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# ISOLATION AND CHARACTERIZATION OF PLASMA MEMBRANE FROM LACTATING BOVINE MAMMARY GLAND

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**Plasma membranes were isolated from lactating bovine mammary gland. Two crude membrane fractions; medium/*d* 1.033 (light membrane) and 1.033/1.053 interfaces (heavy membrane), were obtained by Ficoll density gradient centrifugation of osmotically washed microsomal fraction. Two crude membranes were further purified separately by sucrose density gradient centrifugation. Both light and heavy membranes banded at a sucrose density of 1.14. The purified membranes appeared as heterogeneous smooth membrane vesicles on electron microscopy. The contaminating suborganelles were not detected. The yield of the purified membranes relative to the homogenate was 1.2%. The degree of purity of the membranes was shown by a great increase in the specific activity of 5′-nucleotidase over the homogenate of 20-fold for light membrane and of 16-fold for heavy membrane. The relative activities of $Mg^{2+}$-ATPase, $(Na^+ + K^+)$-ATPase, γ-glutamyl transpeptidase, phosphodiesterase I, akaline phosphatase and xanthine oxidase were also high (12–18-times) and nearly 20% of these enzymes was recovered. The activity of marker enzyme for mitochondria, endoplasmic reticulum and Golgi apparatus was very low, while that of acid phosphatase for lysosome was relatively high (5-times). DNA and RNA contents were very low. The major polypeptides rich in other suborganelles were not detected profoundly in the membrane fraction and the polypeptide compositions in both light and heavy membranes were similar upon SDS-polyacrylamide gel electrophoresis.**

## Introduction

The envelopment of milk lipid droplets in plasma membrane at secretion produces one of the most special dispositions of biological membranes in all of nature [1]. Secretion of milk involves not only discharge of lactose, protein and fat globules from the cell but also discharge of large quantities of cellular membranes [2]. Upon secretion of milk from the cell, the secretory vesicles containing the materials such as lactose and protein are likely to fuse with the plasma membrane and, coincidently, the contents of secretory vesicle are discharged into the lumen. On the other hand, a milk lipid droplet, after it is enveloped by the plasma membrane, seems to be projected from the cell into the lumen. It is thus considered that the contents of the secretory vesicle and the milk lipid droplets are secreted probably by the same membrane [1]. Such observations were first made by Bargmann et al. [3,4] by means of electron microscopy and, thereafter, supported by further morphological and biochemical investigations [5–10], as discussed in recent reviews [1,11,12]. The marker enzymes such as 5′-nucleotidase in the plasma membrane of the

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; INT, 2-*p*-iodophenyl-3-nitrophenyl-5-phenyltetrazolium chloride; EGTA, ethyleneglycolbis(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

secretory cell are also contained in milk fat globule membrane [5,10] and the lipid composition of both materials is apparently similar [2,6]. However, very little is yet known as to whether protein compositions of both materials are similar [6]. The comparison has been made using crude plasma membrane [6]. In order to resolve the origin of milk fat globule membrane and mechanism of milk secretion, it is first necessary to isolate the plasma membrane in high purity.

Recently, an isolation procedure for highly purified plasma membrane has been developed in liver, lymph node, brain, spleen and so forth (see Ref. 13 for review). Lauter et al. [14] stressed from their comparative studies that the method for the isolation of plasma membrane fractions must be explored taking differences of species, tissues and/or physiological and pathological conditions into consideration. Keenan et al. [6] isolated a plasma membrane from the lactating bovine mammary gland by applying the procedure of Neville [15] as modified by Emmelot et al. [16,20]. However, the yield was very low and the purity was not specified except that mitochondria were occasionally observed upon electron microscopy. Keenan et al. [2] described thereafter that application of the procedure of Touster et al. [17] was also unsuccessful. We think that the method for the isolation of plasma membrane from the lactating mammary gland should be developed taking into account the fact that the mammary gland is rich in connective tissue.

In the present paper, we report the method for isolating plasma membranes with high purity from the lactating mammary gland of the cow. The purified plasma membranes were characterized by the determination of activities of marker enzymes, electron microscopic observation, chemical assay and electrophoresis.

## Materials and Methods

### *Materials*

Mammary tissue was obtained from lactating Holstein cows at a slaughter house and chilled immediately on ice during transportation to the laboratory.

### *Chemicals*

Ficoll 400 was purchased from Pharmacia Fine Chemicals, Uppsala; Hepes, *p*-nitrophenyl-*p*-D-glucuronide and rotenone from Nakarai Chemicals, Kyoto; sodium *p*-nitrophenyl-5′-thymidylate from Calbiochem, CA; disodium *p*-nitrophenyl phosphate from Koch-Light, Colnbrook, U.K.; *N*-acetylglucosamine, L-γ-glutamyl-*p*-nitroanilide, cytochrome *c* (from horse heart), disodium ATP, DNA and RNA from Sigma Chemicals, St. Louis; sodium 5′-AMP from Kyowa Hakko, Tokyo; 2-*p*-iodophenyl-3-nitrophenyl-5-phenyltetrazolium chloride (INT) and EGTA from Tokyo Kasei Kogyo, Tokyo; sodium glucose 6-phosohate, NADH, and dipotassium UDP-D-galactose from Boehringer Mannheim; [1-$^{14}$C]tyramine from New England Nuclear, Boston; UDP-D-[U-$^{14}$C]galactose from Amersham International. All other chemicals were analytical grade. All solutions were prepared with deionized and glass-distilled water throughout.

### *Isolation of plasma membrane*

About 100 g fresh weight of the lactating mammary tissue was used for each preparative run. All operations were carried out at 0–4°C with prechilled materials. The centrifugal fields referred to the bottom of the tubes. Media were 0.25 M sucrose/1 mM carbonate buffer (pH 7.5) (medium A) for procedure A, 0.25 M sucrose/5 mM Tris-HCl buffer (pH 8.0) (medium B) for procedure B, and 0.25 M sucrose/10 mM Hepes buffer (pH 7.5), containing 1 mM $MgCl_2$ (medium C) for procedure C.

