Nagoya J. med. Sci. 34: 241-258, 1972

STUDIES ON STRUCTURE AND FUNCTION OF THE BIOLOGICAL MEMBRANE SYSTEMS

III. THE MODES OF ACTION OF CORTICOSTERONES ON THE ISOLATED SUBCELLULAR MEMBRANES*

YOSHIAKI ITATSU

Department of Pathology, Nagoya University School of Medicine
(Director: Prof. Kentarō Takagi)

ABSTRACT

The modes of action of corticosterones on the isolated subcellular compartments of mitochondria, lysosomes and red cell ghosts were studied by using chiefly hydrocortisone and 11-desoxycorticosterone (11-DOC) at higher concentrations than physiological ones (below 10^{-6} M). The changes of those biological membrane systems were examined both electron-microscopically and biochemically. The ultrastructural alterations of the membrane structures corresponded to the changes of the enzyme activities in both radical and ion reactions which were directed or controlled in the subcellular membranes.

$10^{-4}-10^{-3}$ M 11-DOC induced the following changes; (a) respiratory inhibition and uncoupling of oxidative phosphorylation in mitochondria, (b) configurational changes in mitochondrial inner membrane to orthodox type involving swelling of mitochondria, (c) release of acid hydrolytic enzymes and fragmentation of lysosomal membranes, (d) inhibition of activities of Na^+, K^+-dependent or Mg^{2+}-activated ATPase, (e) dislocation and micelles formation of lipid in the membrane and electron dense body formation on the surface of the membrane, (f) fusion and aggregation of membrnes.

Hydrocortisone or cholesterol showed similar effects, to less degrees, as a result of non specific actions at high concentrations (10^{-3} M), but hydrocortisone had a tendency to stabilize the lysosomal membranes. The different mode of action of 11-DOC and hydrocortisone on the membrane may induce the different changes of the high ordered structures in the membraneous polymers which are composed of proteins and lipids.

INTRODUCTION

During the last decade, the properties and functions of the cellular membranes have been one of the most actively explored fields in biological sciences.

板津義昭

Received for publication June, 14, 1971

* A part of this report was presented at the 59th general meeting of the Japanese Pathological Society at Kyoto, 1970.

The cellular membranes are now well established to be highly organized with dynamic structures, based upon the interaction of their lipids and proteins, in which many enzymes are located or enmeshed and essential parts of the control mechanisms are affected on the membranes by their activities. Considerable information is now available from electron microscopy, combined with biochemical and biophysical analysis. Some attempts[44) 34)] have been made to explain hormonal effects on the membrane-linked enzymes' activities.

The author tried to study the action of hormones on the membrane systems, with relation to the corresponding changes in fine structure and biochemical function.

Recently, it has been assumed that the effects of many hormones on their target cells appear to be mediated through activation of adenyl cyclase, the enzyme which is bound to the surface membrane[6)]. By stimulation of the adenyl cyclase activity it increases to catalyze the conversion of adenosine triphosphate (ATP) to adenosin-3′, 5′-mono-phosphate (cyclic AMP) and inorganic pyrophosphate (PPi). The cyclic AMP so formed is believed to bring about changes characteristic of the hormone as secondary messenger[42)]. If these hormonal effects on the target cells are named "specific action" of the hormone, it is also supposed to happen that hormonal effects are not mediated by a direct hormone-enzyme interaction, but that hormones act upon a higher order of the cellular compartment, probably, upon the framefork in which the enzymes are enmeshed. The author named the above mentioned latter effects "non-specific action" of the hormone, influenced upon the membrane-bound protein's activities which may constitute the controlled systems through the membrane's function and structure.

Although there are also a few reports on the role of cyclic AMP to implicate the adenyl cyclase system in the mediation of the effects of the steroid hormones[43) 38) 40)], corticosterones appear to have no evident target cell, so far as mineral- and glucocorticoid are concerned. The "non-specific" effects of corticosterones on biological membrane systems were studied, using pharmacological or higher concentrations than physiological one (usually below 10^{-6} M).

The following abbreviations are used in this paper:
ATP: adenosine triphosphate; ADP: adenosine diphosphate; Pi: inorganic phosphate
ACTH: adrenocorticotropic hormone; 11-DOC: 11-desoxycorticosterone;
RCG: red cell ghost;

$$\text{Respiratory Control Index (R.C.I.)} = \frac{\text{Rate of oxygen consumption with ADP}}{\text{Rate of oxygen consumption without ADP}} = \frac{\text{Rate of state 3 respiration}}{\text{Rate of state 4 respiration}}$$

$$\text{ADP/O} = \frac{\mu \text{ moles of ADP added}}{\mu \text{ moles of oxygen consumption in state 3}}$$

MATERIALS AND METHODS

1) Reagents

Most of the chemicals used in the incubation mediums and protein determinations were of analytical grade and were purchased from the usual commercial sources. 11-Desoxycorticosterone (11-DOC), Hydrocortisone (HC) and other steroid hormones were purchased from Sigma Chemical Co. ACTH was purchased from Daiichi Pharmaceutical Co. Steroid chemicals were dissolved in 100% ethanol to give 100 mM solution, and were applied at the diluted concentration of 10^{-5}–10^{-2} M in ethanol. Since steroid chemicals are virtually insoluble in saline, it was necessary to standardize the mode of addition of steroid hormones to the cellular membranes. Reproducible results were obtained when an ethanolic solution of steroid hormones (1–2%) was pippeted into a measured volume (3–5 ml) of the cell or subcellular suspensions and on the other hand an equal volume of ethanol was added in a similar manner to such suspensions as described above in control experiments. ACTH was completely soluble in the reaction medium and applied at the diluted concentration of 10^{-2}–1 unit in the incubation medium comparing with control or steroid experiments.

2) Fractionation Procedure

Mitochondria were isolated from rat livers by the modified method of Chance-Hagihara[2]. Rat livers were homogenized in a polytefron type homogenizer, suspended in 0.1 mM EDTA 0.21 M Mannitol and 0.07 M Sucrose, and washed and centrifuged at 8,000 × g three times.

Lysosomes were isolated from rat livers by the method of Ragab, H. *et al.*[33] Rat livers were homogenized in a polytron type homogenizer, suspended in 1 mM EDTA, 0.25 M Sucrose (pH 7.4), and then, centrifugal fractionation of homogenate was done, using a density gradient centrifugation method. The gradients were prepared in three discrete layers which were as follows, from bottom to top;

Gradient I. 0.6 M sucrose 70 ml, 0.45 M sucrose 60 ml, 0.30 M sucrose 50 ml in 0.5 mM EDTA (pH 7.4).

Gradient II. 0.7 M sucrose 70 ml, 0.6 M sucrose 60 ml, 0.45 M sucrose 50 ml in 0.5 mM EDTA (pH 7.4).

Red cell ghosts were prepared from rabbit's blood, with 0.1 M Na_3-citrate added as anticoagulant (5 ml/40 ml whole blood), and were isolated by the method of Marchesi and Palade[24].

3) *Radioactive Assay*

To the suspensions of red cell ghosts (0.5 mg/ml), 10 μci/0.1 mg. albumin of I^{131}-human serum albumin (Daiichi Radioisotope Co.) was added and the reaction mixtures were incubated at 37°C for 10 min. and then, after centrifuga-

tion at 1,5000×g, the pellets were washed with the following solution, three times. The final membrane-supensions were measured for I^{131}-radioactivities in a well-type Scintillation counter. The reaction mixtures contained 140 mM NaCl, 6 mM KCl, 2 mM $MgCl_2$, 3 mM glucose and 10 mM Tris-HCl (pH 7.4)

4) Electron Microscopy

The ultrastructural changes of the subcellular specimen were examined both with thin sections and negatively stained preparations. The specimens were fixed with 2% glutar aldehyde in 0.05 M cacodylate or phosphate buffer with 0.25 M sucrose added (pH 7.4). After 1 hour, they were washed in the 0.05 M cacodylate buffer (pH 7.4) with 0.25 M sucrose solution over 5 hours, and the pellets of the specimens were collected by centrifugation and then post-fixed in 1% osmium tetroxide, buffered with cacodylate or phosphate (pH 7.4). The post-fixed specimens were dehydrated in graded alcohols and embedded in Epon mixture (Luft.[23]). The ultra-thin sections were obtained with glassknives by a Sorvall MT-2 Ultramicrotome. The sections were mounted on carbon-coated grids, and contrasted with uranyl acetate and Reynolds' lead[36]. Photographs were taken with initial magnifications of 5,000–20,000–50,000, with a Hitachi 11 DS electron microscope, operating at 50–75 kv. Negative staining of the specimens was pursued by means of the procedure of Harris[17] with 2% phospho-tungstate, adjusted to pH 7.4 with KOH or NaOH.

