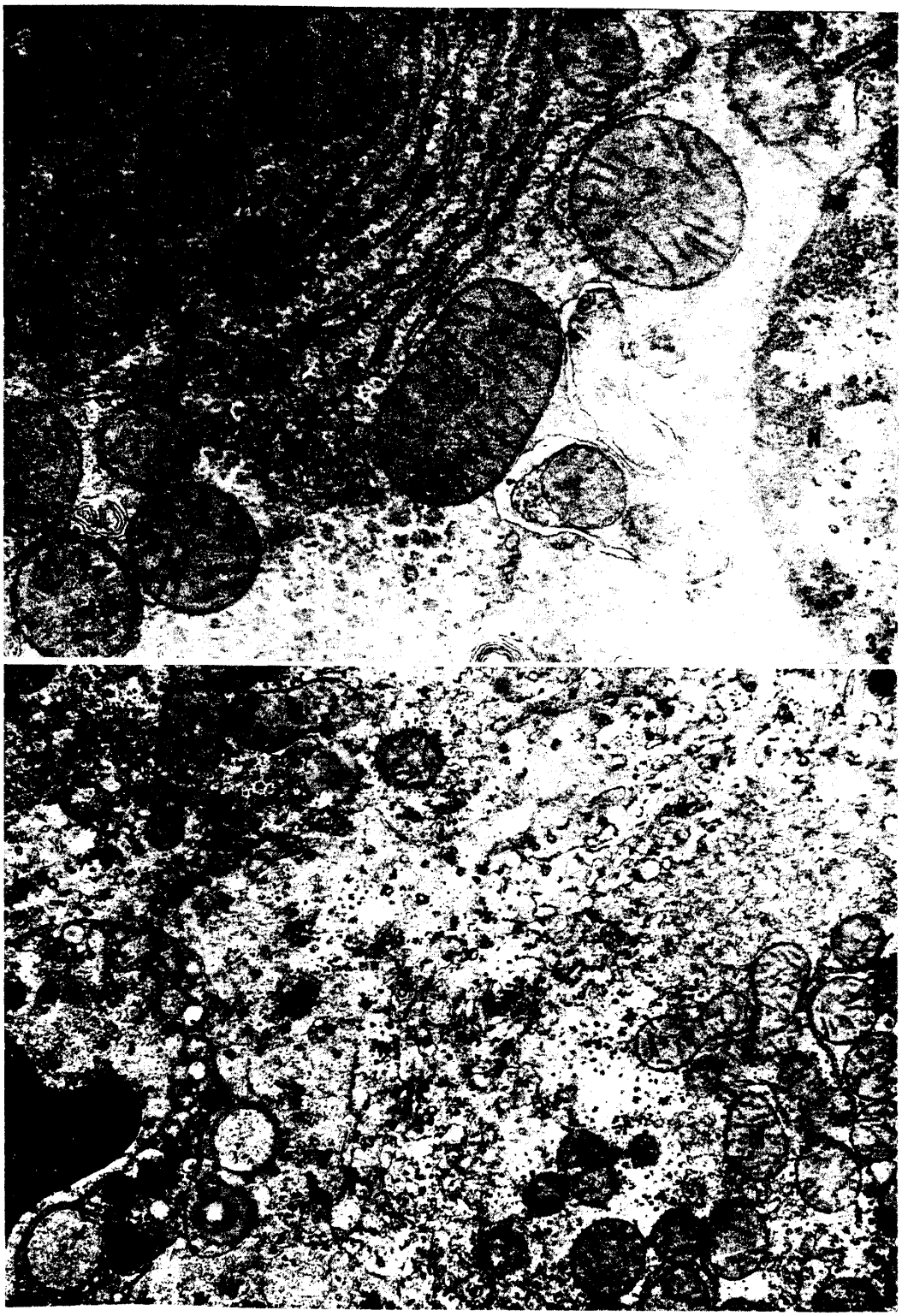
Fig. 17 An electron cytochemical demonstration of magnesium ion-activated adenosine triphosphatase of isolated microsomes of rat liver cell. Fixation: 5% glutaraldehyde, Post-fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. Ribosome is