*Preparation of the homogenate.* Before disruption, about 100 g of the tissue were finely minced with scissors (pieces less than 5 $mm^3$) and rinsed three or four times with 3 vol. of appropriate medium (medium A, B or C). Portions corresponding to about 50 g minced tissue were roughly disrupted in 200 ml of the medium using a Waring blendor (Nihon Seiki) twice for 30 s at intervals of 30 s at medium speed. The broken cell suspension was filtered through one layer of pre-wetted surgical gauze. The undisrupted cell with connective tissue corresponding 100 g wet tissue was collected in 200 ml of medium, blended and filtered. The operation was repeated. The filtrate was combined and in total about 1000 ml of the broken cell suspension were homogenized in an all-glass homogenizer (50 ml capacity) of the Potter-Elvehjem

type with a loose Teflon pestle (clearance of 0.40 mm), normally using five up-and-down strokes at 1000 rpm. The homogenate was filtered through pre-wetted gauze, first through a double layer, secondly four layers and then eight layers.

*Preparation of microsomal fraction.* The crude microsomal fraction was prepared for procedures B and C. After centrifugation of the homogenate for 15 min at $1500 \times g$ in a Hitachi RPR-10-2 rotor, the fatty layer floating was sucked off and the supernatant (sup-1) was recovered by decantation. The supernatant was centrifuged for 20 min at $9000 \times g$ in a Hitachi RPR-10-2 rotor. The precipitate was suspended in 80 ml medium B or C by two strokes in a Potter-Elvehjem homogenizer at 1000 rpm and centrifuged as above. The resultant supernatants (sup-2) were combined and centrifuged for 60 min at $106000 \times g$ in a Hitachi RP-30 rotor. After removal of supernatant (sup-3) by decantation, the precipitate (ppt-3) was recovered as the crude microsomal fraction. The respective precipitates (ppt-1 and ppt-2) obtained by centrifugation at $1500 \times g$ and $9000 \times g$ was recovered as reference fractions without further purification.

*Procedure A: Isolation of plasma membrane from the nuclear fraction.* The homogenate in medium A was centrifuged for 10 min at $1500 \times g$. The precipitate was suspended with medium A. The centrifugation of the suspension (680 ml) was carried out by the procedure of Emmelot et al. [16,20]. An upper layer (yellow in appearance) of the precipitated material was suspended. The suspension (340 ml) was centrifuged for 15 min at $1000 \times g$. The fluffy layer of plasma membrane above tightly packed material was suspended and centrifuged for 10 min at $1000 \times g$. Washing was thrice repeated as before. The precipitate containing crude plasma membrane (7.8 ml) was mixed vigorously with 21.6 ml 81% (w/v; *d* 1.30) sucrose solution to bring it to *d* 1.22. The discontinuous sucrose density gradient was built up by adding 9.8 ml membrane suspension, 5 ml 53.4% (w/v, *d* 1.20), 7 ml 48.0% (w/v, *d* 1.18), 6 ml 42.9% (w/v, *d* 1.16), and 4 ml 37.6% (w/v, *d* 1.14) sucrose/1 mM carbonate buffer (pH 7.5) and centrifuging for 90 min at $900000 \times g$ in an RPS 25-2 swing rotor. Membranes were mostly recovered from the *d* 1.14/1.16 interface, pelleted by centrifugation at $106000 \times g$ for 30 min and suspended in medium A.

*Procedure B; Isolation of plasma membrane from the microsomal fraction without osmotic washing.* The microsomal fraction was suspended in medium A or B, and a portion (7 ml) was overlayered on the top of the Ficoll density gradient consisting of 9 ml *d* 1.033, 9 ml *d* 1.053 and 7 ml *d* 1.090 Ficoll/1 mM carbonate buffer (pH 7.5) or 5 mM Tris-HCl buffer (pH 8.0). After centrifugation at $90000 \times g$ for 3 h, the membranes were recovered from the interface of medium/1.033. The membrane materials were suspended in medium (7.8 ml) and mixed with 21.6 ml *d* 1.30 sucrose solution. Centrifugation through the sucrose density gradient was performed as described in procedure A. Membranes were recovered from both *d* 1.14/1.16 and 1.16/1.18 interfaces.

*Procedure C: Isolation of plasma membrane from the osmotically washed microsomal fraction.* The steps of purification of plasma membrane by procedure C are as follows. The membraneous material was suspended on a Potter-Elvehjem homogenizer with a Teflon pestle of 0.2 mm clearance unless stated otherwise. The microsomal fraction was washed with 10 mM Hepes buffer (pH 7.5) and subsequently with 1 mM Hepes buffer (pH 7.5) by centrifugation at $106000 \times g$ for 60 min, as described by Wallach et al. [18,19]. The washed microsomes were suspended in 50 ml 1 mM Hepes buffer (pH 8.2) containing 1 mM $MgCl_2$ (buffer A) with six up-and-down strokes at 1000 rpm and dialyzed against the same buffer for 60 min. The dialyzed microsomes (8 ml) were then layered upon 12 ml of 10% (w/v, *d* 1.033) and 12 ml of 15.5% (w/v, *d* 1.053) Ficoll in 10 mM Hepes buffer (pH 7.5) containing 1 mM $MgCl_2$. Tubes were centrifuged at $100000 \times g$ for 8–12 h in a Hitachi RPS 25-2 or RPS 27-2 swing rotor. Crude membrane materials at medium/*d* 1.033 (light fraction) and *d* 1.033/1.053 interfaces (heavy fraction) were collected separately by aspiration. The precipitate (Ficoll PPT) was also recovered. Each membrane fraction was diluted with about 150 ml buffer A and pelleted by centrifugation for 90 min at $106000 \times g$. The resultant crude plasma membrane fractions were suspended separately into about 25 ml buffer A, homogenized with two up-and-down strokes at 1000 rpm, and over-

layered (7 ml) on the top of a second discontinuous sucrose density gradient consisting of 9 ml 37.6% (w/v, *d* 1.14), 9 ml 42.9% (*d* 1.16) and 7 ml 48.0% (*d* 1.18) sucrose in buffer A. After centrifugation at 100000 × *g* for 120 min in a RPS 25-2 or 27-2 rotor, the upper band at medium/*d* 1.14 interface was collected by aspiration and pelleted by centrifugation at 106000 × *g* for 90 min after appropriate dilution with 10 mM Hepes buffer (pH 7.5) containing 1 mM $MgCl_2$. The light and heavy plasma membranes were separately resuspended in medium C (protein concentration 0.2–0.3%). Materials at 1.14/1.16 and 1.16/1.18 interfaces were also recovered and treated by the same way as plasma membrane.