5) Biochemical Studies

Protein determinations of each fraction prepared as described previously under Fraction procedure were made by a modification of the Biuret procedure as mg. protein per ml [11]. The proteins, released from the isolated lysosomes, were also measured by this Biuret procedure.

Mitochondrial respiration and Oxidative phosphorylation

The oxygen consumption by mitochondrial respiration was measured by using a Beckman's polarographic oxygen electrode (Clark-type) and a potentiometric recorder. Respiratory control index (R.C.I.) and ADP/O ratio were calculated by the method of Chance-Williams[3)4)]; Respiratory control ratio of a sample was expressed as the ratio of the respiratory rate after addition of ADP (respiratory state 3) to that before its addition (respiratory state 4). The ADP/O ratio was estimated from the amount of ADP added and of oxygen consumed in the respiratory state 3. The final volume of the reaction vessel was adjusted to 5.0 ml by the addition of mitochondrial suspension to the air saturated reaction medium, which contained 0.3 M mannitol, 10 mM KCl, 10 mM KH_2PO_4 (pH 7.4), 2.5 mM $MgCl_2$, 0.25 mM EDTA, and 8 mM succinate or 4 mM pyruvate plus 0.4 mM malate as substrate. The oxygen consumption of mitochondria in the presence of 2% ethanol was taken as control, because the effect of 2% ethanol on the oxygen consumption of mitochondria was almost

negligible in comparison with the remarkable effect of the steroids dissolved in the same volume of ethanol. Other specific conditions employed are given in the table or legends for figures.

ATPase Assay

The reaction solution contained the washed membrane suspensions (4 mg. protein/ml.), the reagents for test (in 10^{-3} M and 10^{-5} M corticosterones) 2 mM ATP (Mg-salt, pH 7.4), 5 mM $MgCl_2$, 140 mM NaCl, 20 mM KCl, 1 mM EDTA and 20 mM Tris buffer (pH 7.4). When the Na^+, K^+-independent-Mg^{++}-ATPase was assayed, 140 mM NaCl was replaced by 0.25 M sucrose in the reaction solution, as described above. The reaction was initiated by adding the membrane suspensions to the assayed tubes at 37°C. and after incubation at 37°C for 30 min., the reaction was stopped with ice-cold 10% trichloracetate or phosphotungstate. The protein precipitate was centrifuged off and the released inorganic phosphate (Pi) of the supernatant was quantitated by a modification of Martin-Doty's method[26]. In each experiment the appropriate control tests were performed in which either one of the following factors was omitted or conditioned; membrane supension (enzyme), ATP (substrate), Test reagents (11-DOC and HC) and at 0 time.

Acid phosphatase and β-Gluculonidase Assay

The suspensions of the isolated lysosomes were incubated in 0.25 M sucrose and 0.5 mM EDTA (pH 7.4) at 37°C for 30 min., and then the reaction mixtures were centrifuged at 20,000 ×g. for 10 min.. The supernatants were measured their total volume and then, concerning the acid hydrolytic enzymes released from lysosomal fractions, acid phosphatase was estimated by a minor modification of Neil and Horner's method[29][19], while β-Gluculonidase was measured by PMG method[25].

EXPERIMENTAL RESULTS

(1) *Alterations in mitochondrial function and structure*;

As indicated in Table 1, mitochondrial respirations were disturbed by various concentrations of steroids. The higher concentrations of the steroids were supplied in mitochondrial treatment, the lower R.C.I. and ADP/0 were taken, in the following turns; (11-DOC > hydrocortisone > cholesterol). These were lower than the non-treat control mitochondrial one. When the mitochondria were pre-treated with 10^{-3} M 11-DOC before being supplied with 4 mM pyruvate and 0.4 mM malate and 0.2 mM ADP, the mitochondrial respiration was inhibited intensely (Fig. 1). However, when the isolated mitochondria were treated with 10^{-3} M 11-DOC, before being supplied with 8 mM succinate and 0.2 mM ADP, oxygen consumption was only slightly retarded and the rate of oxidation of the substrate transitioned indistinctly from state 3 to state 4, in which almost

TABLE 1. The Effects of Various Steroids on the Respiration of the Isolated Mitochondria (Rat liver)

Steroids	Addition* (mM)	$-\Delta O$ (mμ Atoms/min./11 mg. prot. mito.)** State 4	State 3	R.C.I.	ADP/O
Control	(2% Ethanol)	9	57	6.2	2.3
Choiesterol	0.1	11	64	5.6	1.9
	1.0	17	63	3.7	1.9
	10.0	15	54	3.6	1.7
11-DOC	0.02	17	68	4.0	2.0
	0.1	23	71	3.1	1.9
	0.4	23	43	1.9	1.2
	1.0	29	32	1.1	uncoupling
Hydrocortisone	0.02	11	63	5.5	2.1
	1.0	17	63	3.7	1.9
	10.0	19	63	3.2	1.7

* 8 mM Succinate was supplied as substrate.

** calculated as 284 [O_2] μM were solubilized in distilled water at 25°C.

The medium contained 11 mg. protein of mitochondria and was composed of 0.3 M manitol, 10 mM KCl, 10 mM KH_2PO_4 (pH 7.4), 2.5 mM $MgCl_2$ and 0.25 mM EDTA. The final volume was 5 ml, in which time course of oxygen consumption in the prescnce of 2% ethanol as control was measured by the oxygen electrode and was compared with that in the presence of steroids dissolved in 2% ethanol.

the oxygen consumption remained invariable.

In the case of treatment with 0.5 unit ACTH, the rate of oxygen consumption was almost similar to that of the intact mitochondria but both R.C.I. and ADP/O were lower compared with those of the intact mitochondria, when succinate was used as substrate, or pyruvate and malate.

This might mean that steroid hormones played an inhibitory role on the coupling mechanism among the sites of mitochondrial respiratory chain with some significantly different effects from the true uncoupling reagent; dinitrophenol (DNP).

The treatment of such hormones as 11-DOC and ACTH could induce some remarkable changes in the ultrastructure of the mitochondria. In the presence of substrate and inorganic phosphate, the isolated mitochondria demonstrated the particular structure of the inner membrane, so-called energized-twisted configuration which was characterized by twisted tubular inner membrane system enveloping matrix space. This configuration could be also observed even in the presence of ethanol at low concentration within 2 percent which was usually added to the reaction mixture as control preparation (Photo 1). When mitohondria were treated with hormones, the inner membrane of mitochondria lost the capacity to demonstrate the energized-twisted configuration, even the

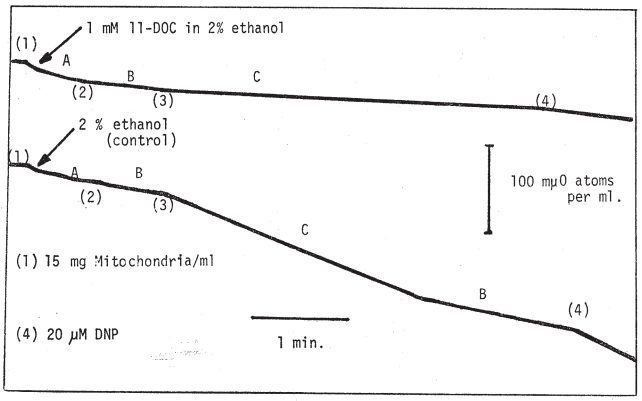


FIG. 1. Polarographic traces of hormonal effects on O_2 consumption of the isolated Mitochondria (male rat liver)

(The oxygen consumption was measured polarographically with oxygen electrode (Beckman's oxygen sensor) at 25°C. The incubation medium contained 15 mg. protein per ml of mitochondria and was composed of 0.3 M mannitol, 10 mM KCl, 10 mM KH_2PO_4 (pH 7.4), 2.5 mM $MgCl_2$ and 0.25 mM EDTA. 4 mM pyruvate and 0.4 mM malate (2) were used as substrate for respiration and 0.2 mM ADP (3) was supplied as phosphate acceptor for oxidative phosphorylation. Pretreatments by the reagents were indicated by the arrows, soon after 15 mg. protein per ml of mitochondria were supplied in the medium (5 ml finally).