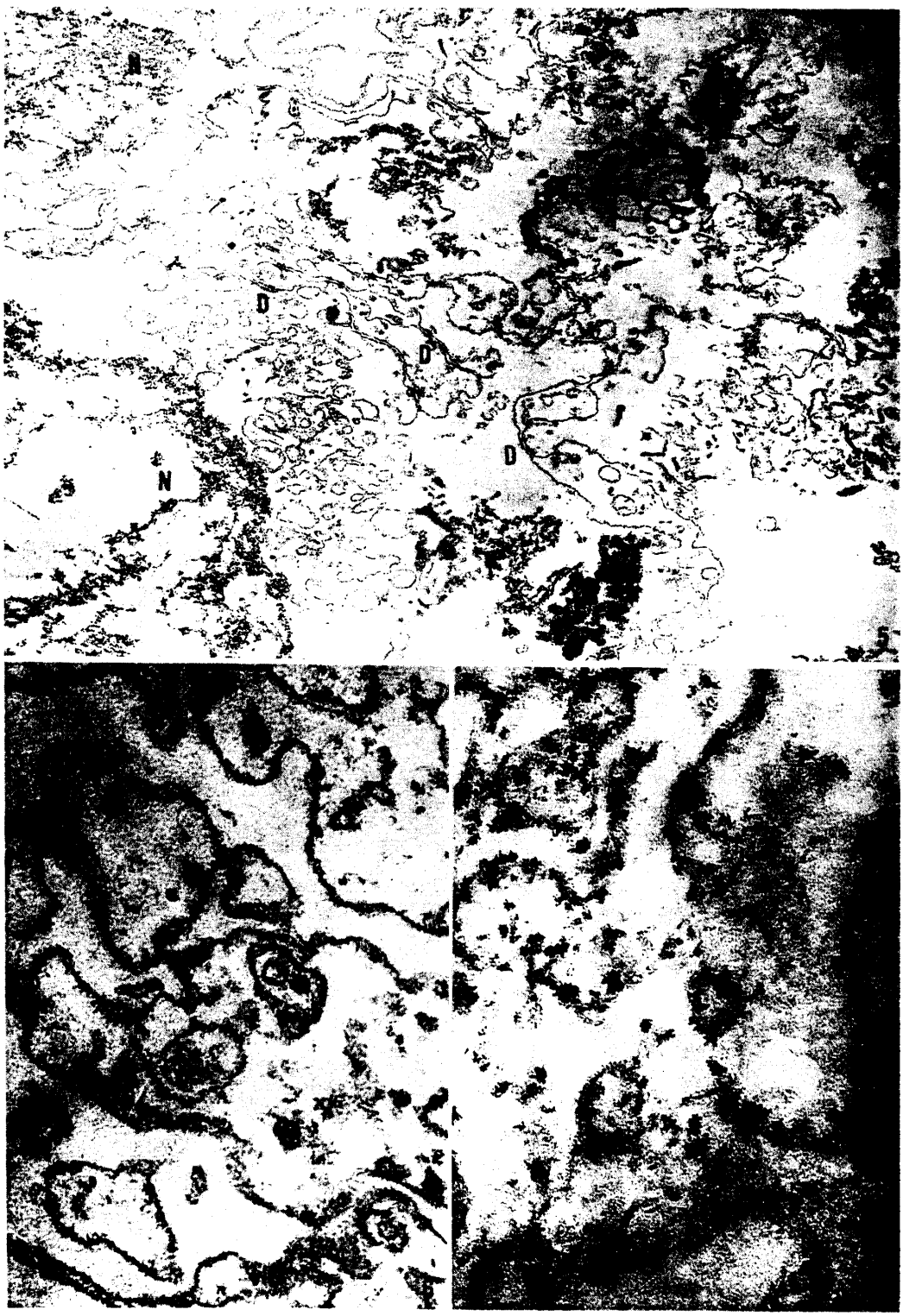
seen, but reaction product is not. × 56,000