*Enzymatic determinations*

All enzymatic activities were determined within 3 days after preparation. Samples were stored in ice. The assay temperature for all enzymes was 37°C. The reaction mixture was immediately chilled in ice after the stopping of the reaction by adding 9% trichloroacetic acid except that 0.5 N NaOH and 2 N acetic acid were used for alkaline phosphatase and γ-glutamyltranspeptidase, respectively.

Acid phosphatase (EC 3.1.3.2) was assayed [21] in 0.1 M acetate buffer (pH 4.8) and 5 mM *p*-nitrophenyl phosphate. Alkaline phosphatase (EC 3.1.3.1) was assayed [22] in 0.05 M ethanolamine-HCl buffer (pH 10.1 at 37°C), 5 mM *p*-nitrophenyl phosphate and 10 mM $MgCl_2$. γ-Glutamyl transpeptidase (EC 2.3.2.2) was assayed [23] in 50 mM Tris-HCl buffer (pH 9.0), 50 mM glycylglycine, 75 mM NaCl and 2.5 mM L-γ-glutamyl-*p*-nitroanilide. Succinate dehydrogenase (EC 1.3.99.1) was assayed [24] in 50 mM potassium phosphate buffer (pH 7.4), 0.1% INT, 50 mM sodium succinate and 25 mM sucrose. Xanthine oxidase (EC 1.2.3.2) was assayed [25] in a reagent mixture containing 0.1 M phosphate buffer (pH 7.8), 0.86 mM $Na_2EDTA$, 4.2 mg gelatin, 17.1 μg phenazine methosulfate, 1.03 mg nitro-BT-tetrazolium salt and 0.14 mM xanthine. Glucose-6-phosphatase (EC 3.1.3.9) was assayed [26] in a reagent assay mixture which was made by combining 0.1 M sodium glucose 6-phosphate (pH 6.5), 35 mM histidine (pH 6.5), 10 mM $Na_2EDTA$ (pH 7.0) and distilled water in a volume ratio of 2:5:1:1. Phosphodiesterase I (EC 3.1.4.1) was assayed [26] in 0.1 M Tris-HCl buffer (pH 9.0) and 5 mM *p*-nitrophenyl-5′-thymidylate. 5′-Nucleotidase (EC 3.1.3.5) was assayed [27] in a reagent mixture containing 10 mM AMP (pH 7.0), 0.1 M Tris-HCl buffer (pH 8.5) and 10 mM $MgCl_2$. $Mg^{2+}$-ATPase (EC 3.6.1.3) was assayed [28] in a reaction mixture containing 120 mM KCl, 0.5 mM EGTA, 30 mM histidine/imidazole buffer (pH 7.0), 5 mM $MgCl_2$ and 2 mM ATP. $(Na^+ + K^+)$-ATPase (EC 3.6.1.4) was assayed [29] in a reaction mixture containing 50 mM imidazole-HCl buffer (pH 7.1), 120 mM NaCl, 12 mM KCl, 5 mM $MgCl_2$, 0.5 mM $Na_2EDTA$, and 3 mM ATP in the presence or absence of 0.2 mM ouabain. NADH-cytochrome *c* reductase (EC 1.6.2.1) was assayed [30] in a reaction mixture containing 0.01% NADH, 40 mM potassium phosphate buffer (pH 7.5), 0.01% cytochrome *c* and 10 mM sodium azide in the presence or absence of 2 μM rotenone. The appearance of reduced cytochrome *c* was recorded at 550 nm. β-Glucuronidase (EC 3.2.1.31) was measured [31] in the reaction mixture containing 8 mM *p*-nitrophenyl-*p*-D-glucuronide and 300 mM acetate buffer (pH 4.0). Galactosyltransferase (EC 2.4.1.22) was assayed [32,33] in a total volume of 0.2 ml containing 0.162 mM UDP[U-$^{14}$C]galactose (50000 dpm), 50 mM sodium cacodylate buffer (pH 6.5), 5 mM $MgCl_2$, 2.4 mM $MnCl_2$, 0.5% Triton X-100 and 2.25 mM *N*-acetylglucosamine. The reaction was stopped by addition of 50 μl 0.3 M $Na_2EDTA$ (pH 7.4). The reaction mixture was passed through a column of Dowex 1-X8 [32]. Monoamine oxidase (EC 1.4.3.4) was assayed [34] in the mixture containing 0.1 M sodium-potassium phosphate buffer (pH 7.8) and 0.3 μM [$^{14}$C]tyramine (50000 dpm). The reaction was stopped by the addition of 0.5 ml 2 M citric acid followed by 10 ml anisole containing 0.6% diphenyloxazole.

Inorganic phosphate released from the substrate by 5′-nucleotidase, ATPases and glucose-6-phosphatase was determined by the method of Chen et al. [35] using ascorbic acid and ammonium molybdate in sulfuric acid. The absorbance of *p*-nitrophenol and of *p*-nitroaniline was measured at 410 nm. The radioactivity was counted on a Packard TriCarb Instrument.

*Chemical assays*

Protein was determined according to Lowry et

al. [36], using bovine serum albumin as standard. Membrane preparations to be analyzed for nucleic acid were extracted by the procedure described by Flec and Munro [37]. DNA was assayed by procedure of Burton [38], using calf thymus DNA as standard. RNA was estimated according to the procedure of Flec and Munro [37] with ultraviolet reading at 260 nm, using yeast RNA as standard. RNA content was not corrected for the contamination of protein.

*Electron microscopy*

Aliquots of membrane were pelleted by centrifugation and the pellets were fixed for 60 min at 0°C in 2.5% glutaraldehyde buffered with 0.1 M sodium phosphate buffer (pH 7.0). The fixed pellets were washed several times, post-fixed wih 1% $OsO_4$ in the same buffer for 2 h at 0°C, and stained en bloc with 0.5% uranyl acetate for 1 h at 0°C. Materials were embedded and sectioned according to Brown et al. [39]. Sections were observed with a Hitachi HU-12 electron microscope operating at 75 kV, using a 50 $\mu$m objective aperture.