A: state 1 (Endogenous respiration)
B: state 4 respiration
C: state 3 respiration

presence of both substrate and inorganic phosphate. They appeared swollen and in so-called orthodox configuration which consisted of small intracristal space, large volume and low electron density of matrix space (Photo 2, 3). There were also seen swellings of mitochondria at high concentration of hormones.

Both treatment of 11-DOC and ACTH had similar effects on ultrastructure of mitochondria. When treated with 10^{-4} M 11-DOC, it was characteristic for mitochondria to show a tendency to aggregate or join together through some electron dense material on the surfaces of the outer membranes (Photo 3). The mitochondria which were treated with 10^{-3} M 11-DOC appeared more swollen than those treated with 10^{-4} M 11-DOC.

ACTH at 1 unit of the concentration could induce marked mitochondrial swelling, dislocation of the outer membrane and aggregation among the mitochondria (Photo 3).

(2) *Alteration in lysosomal function and structure*;

After being treated with 2% ethanol at 37°C for 30 min. (in control experiment), the isolated lysosomes showed the released activation of acid phos-

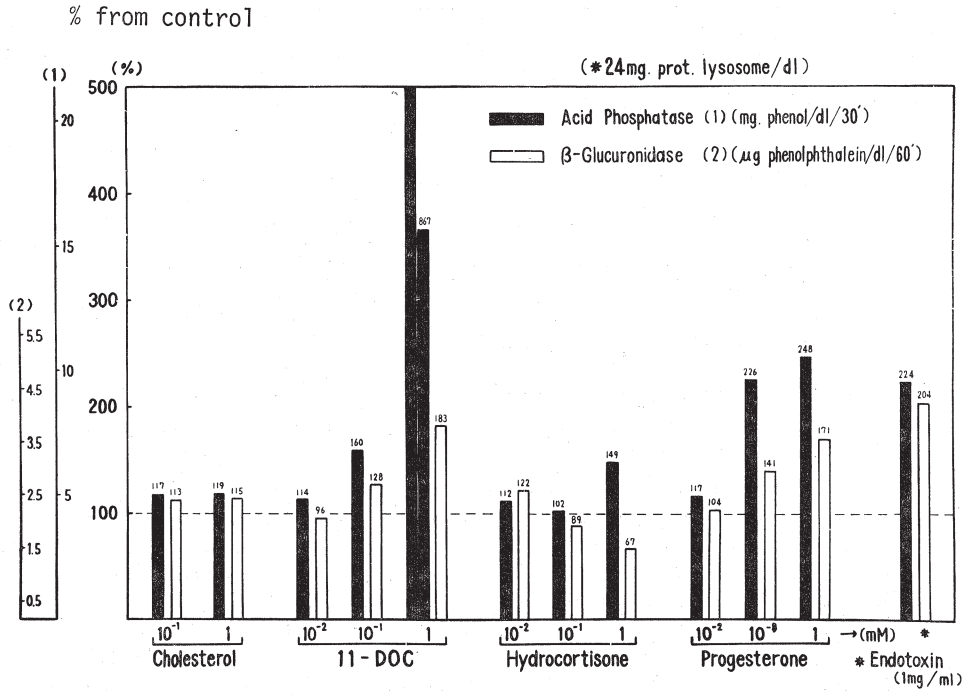


FIG. 2. Release percent of acid hydrolase from the isolated lysosomes* (male rat liver) after hormonal treatment (30 min.)

* The isolated lysosomes were incubated in 0.25 M sucrose and 0.5 mM EDTA (pH 7.4), at 37°C for 30 min., then, centrifuged at 20,000 ×g. and the released enzymes' activities of the supernatant were measured and calculated.

phatase (4.2 mg. phenol/24 mg. lysosome protein/dl/30 min.) and of β-gluculonidase (2.25 mg. phenolphthalein/24 mg. lysosome protein/dl/60 min.), which were indicated in 100% respectively, in Fig. 2. After the treatment with steroids for 30 min. at 37°C, the lysosomal pellets released protein into the supernatant and the total released proteins were estimated by the Biuret procedure[12]. When treated with 10^{-3} M 11-DOC, the lysosomes (7 mg. protein) released maximum 3.8 mg. protein into the supernatant, while the lysosomes (7 mg. protein) of control experiment released 1.3 mg. protein during incubation at 37°C for 30 min. The height of the columns on Fig. 2 represents the activities of the released lysosomal enzymes, such as acid phosphatase and β-gluculonidase, after 30 min-treatment by various steroids, while the centrifuged sediments of lysosomal remnants were observed electron-microscopically with thin sections (Photo 4, 5, 6, 7). When treated with 10^{-5} M 11-DOC, the lysosomal pellets showed destruction of the lysosomal membranes with their fragmentation or

small vesicles formation (Photo 7), but in the case of treatment with 10^{-5} M hydrocortisone or 0.01 unit ACTH the lysosomal destruction was less remarkable (Photo 5, 6). Generally, these hormonal releases of the lysosomal acid hydrolases might be induced by non-specific actions of hormones because a large amount of progesterone or endotoxin induced also activation of these enzymes in the supernatant. However, cholesterol did not release much acid hydrolases, and hydrocortisone had more tendency to inhibit than to enhance released activation of these enzymes. The mode of action of steroids on lysosomal membranes was slightly different and characteristic according to the difference of their molecular constitutions.

(3) *Alterations in function and structure of plasma membranes*;

Table 2 shows that red cell ghosts enhanced up-take of I^{131}-albumin when the medium was conditioned under the absence of Na^+ or treatment with 11-

TABLE 2. Influences on the Up-take of I^{131}-Bovine Serum Albumin in the Rabbit's Red Cell Ghosts

Medium condition**	Addition		c.p.m. $\times 10^3$	(%)	up-taken I^{131}-albumin*
	11-DOC (mM)	Cortisol (mM)			
140 mM Na^+ K^+ Free	0	0	12.8	102	67.5
20 mM K^+ Na^+ Free	0	0	32.7	260	172.3
140 mM Na^+ 20 mM K^+	0	0	12.6	100	66.4
	10^{-2}	0	22.3	177	117.5
	10^{-1}	0	15.0	119	79.0
	1	0	4.4	35	23.2
	0	10^{-2}	9.7	77	51.1
	0	10^{-1}	9.6	75	49.8
	0	1	11.2	89	59.1

* represented as mμ g. prot./R.C.G. mg. prot./10 min.

** pre-incubated for 10 min. in the same medium, added to 6 mM ATP, 6 mM $MgCl_2$, 3 mM glucose, mM 20 PO_4^{--}, and 20 mM Tris-Buffer (pH 7.4), finally.