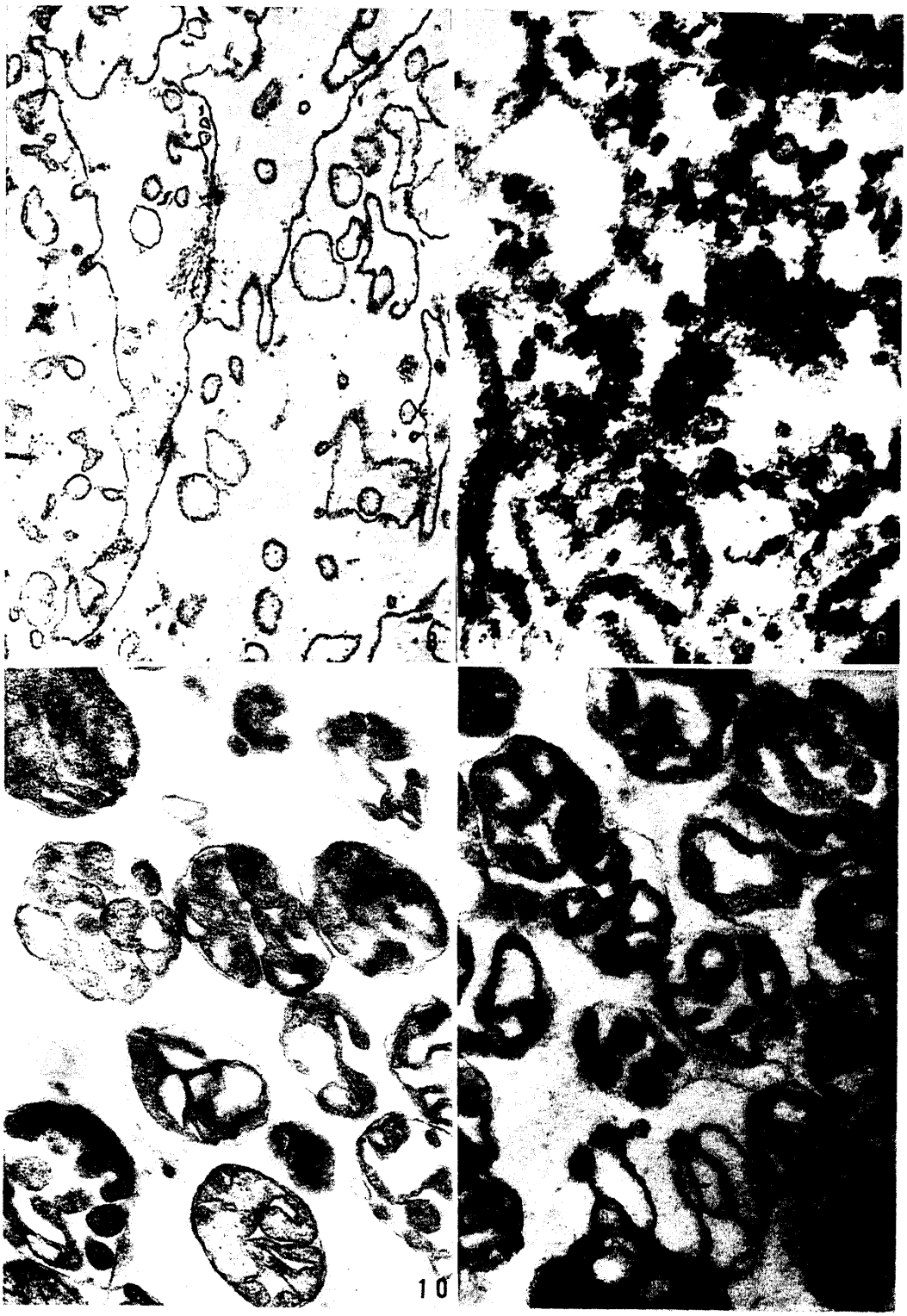
Fig. 18 An electron cytochemical demonstration of magnesium ion-activated adenosine triphosphase of isolated nuclei of rat liver cell. Fixation: 5% glutaraldehyde, Post-fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. Reaction product is not seen. × 10,000

Fig. 19 An electron cytochemical demonstration of magnesium ion-activated adenosine triphosphatase of isolated nuclei of AH 130 cell. Fixation: 5% glutaraldehyde, Post-fixation: 1% OsO_4, Staining: uranyl acetate and lead citrate. Reaction product is not seen. × 12,800


M
M
M
2




N
D
D
D
N


10