*Electrophoresis*

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by the procedures of Weber and Osborn [40] and Laemmli [41]. The concentration of acrylamide was 10% and the ratio of acrylamide and methylenebisacrylamide was 37:1 (w/w). Samples were solubilized in 10 mM Tris-HCl buffer (pH 6.8) or phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol and 10% sucrose, and heated for 3 min in a boiling water bath prior to loading on gels. Protein was stained with Coomassie brilliant blue R-250 [42] and carbohydrate with periodic acid-Schiff reagent [43].

## Results

In order to check the purity of the isolated plasma membrane, the marker enzymes for each subcellular organelle were assayed [13,44]: 5′-nucleotidase, $Mg^{2+}$-ATPase, $(Na^+ + K^+)$-ATPase, phosphodiesterase I, γ-glutamyltranspeptidase, alkaline phosphatase and xanthine oxidase for plasma membrane, acid phosphatase and β-glucuronidase for lysosome, glucose-6-phosphatase and NADH-cytochrome *c* reductase for endoplasmic reticulum, succinate dehydrogenase and monoamine oxidase for mitochondria, and galactosyltransferase for Golgi apparatus. Alkaline phosphatase and xanthine oxidase are known to be rich in the milk fat globule membrane [1].

*Isolation of plasma membrane*

Before adopting the final method (procedure C), we tried to isolate plasma membrane from the nuclear fraction of the homogenate, according to the procedure of Emmelot et al. [16,20] (procedure A). The plasma membrane was gathered at the *d* 1.14/1.16 interface. The yield of plasma membrane was very low (approx. 3.2 mg from 100 g wet tissue). The relative specific activities of 5′-nucleotidase and $Mg^{2+}$-ATPase were about only 3–4-times compared with those of the homogenate. In addition, the contamination of mitochondria, lysosome and endoplasmic reticulum was considered to be large since the relative activities to the homogenate of their respective marker enzyme were high (3–4-times). Keenan et al. [6] applied this procedure for the isolation of plasma membrane of the secretory cells of a bovine mammary gland. They did not describe the results of enzymatic activities of the preparation. Besides, the yield was very low. We concluded that this procedure is not suitable for the isolation of the plasma membrane from a nuclear fraction, since the secretory cells of the mammary gland are rich in the connective tissue and therefore it is not easy to disrupt the tissue by only a Potter-Elvehjem-type or a Dounce-type homogenizer equipped with a Teflon pestle. It was necessary to use a blender or a similar tool for disrupting the mammary secretory tissue. In addition, Lauter et al. [14] pointed out that Neville's method [15] which was improved by Emmelot et al. [16,20] yielded acceptable plasma membrane preparations from rat and guinea-pig liver but not from cat and rabbit liver. The present data endorsed the suggestion of Lauter et al. [14].

Secondly, plasma membrane was isolated from the microsomal fraction according to the method of Touster et al. [17,26]. In addition, Ficoll gradient centrifugation was adopted according to House et al. [45] and then sucrose density gradient centrifugation was followed. By this procedure

(procedure B), the membrane fraction was finally collected at *d* 1.14/1.16 and 1.16/1.18 interfaces. The activities of the enzymes in both membrane fractions were almost similar. Specific activities of the marker enzymes, 5′-nucleotidase, $Mg^{2+}$-ATPase and phosphodiesterase I, for plasma membrane increased with the purification processes and their activities in both membrane fractions were 8–12-times those in the homogenate. The contamination of lysosome (7-times) and endoplasmic reticulum (4-times) was still high but that of mitochondria was very low (0.7-times over the homogenate). Yield of both the membrane fractions, expressed as the amount of protein, was 25 mg per 100 g wet tissue. However, the relative specific activities of the marker enzymes for plasma membrane were still lower than those in the membrane obtained from liver by recently developed techniques. By a method similar to procedure B without Ficoll gradient centrifugation, Shin et al. [46] have isolated plasma membrane from lactating rat mammary gland. Their preparation had a relative specific activity 15- and 17-fold over that of the homogenate for 5′-nucleotidase and ($Na^+ + K^+$)-ATPase, respectively. Huggins et al. [47] also isolated the plasma membranes of high purity by flotation on a discontinuous sucrose density gradient and further digitonin-shift purification of a microsomal fraction of rat mammary gland; the relative specific activity of 5′-nucleotidase was 73- and 37-fold for their $F_1DF_3$ and $F_2DF_3$ fractions, respectively. The yield of the membranes was not specified.

In order to increase the purity, the microsomal fraction was washed by osmotic shock according to Wallach et al. [18,19] before purification by Ficoll and sucrose density gradient centrifugation (procedure C). By this procedure a plasma membrane with high purity was isolated in good yield from the mammary gland. By prolonged centrifugation adopted in this procedure on a Ficoll density gradient, the membrane was separated into two fractions: medium/*d* 1.033 and 1.033/1.053 interfaces. The two crude membrane fractions were further purified by sedimentation through a sucrose density gradient. Each membrane was gathered mostly at the interface of the medium/*d* 1.14. Materials at the *d* 1.14/1.16 interface were low in protein yield (1/2–1/3), while they were low in specific activities of plasma membrane marker enzymes (2/3–1/2) and almost equivalent in the somewhat high activity of the marker enzymes for other suborganelles, when compared with those of corresponding membrane fractions. The recovery of the marker enzymes for plasma membrane at *d* 1.14/1.16 and 1.16/1.18 interfaces was very low (Table II). The membrane gathered at medium/*d* 1.033 in Ficoll and at medium/*d* 1.14 in sucrose was designated the light membrane, while the membrane obtained from the interface of *d* 1.033/1.053 in Ficoll and medium/*d* 1.14 in sucrose was the heavy membrane.

Washing of the microsomal fraction with osmotic shock markedly improved the purity of the membrane, as discussed by Ferber et al. [19]. As compared with crude microsome, the protein content decreased by about 54% in the washed and dialyzed microsomes. In addition, the activity of the marker enzymes for plasma membrane increased by 20–40% except for γ-glutamyltranspeptidase (1%). Purification by sucrose density gradient sedimentation caused an increase in the specific activity of plasma membrane marker enzymes but a decrease of the recovery of the membranes. Substitution of Hepes for Tris as medium resulted in some increase in the activities of the enzymes (results not shown) in agreement with Ferber et al. [19]. Carraway et al. [48] also prepared plasma membrane by discontinuous sucrose gradient from the washed microsomal fraction of mammary tumor cells. Their preparations showed only a 10-fold purification over the homogenate with respect to ATPase and 5′-nucleotidase. A combination of osmotic washing and prolonged centrifugation appeared to improve the purity of plasma membrane.