DOC (10^{-5} M), but that hydrocortisone inhibited this up-take. The influences of corticosterones on the activities of (Na^+, K^+)-dependent or (Na^+, K^+)-independent, Mg^{++}-activated ATPase of the sonicated red cell ghosts are demonstrated in Table 3. 11-DOC inhibited the activities of (Na^+, K^+)-dependent, Mg^{++}-ATPase, more intensely than hydrocortisone, and at 10^{-3} or 10^{-5} M concentration, both of these corticosterones inhibited the activities of the membrane-Mg^{++}-activated ATPase, whether they depended on Na^+ and K^+ or not. The ultrastructural changes of red cell ghosts induced by 10^{-4} M 11-DOC re-

TABLE 3. Activities of (Na^+, K^+)-dependent and (Na^+, K^+)-independent Mg^{++}-activated ATPase of the sonicated preparation of the red cell ghosts; The assay media contained 140 mM NaCl, 20 mM KCl, 6 mM $MgCl_2$, 2 mM Mg-ATP and 20 mM Tris buffer (pH 7.4). Na^+, K^+-independent, Mg^{++}-ATPase was assayed in the medium, which replaced 140 mM NaCl by 0.25 M sucrose

Treatment	Concentration	(Na^+K^+)-dependent, Mg-ATPase*	(Na^+K^+)-independent, Mg-ATPase*
Control	0	403±120	223± 8
11-DOC	10^{-5} M	216± 4	203±10
	10^{-3} M	205± 14	207±18
Hydrocortisone	10^{-5} M	325± 32	196±24
	10^{-3} M	252± 12	208±29

* Represented by released Pi (mμ moles/mg. prot. R.C.G./30 min.)

vealed flattened deformity, and aggregation with complex wave-like configurations (Photo 8). The fibrillar material was observed on the inner surface of the unit membranes (arrow). Occasionally, the aggregated membranes were found to be rearranged with their components (Photo 9). When negative staining of the red cell ghosts incubated with 10^{-3} M 11-DOC for 10 min. at 37°C was done the outer surface of the membranes revealed the globular lattice structures and grooves (Photo 10–13 a), and they appeared much clearer than in the treatment by 10^{-3} M hydrocortisone. When treated, especially with 10^{-3} M 11-DOC, the membrane released its component from the surface, which were rearranged to form the micelle's structure (Photo 13 b).

DISCUSSION

According to Davson-Danielli[5], the cellular membrane is viewed as a hydrophobically bounded phospholipid bilayer continum, to which the extended protein are attached electrostatically. However, Green and Fleisher[12] showed that in biological membranes, phospholipid molecules are bounded hydrophobically to protein. The biochemical and electron microscopic study of a variety of biological membranes has led us to the view of a membrane not as a continuum but as a composite of nesting lipoprotein repeating units[9) 13) 27)].

On electron microscopic studies osmium tetroxide (OsO_4) or phosphotungstic acid (PTA) are widely used as a fixative and as a stain, and these heavy metal atoms are considered to be deposited in the sample and to produce electron dense areas. Various sites such as the double bond of unsaturated fatty acid molecules, polar regions of phospholipids and layers of protein have all been suggested as sites of OsO_4 deposition. Stoeckenius[41] concluded from his accumulated evidence that the osmic acid is taken up in the region of the phospholipid polar groups rather than the alkyl chain region.

The properties and functions of the cellular membranes, undoubtedly, are based upon the interaction of their lipids and proteins. The plasma membrane consists of approximately 40% lipid and 60% protein[47]. Most of the membrane lipids consist of neutral lipids, phospholipid and cholesterol. The proteins are organized in high dimensions and modified, in dynamic functions and structures, by hydrophobic bond or coulobic forces of associated lipids, which form membrane structure or micelle, based essentially on the physico-chemical properties. The membrane-bound protein is structurally organized and shows activities of enzymic and other parameters[34]. As is evident from the recent reports on cytochemical works with electron microscopy[24) 35)] and biochemical studies of isolated membranes[8) 6)], the available evidence shows that the Mg^{2+}-ATPase, Na^{+}, K^{+}-Mg^{2+}-ATPase or adenyl cyclase are associated with the plasma membrane.

The observed ATP-splitting activity reflects a more complex process or processes concerned not only with active transport, but also with contraction and relaxation of mitochondria, myofibrils and, perhaps, membrane systems in general.

The steroid hormones are suspected to combine or act on the membraneous polymers in a similar manner, as cholesterol is taken up within the cell membrane. The steroid molecule and nucleus can be pictured as rather cylindrical in shape and as intercalated between the hydrocarbon chains of the phospholipids, according to the usual shema, with-OH group "anchored" in the aqueous phase or complexed with the base of a phospholipid. (Fig. 3). It may be assumed that the polar head group region of the lipids forms a relatively rigid "floor" to which the -OH group of the steroid is anchored[20]. Cholesterol molecule has only one polar region at A-ring, but corticosteroid contains polar regions, both at A-ring and at the converse side of steroid molecule. Cortisol or corticosterone contain "-OH" at C_{11}-(α), and are related to gluconeogenetic metabolism and membrane "stabilizer", but 11-DOC lacks in hydroxyl of C_{11}

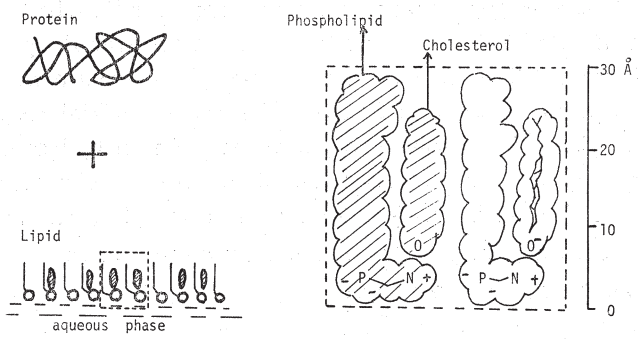


FIG. 3. Diagramatic representation of membane lipids*
(*cited by F. A. Vandenheuvel.)

and is related to mineral metabolism and membrane "labilizer". The molecular structures of corticosterones are characteristic of ketol side chain (-CO-CH_2-OH) at C_{17} which has a powerful reducing process.

ACTH is hydrophilic and composed of a poly-peptide-chain of 39 amino-acids, in which four basic aminoacids (-Lys-Lys-Arg-Arg-) are suspected electrostatically to interact with the membrane's protein. On the other hand, steroid hormones reveal hydrophobic, but fundamentally, amphilic properties and do not solubilize the membranes so surface-actively as a detergent as deoxycholate.

1) Mitochodrial Membrane and Oxidative Phosphorylation

It has been investigated that isolated liver or heart mitochondria undergo reversible ultrastructural transformations during changes in the respiratory states[14) 15)]. Major transformations were observed in the manner of folding of the inner membrane and in the volume and in the electron density of the matrix[15)]. It was postulated that conformational energy generated by electron transport is the immediate source of energy for the synthesis of ATP during oxidative phosphorylation[16)]. When mitochondria in the energized configuration are exposed to the action of uncouplers, they undergo a rapid transition into the non-energized configuration. Mitochondrial respiration is controlled by substrate, oxygen concentration, supply of inorganic phosphate and especially supply of ADP or AMP.

Respiratory Control Index is a most sensitive indicator for the tightly coupled state between mitochondrial structure and function. Treatment with 0.1 unit ACTH induced the lowered efficiency of both respiratory control and oxidative phosphorylation, but preserved a loosely coupled state. However, treatment with 10^{-3} M 11-DOC lost the efficiency of respiratory control and induced respiratory release (uncoupling). This process inhibited phosphorylation of ADP, induced depletion of ion and is followed by matrix swelling.

When pyruvate and malate were used as substrate, respiratory control was not so sensitive as when succinate was used as substrate. However, as illustrated in Fig. 1, the electron transfer system was found to be affected by inhibitory effect of 10^{-3} M 11-DOC, but this effect disappeared by adding to succinate as substrate. The results demonstrate that treatment with 11-DOC (10^{-3}–10^{-4} M) caused multiple defects in the mitochondrial respiratory chain and uncouples oxidative phosphorylation, as reported on hydrocortisone also[21)]. It may be that inhibited or disturbed loci of respiratory chains by corticosteroids seem to accord with lipid-associated portion which is hydrophobically bound to the repeating units of the inner membrane and that ACTH may interfere with polar sites of membrane.

2) Lysosomal Membrane and Released Activation of Acid Hydrolase

As pointed out by de Duve and Wattiaux[7)] the cytochemical definition of

lysosomes as particles that are bounded by a single "unit membrane" and have a positive staining reaction for acid phosphatase may be considered for most practical purposes as the equivalent to the biochemical definition[30]. Treatments with surface active agents act primarily by causing an injury to the particles' membranes. which then allow the external substrates to penetrate within the particles as well as the internal enzymes to leak out, thus accounting for the simultaneous activation and solubilization of the enzyme[1]. The lipid micelles may be replaced by globular proteins and in this way enzymes may be an integral part of the structure of the lysosomal membrane[22]. The normally latent enzymes of lysosomes may exist in different iso-enzyme forms and certain of these isozymes may be strongly membrane-bound, while others are freely diffusible within lysosomes.