*Enzymatic and chemical profiles of the purified membranes*

The enzymatic profiles of various membrane fractions obtained by the procedure C are shown in Table I. The relative specific activities of the marker enzyme of the light membrane were 15–21-times over those of the homogenate and those of the heavy membrane were 12–17-times. The relative specific activity of 5′-nucleotidase was the highest among the plasma membrane marker enzymes determined. The average recovery of these

TABLE I

ENZYMATIC ACTIVITIES AND PROTEIN CONTENT OF PLASMA MEMBRANES PREPARED FROM THE WASHED MICROSOMAL FRACTION

Specific activity is expressed as μmol substance released or utilized per mg protein per min. Values are given ±S.D. with preparations from different animals. Relative activity (in parentheses) is the specific activity of each fraction relative to that of the homogenate.

| Enzymes | Specific activity (μmol/mg protein per min) | | | | | |
|---|---|---|---|---|---|---|
| | Homogenate | Ficoll medium/1.033 | Ficoll 1.033/1.053 | Light membrane | Heavy membrane | Number of preparations |
| 5'-Nucleotidase | 0.0330±0.0064 | 0.4415±0.1025 (16.0±2.7) | 0.2979±0.0828 (11.0±3.1) | 0.6550±0.2291 (21.3±2.6) | 0.5238±0.1974 (17.2±3.8) | 8–11 |
| $Mg^{2+}$-ATPase | 0.0412±0.0114 | 0.4424±0.1496 (12.0±3.5) | 0.3960±0.1518 (11.9±1.9) | 0.5894±0.1971 (15.2±4.4) | 0.5219±0.1914 (13.6±4.9) | 8–11 |
| Phosphodiesterase I | 0.0292±0.0130 | 0.3786±0.0874 (13.6±2.0) | 0.2157±0.0776 (9.9±2.5) | 0.5375±0.2233 (17.5±2.5) | 0.4251±0.1825 (13.9±3.0) | 7–11 |
| γ-Glutamyltranspeptidase | 0.3293±0.0743 | 3.8643±0.8415 (13.0±3.2) | 2.6575±0.5787 (8.9±2.0) | 5.4113±1.3142 (16.5±2.1) | 4.0952±1.3307 (12.4±2.4) | 6–9 |
| Alkaline phosphatase | 0.0362±0.0142 | 0.6426±0.1626 (19.1±5.2) | 0.4791±0.0975 (14.5±4.3) | 0.2378±0.0132 (7.6±2.7) | 0.1790±0.0449 (4.9±1.3) | 4–6 |
| Acid phosphatase | 0.0132+0.0032 | 0.0621+0.0205 (5.1+2.0) | 0.0576+0.0168 (4.6+1.7) | 0.0836+0.0217 (6.3+1.3) | 0.0867+0.0387 (6.3+1.8) | 9–11 |
| β-Glucuronidase | 0.0008±0.0001 | 0.0006±0.0003 (0.73±0.4) | 0.0005±0.0001 (0.60±0.2) | 0.0006±0.0002 (0.62±0.1) | 0.0003±0.00002 (0.36±0.1) | 3–4 |
| Glucose-6-phosphatase | 0.0021±0.0010 | 0.0065±0.0023 (5.7±3.4) | 0.0063±0.0028 (4.6±2.5) | 0.0069±0.0032 (4.1±2.2) | 0.0077±0.0032 (4.7±2.4) | 9–11 |
| NADH-cytochrome *c* reductase (+rotenone) | 0.2656±0.0248 | 0.0753±0.0096 (0.29±0.01) | 0.0882±0.0273 (0.33±0.08) | 0.0444±0.0019 (0.17±0.02) | 0.0411±0.0065 (0.16±0.01) | 3–4 |
| Galactosyltransferase | 0.0085±0.0022 | 0.0441±0.0133 (5.2±1.6) | 0.0594±0.0205 (7.0±0.9) | 0.0069±0.0030 (0.81±0.11) | 0.0077±0.0052 (0.91±0.40) | 4 |
| Succinate dehydrogenase | 0.0033±0.0011 | 0.0005±0.0002 (0.15±0.10) | 0.0004±0.0002 (0.13±0.06) | 0.0003±0.0001 (0.09±0.04) | 0.0002±0.0001 (0.08±0.03) | 10–11 |
| Monoamine oxidase | 0.0016±0.0001 | 0.0024±0.0005 (1.50±0.28) | 0.0022±0.0009 (1.40±0.58) | 0.0020±0.0007 (1.24±0.45) | 0.0015±0.0004 (0.94±0.26) | 4 |
| Protein (mg/100 g wet tissue) | 3609±599 | 40.3±6.1 (1.14±0.06) | 47.4±6.6 (1.36±0.23) | 23.7±5.3 (0.65±0.09) | 21.4±6.2 (0.58±0.10) | 11 |

TABLE II

DISTRIBUTION OF MAJOR MARKER ENZYMES AND PROTEIN IN SUBFRACTIONS DURING PURIFICATION STEPS OF PLASMA MEMBRANE

Values are the means of two preparations from different animals and 100% equals to the total activity or amount in the homogenate per 100 g wet tissue. Amount of protein in the homogenate is given in mg. Total activity of each enzyme in the homogenate is expressed as μmol substrate released or utilized. ppt-1, nuclear pellet; ppt-2, mitochondrial and lysosomal pellet; sup-3, cytoplasmic fraction; and ppt-3, crude microsomes.

| | Total activity | ppt-1 | ppt-2 | sup-3 | ppt-3 | Ficoll density gradient | | | Sucrose density gradient | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Ficoll < 1.03 fraction | | | Ficoll 1.03/1.05 fraction | | |
| | | | | | | < 1.03 | 1.03/1.05 | pellet | < 1.14 | 1.14/1.16 | 1.16/1.18 | < 1.14 | 1.14/1.16 | 1.16/1.18 |
| 5′-Nucleotidase | 88.2 | 0.8 | 5.8 | 7.8 | 51.4 | 19.2 | 13.9 | 12.8 | 15.0 | 1.1 | 0.1 | 6.9 | 2.8 | 0.4 |
| Phosphodiesterase I | 68.6 | 4.7 | 6.9 | 9.1 | 56.1 | 15.8 | 12.4 | 11.3 | 11.5 | 1.1 | 0.2 | 7.1 | 3.2 | 0.5 |
| $Mg^{2+}$-ATPase | 133.2 | 1.4 | 16.4 | 24.6 | 56.1 | 15.3 | 13.3 | 8.7 | 10.1 | 0.8 | 0.2 | 6.7 | 1.5 | 0.2 |
| Acid phosphatase | 38.5 | 5.8 | 17.9 | 34.1 | 29.1 | 4.1 | 4.6 | 15.5 | 3.5 | 0.1 | 0.1 | 2.3 | 3.7 | 0.3 |
| Succinate dehydrogenase | 5.2 | 13.3 | 14.6 | 7.4 | 20.0 | 0.4 | 0.1 | 20.6 | 0.1 | 0.01 | 0.01 | 0.1 | 0.2 | 0.1 |
| Glucose-6-phosphatase | 9.2 | 0.9 | 1.7 | 23.6 | 21.2 | 2.6 | 1.4 | 9.0 | 0.9 | 0.1 | 0.1 | 0.1 | 0.5 | 0.4 |
| Protein | 3040.8 | 8.7 | 7.9 | 38.5 | 19.7 | 1.2 | 1.3 | 8.3 | 0.6 | 0.1 | 0.01 | 0.4 | 0.3 | 0.1 |

enzymes was about 27–33% in both the membrane fractions after Ficoll density gradient but decreased to about 17–23% after a sucrose density gradient. Specific activity and the recovery of these marker enzymes were higher in the light membrane than in the heavy membrane. The specific activities of $(Na^+ + K^+)$-ATPase, which were measured in one preparation, was 0.0475 μmol/mg protein per min for homogenate, 0.9975 for light membrane, and 0.9232 for heavy membrane in the presence of ouabain and the respective values in the absence of ouabain were 0.539, 1.050 and 0.936. The activity of $(Na^+ + K^+)$-ATPase was also high but almost similar for light and heavy membranes. In addition, $(Na^+ + K^+)$-ATPase of both membranes was insensitive to ouabain, in concord with the previous results [49].

Characteristic marker enzymes for various subcellular organelles were determined. The relative specific activity of succinate dehydrogenase which is a marker for mitochondrial inner membrane was one-tenth compared with that of the homogenate, while the activity of monoamine oxidase, which is a marker for mitochondrial outer membrane, was somewhat higher. However, the recovery of these enzymes was only 0.1%, suggesting that the contamination of the mitochondria was very low. The contamination of lysosome and endoplasmic reticulum was possible and the recovery of acid phosphatase and glucose-6-phosphatase was 6.6%. However, the activity of β-glucuronidase, another marker enzyme for lysosome, was very low and that of NADH-cytochrome *c* reductase, a marker for endoplasmic reticulum, was also low. The activity of galactosyltransferase was relatively low, suggesting some small contamination of the Golgi apparatus.

Furthermore, a specific activity of alkaline phosphatase was lower in the purified plasma membrane than in the crude plasma membranes obtained by a Ficoll density gradient (Table I). The differences depended on the presence or absence of sucrose in the medium. The removal of sucrose from the purified membranes by dialysis caused higher recovery of the enzyme activity (data not shown). The activity of alkaline phosphatase seemed to be inhibited by sucrose in the medium, as pointed out by Diaz-Maurino and Nieto [50]. After removing sucrose by dialysis against 1 mM Hepes buffer (pH 7.5) containing 1 mM $MgCl_2$ at 4°C overnight, the activity was recovered: in the case of the light membrane the specific activities were 0.229 and 0.660 μmol/mg protein per min before and after dialysis, respectively. The latter showed a 21-fold purification over the homogenate. The activity of xanthine oxidase, which is rich in bovine milk fat globule membrane, was determined in only one membrane preparation (not included in Table I). The specific activity of this enzyme was 0.044 μmol/mg protein per min

TABLE III

RNA AND DNA CONTENTS OF VARIOUS MEMBRANE FRACTIONS

Values (μg per mg protein) are given the mean ± S.D. in 5–9 preparations from different animals. Values in parenthesis indicate the ratio of RNA or DNA content of each fraction to that of the homogenate. RNA content was measured at 260 nm as described in Materials and Methods and was not corrected for contamination by protein.

| Fraction | RNA | DNA |
|---|---|---|
| Homogenate | 50.7 ± 11.4 (1.00) | 32.6 ± 9.6 (1.00) |
| Nuclear pellet | 42.8 ± 20.5 (0.82) | 84.1 ± 27.9 (2.77) |
| Mitochondria and lysosomal pellet | 61.9 ± 7.2 (1.07) | 37.0 ± 13.1 (1.35) |
| Crude microsomes | 128.2 ± 13.3 (2.22) | 15.9 ± 5.9 (0.59) |
| Ficoll medium/1.033 | 58.6 ± 22.0 (1.21) | 4.2 ± 1.2 (0.11) |
| Ficoll 1.033/1.053 | 81.0 ± 12.0 (1.85) | 4.6 ± 1.8 (0.13) |
| Ficoll pellet | 142.0 ± 26.7 (2.95) | 16.3 ± 4.6 (0.59) |
| Light membrane | 42.9 ± 14.2 (0.88) | 4.2 ± 1.1 (0.14) |
| Heavy membrane | 57.9 ± 21.9 (1.22) | 4.8 ± 1.7 (0.17) |