At the later stage of enzyme release after hormonal treatment, lysosomal particles showed a loss of their electron opaque matrix, and small membraneous vesicles of fragmentations inside the lysosomal "ghost" were observed, especially in the lysosomal pellets which were treated with 10^{-4} M 11-DOC for 30 min, at 37°C. 10^{-3} M 11-DOC treatment induced the markedly increased release percent of acid phosphatase but treatment by 10^{-4} M 11-DOC induced a little release of acid phosphatase as illustrated in Fig. 2. This may mean that acid phosphatase is bound to small membraneous vesicles or fragmentations and is liberated in such soluble forms as "free enzyme" or "enzyme release" from labilized membraneous fragments. Weissman suggested that it might not be necessary to postulate any role for membrane proteins in order to account for the action of steroids on the stability of the lysosomes, since these hormones have comparable actions on dispersed particles of phospholipid[22].

10^{-4} M cortisol inhibited the release of acid phosphatase and β-gluculonidase. This may indicate the "stabilization" of the lysosomal membrane but 10^{-3} M cortisol labilizes the membrane. Otherwise, it was also observed that treatment by progesterone showed more considerable release of these enzymes.

3) Plasma Membrane and Adenine Nucleotide Metabolism

It is self-evident that hemolysis *in vitro* indicates a pathologic alteration in the erythrocyte membrane which permits the escape of hemoglobin but the hemoglobin-free ghost membranes are again reconstituted functionally and structurally under physiologically appropriate conditions.

It was observed that the red cell ghosts took up I^{131}-albumin and invaginate in the presence of exogenous ATP[28].

As illustrated in Table 3, various factors influence the up-take of RCG. Especially, Na^+, K^+ and Ouabain seem to have a major influence on contracting and relaxing mechanisms at or subjacent to the membrane. 11-DOC, above 10^{-4} M, dislocated erythrocyte membrane's proteins or phospholipids so that the corticosteroid excess is thought to disorder the possible mechanism of

ATP-ase coupling to the carrier operation and ion transport as well as to the energy metabolism of the cell.

In terms of the membrane, anaerobic glycolysis must provide ATP as the energy source for the ouabain-sensitive, Na^+, K^+, ion pump of the membrane which is an essential factor in the control of the normal red cell volume[45]. The assumption of the location of the enzyme in the cell membrane is supported by experiments on red blood cells in which it has been shown that the (Na^+, K^+)-activated, Ouabain-sensitive enzyme system, is present in the membrane [10) 18) 48)], and that in the broken red cells, the enzyme is located in the membrane fragments[10) 18) 32) 46) 48)].

The author tried to estimate the difference of ATPase activities of the corticosterone-treated RCG, and the result revealed the inhibitory effects on both Na^+, K^+-dependent-Mg^{2+}-ATPase and Na^+, K^+-independent-Mg^{2+}-ATPase of the sonicated RCG.

Recently, A. S. Rosenthal *et al.*[39] described a possible role for the Ca^{2+}-dependent ATPase and the reticular fibrils of RCG in the maintenace of membrane elasticity and rigidity. On thin section of RCG treated with 10^{-4} M 11-DOC, the labilized or adhesive ghost membranes revealed flattened deformity but the inner aspect of the unit memrane preserved well the fibrillar material (arrows in Photo 9).

The functions of the former enzymes on the integrated membrane structure are thought to depend on membrane lipids and the effects of corticosterones involved the changes in the orientation of lipids, hydrophobically bound to the membrane proteins, and probably induced a partially different change in their relative position towards the membrane proteins. The difference of the chemical structures of corticosterones may be related to the difference of tendency for the apolar groups of the membrane to rearrange in such a way that they cluster together, extruding water and decreasing the free energy of the system as a whole.

ACKNOWLEDGEMENT

The author wishes to express his gratitude to Dr. Junpei Asai for his advice and collaboration throughout this study. He also is indebted to research members of Dept. of Pathology and Biochemistry, to Emer. Prof. Masasumi Miyakawa for their kind comments, technical assistance and encouragements, and to Prof. Kentaro Takagi and Dr. Nagako Itoh for their aids in the preparation of manuscript.

REFERENCES

1) Beufay, H. and de Duve, C., Tissue fractionation studies. 9, enzymic release of bound hydrolases, *Biochem. J.*, **73**, 604-609, 1959.

2) Chance, B. and Hagihara, B., Initiation of succinate oxidation in aged pigeon heart mitochondria, *Biochem. Biophys. Res. Comm.*, **3**, 1-5, 1960.
3) Chance, B. and Williams, G. R., The respiratory chain and oxidative phosphorylation, *Advances Enzymol.*, **17**, 65-134, 1956.
4) Chance, B. and Williams, G. R., Respiratory enzymes in oxidative phosphorylation. (1) Kinetics of oxygen utilization (VI), *J. Biol. Chem.*, **217**, 383-393, 1955.
5) Danielli, J. F. and Davson, H., A contribution to the theory of permeability of thin films, *J. Cellular Comp. Physiol.*, **217**, 383-393, 1955.
6) Davoren, P. R. and Sutherland, E. W., The cellular location of adenyl cyclase in the pigeon erythrocyte, *J. Biol. Chem.*, **238**.
7) de Duve, C. and Wattiaux, R., Functions of lysosomes, *Ann Rev. Physiol.*, **28**, 435-492, 1966.
8) Emmelot, P. and Bos, C. J., Studies on plasma membranes, III, Na^+-K^+-Mg^{2+}-ATPase, and 5'-nucleotidase activity of plasma membranes isolated from rat liver, *Biochim, Biophys, Acta.*, **120**, 369-382, 1966.
9) Fernández-Morán, H., Oda, T., Blair, P. V. and Green, D. E., A macromolecular repeating unit of mitochondrial structure and function, *J. Cell. Biol.*, **22**, 63-101, 1964. Correlated electron microscopic and biochemical studies of isolated mitochondria and submitochondrial particles of beef heart mucsle.
10) Glynn, I. M., Activation of adenosine triphosphatase activity in a cell membrane by external potassium and internal sodium, *J. Physiol.*, **160**, 18 p-19 p, 1962 a.
11) Gornall, A. G., Bardwill, C. J. and David, M. M., Determination of serum proteins by means of the biuret reaction, *J. Biol. Chem.*, **177**, 751-766, 1949.
12) Green, D. E. and Fleisher, S., The role of lipids in mitochondrial electron transfer and oxidative phosphorylation, *Biochim. Biophys. Acta*, **70**, 554-582, 1963.
13) Green, D. E. and Perdue, J. F., Membranes as expressions of repeating units., *Proc. Natl. Acad. Sci.*, **55**, 1295-1302, 1966.
14) Green, D. E., Asai, J., Harris, R. A., and Penninston, J. T., Conformational basis of energy transformations in membrane systems III. Configurational changes in the mitochondrial inner membrane induced by changes in functional states, *Arch. Biochem. Biophys.*, **125**, 684-705, 1968.
15) Hackenbrock, C. R., Ultrastructural bases for metabolically-linked mechanical activity in mitochondria I, Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria, *J. Cell. Biol.*, **30**, 269-297, 1966.
16) Hackenbrock, C. R., II. Electron transport-linked ultrastructural transformations in mitochondria, *J. Cell. Biol.*, **37**, 345-369, 1968.
17) Harris, J. R., The isolation and purification of a macromolecular protein component from the human erythrocyte ghost, *Biochim, Biophys, Acta.*, **188**, 31-42, 1969.
18) Hoffman, J. F., The link between metabolism and the active transport of Na in human red cell ghosts, *Fed. Proc.*, **19**, 127, 1960.
19) Hansen, P. Walloe, Simplification of Kind and King's method for determination of serum phosphatase, *Scandinav. J. Clin. Lab. Invest.*, **18**, 353-356, 1966.
20) Hubbel, W. L. and McConnel, H. M., Motion of steroid spin labels in membranes, *Proc. Natl. Acad. Sci.*, **63**, 16-22, 1969.
21) Kimberg, D. V., Loud, A. V. and Wiener, J., Cortisone-induced alterations in mitochondrial function and structure, *J. Cell. Biol.*, **37**, 63-79, 1968.
22) Lucy, J. A., *Lysosomes in Biology and Pathology* **2**, 313, 1969 edit by Dingle, J. T. and Fell; H. B., North Holland.
23) Luft, J. H., Improvements in epoxy resin embedding methods, *J. Biophys. Biochem, Cyotol.*, **9**, 409-414, 1961.
24) Marchesi, V. G. and Palade, G. E., The localization of Mg-Na-K-activated adenosine

triphosphatase on red cell ghost membranes, *J. Cell. Biol.*, **35**, 385-403, 1967.
25) Marsh, C. A. and Reid, L. H., Chemical synthesis of phenolphthalein β-glucuronide, *Biochim. Biophys. Acta.*, **97**, 597-599, 1965.
26) Martin, J. B. and Doty, D. M., Determination of inorganic phosphate, Modification of the isobutyl alcohol procedure, *Anal. Chem.*, **21**, 965-967, 1949.
27) McConnel, D. G., Tzagoloff, A., MacLennan, D. H., and Green, D. E., Studies on the eletron transfer system LXV, Formation of membranes by purified cytochrome oxydase, *J. Biol. Chem.*, **241**, 2373-2382, 1966.
28) Nagura, H., Itatsu, Y., Takahashi, Y., Katsumata, Y. and Asai, J., Biological membrane systems and substance-transport, *Seikagaku*, **41**, 175, 1969. (In Japanese)
29) Neil, M. W. and Horner, M. W., Studies on acid hydrolases in adult and foetal tissues, Acid p-nitrophenyl phosphate phosphohydrolases of adult guinea-pig liver, *Biochem. J.*, **62**, 217-224, 1964.
30) Novikoff, A. B., *The Cell*, **Vol. 2**, 423, 1961, edit. by Brachet, J. and Mirsky, A. E., New York, Academic Press.
31) Pohl, S. L., Birnbaumer, L. and Rodbell, M., Glucagon-sensitive adenyl cyclase in plasma membrane of hepatic parenchymal cells, *Science*, **164**, 566-567, 1969.
32) Post, R. L., Merrit, C. R., Kinsolving, C. R. and Albright, C. D., Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte, *J. Biol. Chem.*, **235**, 1796-1802, 1960.
33) Ragab, H., Beck, C., Dillard, C. and Tappel, A. L., Preparation of rat liver lysosomes, *Biochim. Biophys. Acta.*, **148**, 501-505, 1967.
34) Reid, E., Chapter 6, Membrane systems, in *Enzyme Cytology*, edit. by Roodyn. D. B., 1967. Academic press, London and New York, p. 321-406.
35) Reik, L., Petzold, G. L., Higgins, J. A., Greengard, P. and Barnett, R. J., Hormone-sensitive adenyl cyclase: Cytochemical localization in rat liver, *Science*, **168**, 382-384, 1970.
36) Reynolds, E. S., The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, *J. Cell Biol.*, **17**, 208-211, 1963.
37) Robertson, J. D., *Cellular Membranes in Development*, edit. by Locke, M., p. 1, Academic Press, New York, 1964.
38) Rosenfeld, M. G. and O'malley, B. W., Steroid hormones: Effect on adenyl cyclase activity and adenosine 3' 5'-monophosphate in target tissues, *Science*, **168**, 253-254, 1970.
39) Rosenthal, A. S., Kregenow, F. M. and Moses, H. L., Some characteristics of a Ca^{2+}-dependent ATPase activity associated with a group of erythrocyte membrane proteins which form fibrils, *Biochim. Biophys. Acta.*, **196**, 254-262, 1970.
40) Ryan, W. L. and Heidrick, M. L., Inhibition of cell growth *in vitro* by adenosine 3', 5'-monophosphate, *Science*, **162**, 1484-1485, 1968.
41) Stoeckenius, W., *The Interpretation of Ultrastructure*, p. 349, edit. by Harris, R. J. C., Academic Press, New York, 1962.
42) Sutherland, F. W., Robison, G. A. and Butcher, R. W., Some aspects of the biological role of adenosine 3', 5'-monophosphate (cyclic AMP), *Circulation*, **37**, 279-306, 1968.
43) Szego, C. M. and Davis, J. S., Adenosine 3', 5'-monophosphate in rat uterus, Acute elevation by estrogen, *Proc. Natl. Acad. Sci.*, **58**, 1711-1718, 1967.
44) Tepperman, J. and Tepperman, H. M., Some aspects of hormones on cells and cell constituents, *Pharmacol. Revs.*, **12**, 301-353, 1960.
45) Tosteson, D. C. and Hoffman, J. F., Regulation of cell volume by active cation transport in high and low potassium sheep red cells, *J. Gen. Physiol.*, **44**, 169-194, 1960.
46) Tosteson, D. C., Active transport, genetics, and cellular evolution, *Fed. Proc.*, **22**, 19-26, 1963.
47) Weinstein, D. B., *Biological Properties of the Mammalian Surface Membrane.* The Wistar Institute Symposium Monograph, No. 8, p. 17., edit. by Manson, L. A., Wistar Institute

Press., 1969.
48) Whittam, R., The asymmetrical stimulation of a membrane adenosine triphophatase in relation to active cation transport, *Biochem. J.*, **84**, 110-118, 1962.

EXPLANATIONS OF PHOTOGRAPHS

PHOTO 1. Energized-twisted configuration of a mitochondrion (×43,000), isolated from male rat liver. Mitochondrial suspensions were pretreated by 2% ethanol (control), and then added to 10 mM Pi, 8 mM Succinate and 0.2 mM ADP. At state 3 of respiration, fixed with 2% glutar aldehyde isotonic solution, then post-fixed with 1% OsO_4 solution.

PHOTO 2. Orthodox configuration of mitochondria at state 3 (×38,000), which were pretreated by 1 unit. ACTH in Fig. 1.

PHOTO 3. Swollen orthodox configuration of mitochondria at state 3 (3×38,000), which were pre-treated by 10^{-4} M 11-DOC in Fig. 1.

PHOTO 4. Lysosomes, isolated from male rat liver and incubated in the medium (0.25 M sucrose and 0.5 mM EDTA, pH 7.4), for 30 min. at 37°C (control), and then, centrifuged at 20,000×g.

PHOTO 5. Isolated lysosomes were added to 0.01 unit ACTH under the same condition as described above.

PHOTO 6. Isolated lysosomes were added to 10^{-2} mM hydrocortisone in 2% ethanol.

PHOTO 7. Isolated lysosomes were added to 10^{-2} mM 11-DOC in 2% ethanol and incubated similarly for 30 min. The lysosomal membranes fell into small vesicle formation and fragmentation. Arrow (a) or inlet (b) show fragmentation of a lysosomal granule.

PHOTO 8. Red cell ghosts of rabbits, treated by 10^{-4} M 11-DOC for 10 min. at 37°C. The membranes are zigzag or flat in shape, or adhere to each other (×23,000).