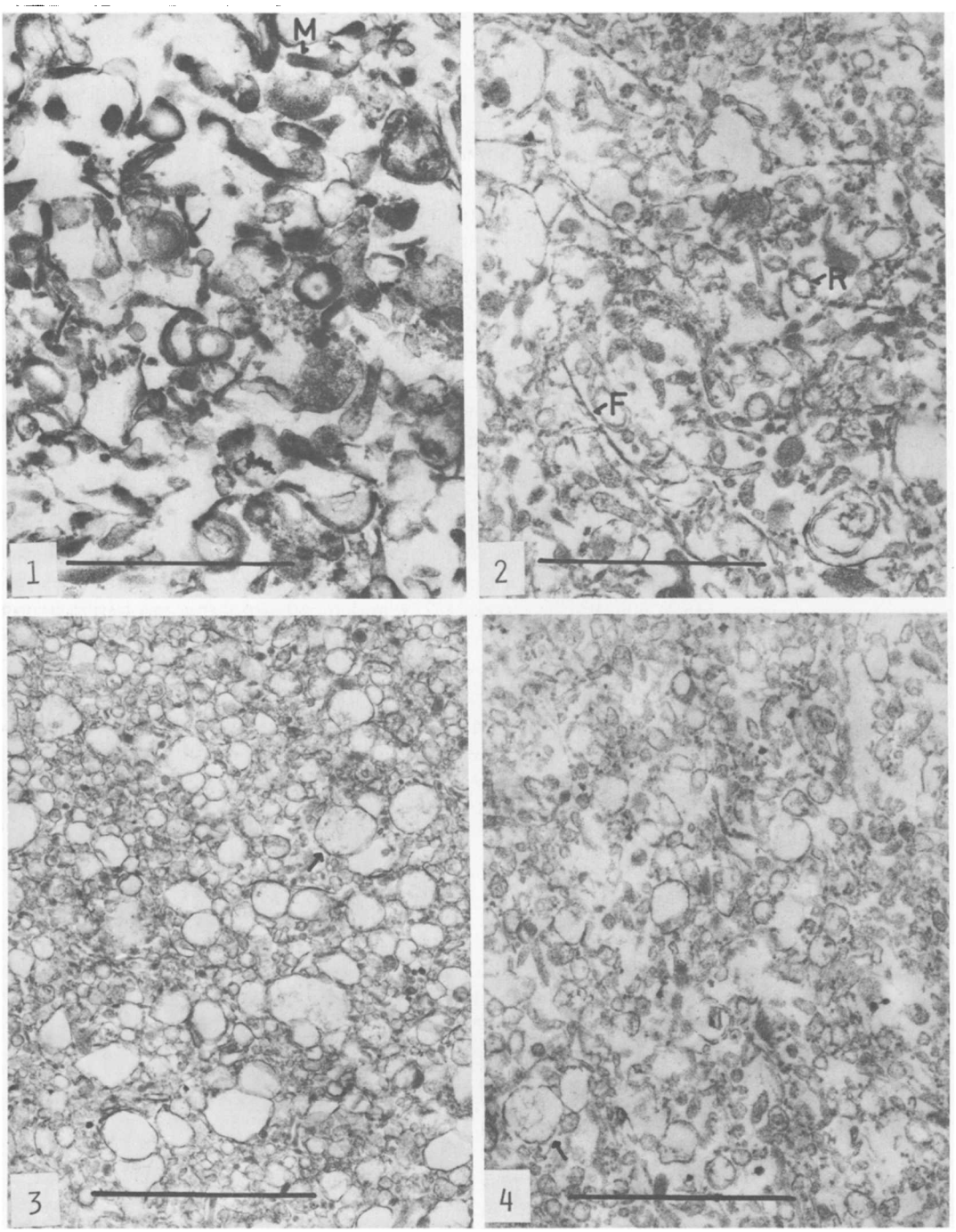


Fig. 1. Characteristic aspect of the various plasma membrane fractions upon electron microscopic observation. 1, Crude light membrane; 2, crude heavy membrane; 3, light plasma membrane; 4, heavy plasma membrane. Crude membrane fractions consist of amorphous and structureless vesicles; fibril (F), rough endoplasmic reticulum vesicle (R) and microvilli-like material (M) are seen. Both light and heavy membrane fractions consist of smooth vesicles; some vesicles showed little adherent material trapped inside the membrane (arrow). Magnification: bar, 2 $\mu$m: $\times$16800.

in a light membrane and 0.030 in a heavy membrane and the relative activity was 8.9- and 6.0-fold over the homogenate. Alkaline phosphatase and xanthine oxidase were recovered to 20% in both membranes, although the relative activity of xanthine oxidase was somewhat lower than those of other marker enzymes.

Of the protein present in the homogenate, about 2.4% protein was recovered in the crude plasma membranes and 1.2% in the purified plasma membranes. About a half of the protein of the former was presumably lost during the subsequent purification. Loss of the protein was somewhat larger in a heavy membrane than in a light membrane.

The degree of the contamination of endoplasmic reticulum and nucleus was estimated from the relative amounts of RNA and DNA in the various membrane fractions. The results are shown in Table III. The DNA and RNA concentrations were low in the both light and heavy membranes. However, RNA content was higher in the heavy membrane than in the light membrane.

*Electron microscopic observation*

Thin sections of the crude and the purified plasma membranes are shown in Fig. 1. In general, the crude plasma membrane fractions contained amorphous and structureless vesicles, microvilli-like materials, fine fibrils and junctional complexes of different sizes. Notable organelles such as mitochondria and nuclei were not seen. The purified membranes consist of many heterogeneous vesicles. Fibrils and junctional complexes observed in the crude membranes disappeared. This may be characteristic in the case of lactating mammary gland. The size of vesicles was generally larger in a light membrane than in a heavy membrane. It is considered that relatively large vesicles were recovered as a light membrane, while relatively small ones were collected as a heavy membrane on Ficoll density gradient centrifugation.

*Electrophoretic property*

For further characterization, electrophoretic profiles of the polypeptides were examined. The patterns obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis of the membrane fractions are shown in Fig. 2, in comparison with those of other subcellular organelle fractions. In general, the major polypeptides rich in the mitochondrial, lysosomal and cytoplasmic fractions were not profoundly detected in the membrane fractions. The patterns of polypeptide composition between the light and heavy membranes were similar. By protein staining, many bands were detected, while three major bands were observed by carbohydrate staining.