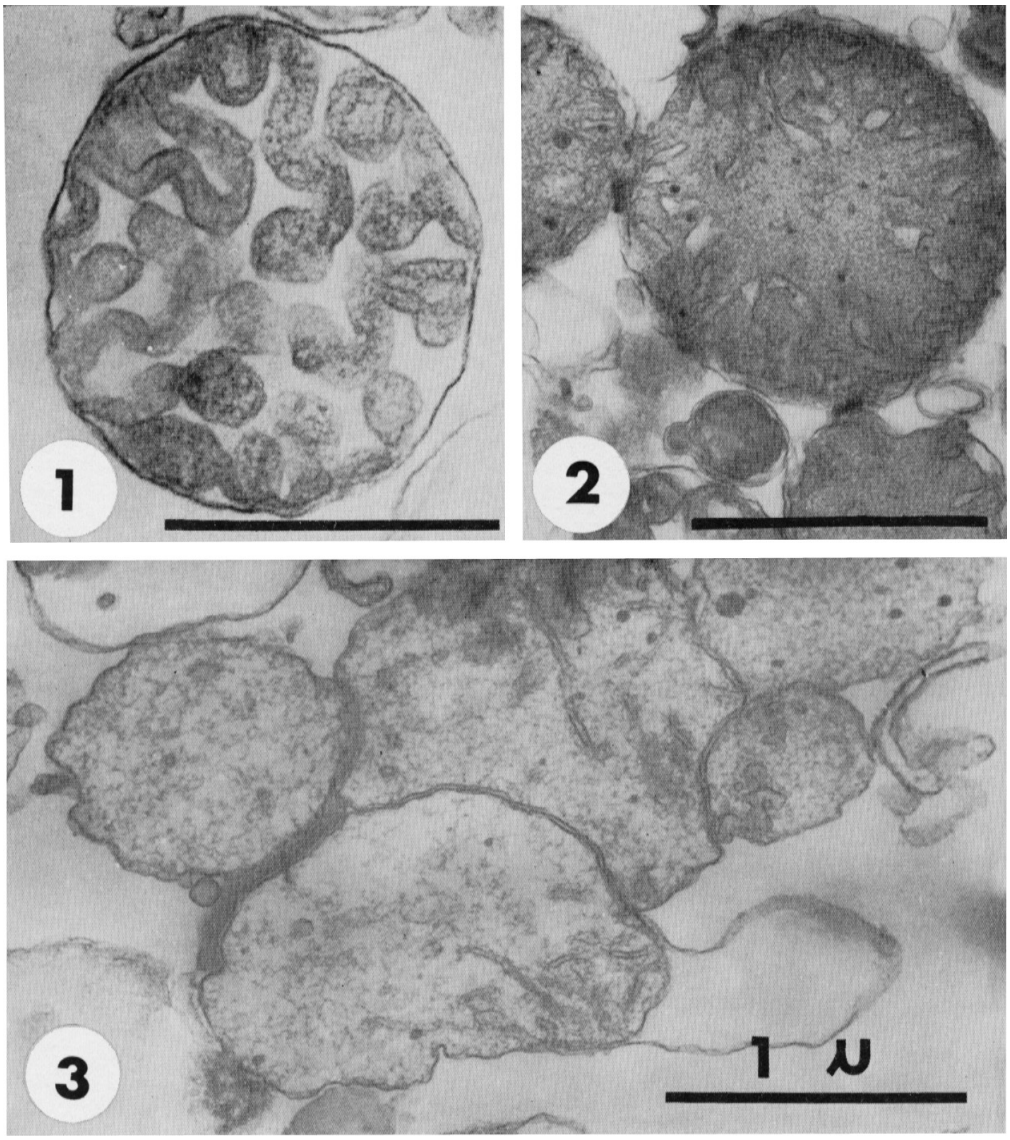
PHOTO 9. Red cell ghosts in the same condition as in Photo 8 aggregated and adherent to one another, have fallen into rearrangement. Arrows indicate the remnant fibrillar material on the inner aspects of the membranes (×24,000).

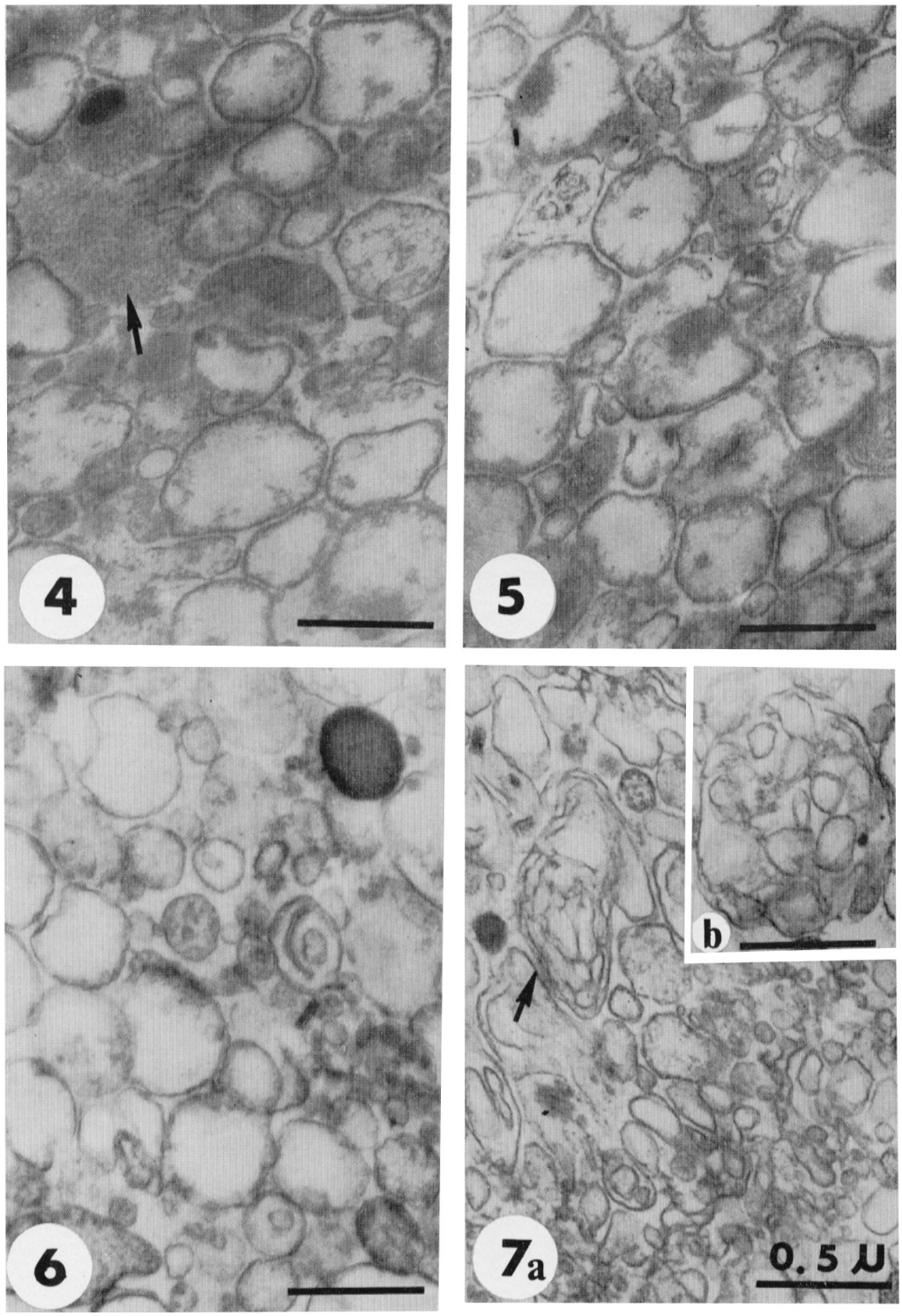
PHOTO 10. After treatment with 10^{-3} M 11-DOC for 10 min. at 37°C, negatively stained with 2% phospho-tungstate (pH 7.4) and observed electron microscopically. The surface of RCG was discovered to be globular and tortuous with deep grooves. Arrows indicate lipid's release from membrane components (×35,000).

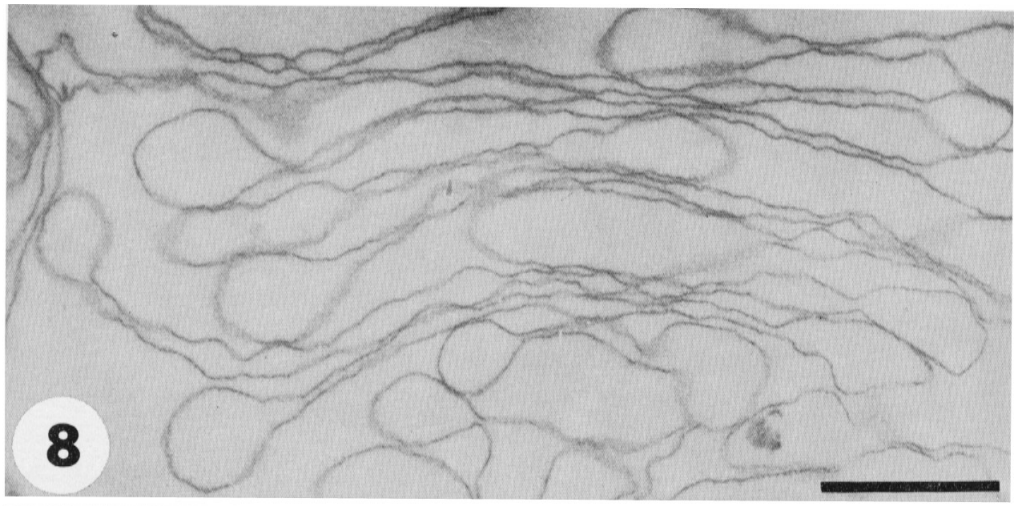
PHOTO 11. The surface configuration of RCG, treated by 2% ethanol for 10 min. at 37°C, (control), negatively stained (×100,000)

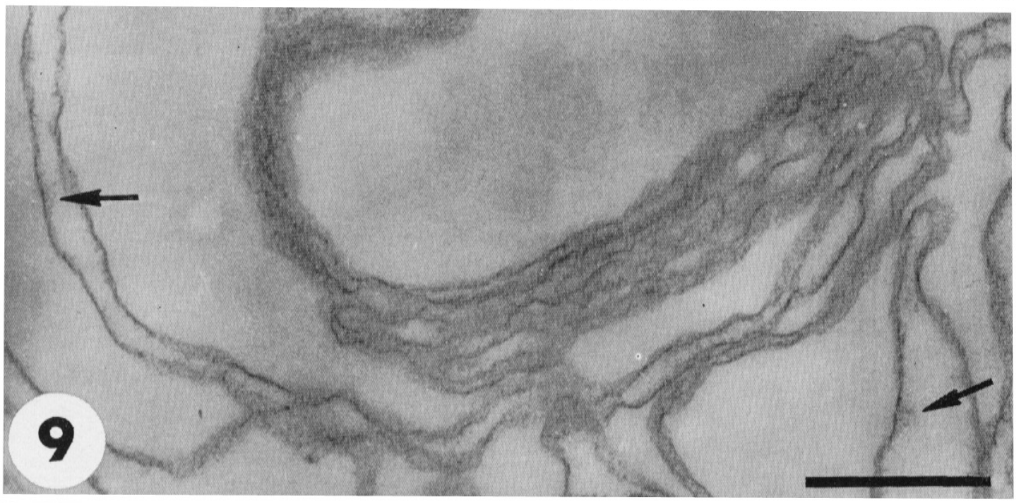
PHOTO 12. Under the same conditions as described above, treated with 10^{-3} M Hydrocortisone in 2% ethanol (×100,000).

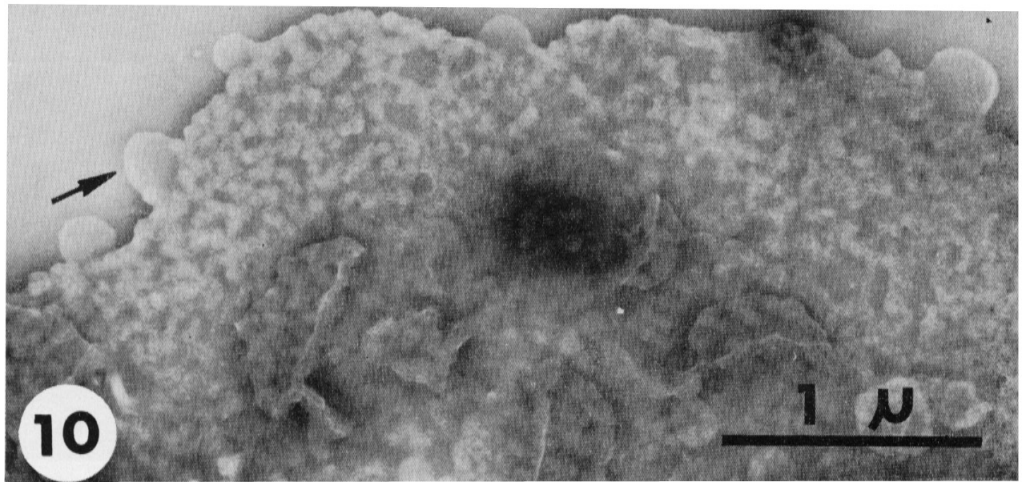
PHOTO 13. Similarly, treated with 10^{-3} M 11-DOC in 2% ethanol. (×100,000). (13 a) Globular lipoproteins and release of the lipid components are seen.
(13 b) reveals rearrangement of the released phospholipid into micelles formation (negatively stained with 2% phosphotungstate (pH 7.4)).

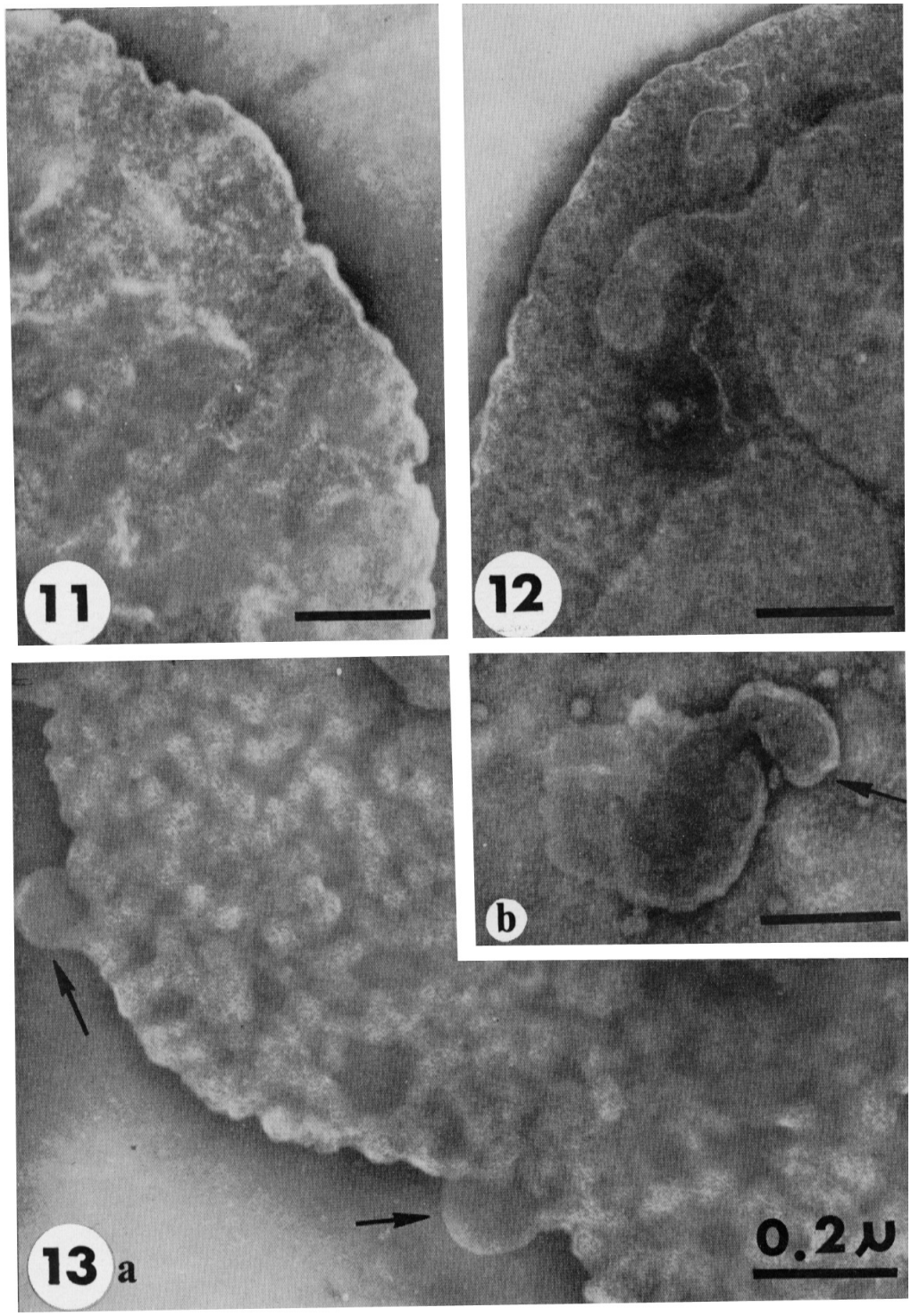

1
2
3
1 μ


4
5
6
7a
b
0.5 μ


8


9


10
1 μ


11
12
b
13 a
0.2 μ