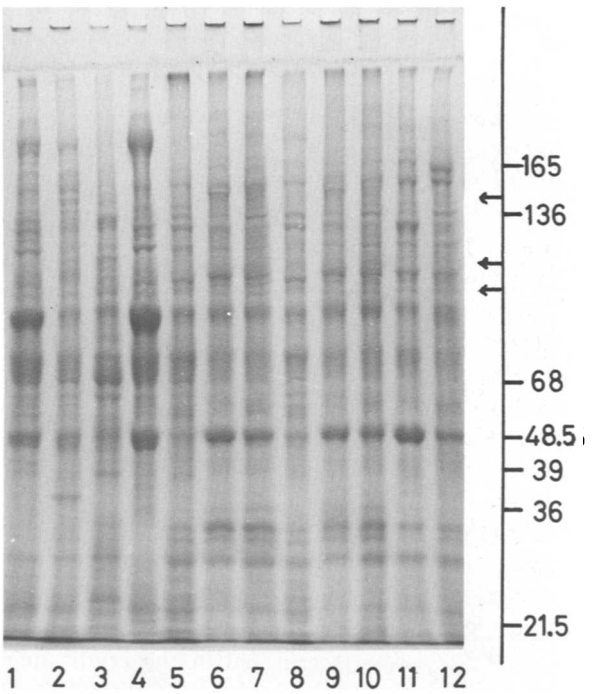


Fig. 2. Protein profiles of the isolated plasma membranes and other suborganelle fractions on polyacrylamide-SDS slab gel electrophoresis. 1, Homogenate; 2, ppt-1; 3, ppt-2; 4, sup-3, 5, washed microsome; 6, crude light membrane; 7, crude heavy membrane; 8, Ficoll ppt; 9, light membrane; 10, heavy membrane; 11, *d* 1.14/1.16 fraction in sucrose of crude light membrane; 12, *d* 1.14/1.16 fraction in sucrose of crude heavy membrane. ppt-1, ppt-2, sup-3, and Ficoll ppt correspond to nucleus, mitochondria and lysosome, cytoplasma and endoplasmic reticulum-rich fraction, respectively. These fractions was used without further purification. Aliquots from each fraction (100 $\mu$g protein) were applied in gel. Gel was stained with Coomassie brilliant blue R-250. For calibration of approximate molecular weights, RNA polymerase (165 000, 155 000, 39 000), bovine serum albumin (68 000), fumarase (48 500), glyceraldehyde-3-phosphate dehydrogenase (36 000), and trypsin inhibitor (21 500) were used. Values shown are molecular weights $\times 10^{-3}$. Arrows indicate the position of bands detected profoundly by periodate-Schiff reagent for carbohydrate stain.

## Discussion

Before adopting procedure C, two methods (procedure A and B) were checked. The plasma membranes obtained by procedure A, which produced the sheet-like membrane, were low in the relative activities of marker enzymes and in the protein recovery. Since it is difficult to homogenize the mammary tissue with a high content of connective tissue, disruption of such tissue should be carried out by more drastic homogenization. By drastic disruption of tissue with Waring blendor, plasma membranes were recovered as vesicular membrane from the microsomal fraction (procedure B). Purity and protein recovery of plasma membrane were improved 2-fold by this procedure. However, the relative activity of 5′-nucleotidase was still low (10-times over the homogenate) as compared with that of liver plasma membrane. Most of the plasma membrane marker enzymes (50–60%) were concentrated in the crude microsomes by procedures B and C (Table II). The contamination due to the trapped intravesicular cytoplasmic proteins is likely to cause the low relative activity of plasma membrane marker enzymes. For removal of such soluble proteins, the two washings by osmotic shock introduced by Wallach and Kamat [18] and prolonged centrifugation on discontinuous Ficoll density gradient were applied as described in the procedure C. By osmotic washing of microsomes, about 54% of the protein was removed and the recovery of marker enzymes for plasma membrane was decreased by 10–40%, while the specific activities of these enzymes were increased by 20–40%. The purity of plasma membrane was increased twice compared with the case of procedure B. The initial washing removed the trapped soluble proteins and by the second washing intravesicular soluble proteins were released because of the transient leakiness of the vesicle membranes caused by osmotic stress at low ionic strength [19].

Purification through a Ficoll density gradient was highly effective. Most of plasma membrane was collected at the medium/$d$ 1.033 interface by centrifugation for 3 h (procedure B) but by the prolonged centrifugation for about 6 h it was separated into two fractions: the medium/$d$ 1.033 and 1.033/1.053 interfaces (procedure C). By the following centrifugation through a sucrose density gradient, most of the plasma membrane of the two fractions was recovered from only the medium/$d$ 1.14 interface. The lower density of the plasma membranes obtained by procedure C was likely to depend on removal of the trapped intravesicular soluble proteins. In addition, the differences between Ficoll and sucrose gradients were as followed; in sucrose density gradient that gave a large osmotic effect the vesicle shrank with progressive centrifugations, but this occurred to a much lesser extent in Ficoll, which exibited a small osmotic effect. Therefore sucrose caused increased density (less than 1.14 g/cm$^3$), while Ficoll resulted in low buoyant density (1.01–1.06 g/cm$^3$), as pointed out by Wallach and Lin [44].

Yields of plasma membranes was about 0.5 mg protein per g wet tissue. This is lower than the yield of 1–3 mg which was obtained from rat liver plasma membrane, possibly because the mammary tissue is rich in the connective tissue.

The relative activities of the marker enzymes of the purified plasma membrane showed high values. The marker enzymes for plasma membrane used in the present study, 5′-nucleotidase, phosphodiesterase I, alkaline phosphatase, $Mg^{2+}$-ATPase, $(Na^+ + K^+)$-ATPase, and γ-glutamyltranspeptidase, are considered to be reliable as the plasma membrane markers of lactating mammary cells. The specific activity of these enzymes found in the crude and purified membranes was 50–65% and 30–45% of that found in the crude microsomes, respectively (Table II). In addition, these enzymes were found in relatively high activities in milk fat globule membrane which is mostly derived from the apical plasma membrane [5,51–54]. Enzymatic activity and the yield of plasma membrane were variable, perhaps reflecting the stage of lactation and the age of the animals.

Acid phosphatase activity was relatively high in the purified plasma membranes. It is considered that this enzyme is not a contaminant from lysosome but may result from the fusion of lysosomal membrane with plasma membrane, because of the significant presence of this enzyme in the secreted milk fat globule membrane. The activity of glucose-6-phosphatase was also somewhat high, while that of rotenone-insensitive NADH-cytochrome $c$ reductase was low. A high glucose-6-phosphatase

activity was likely to be due to the fact that other phosphatases were not inhibited completely with only EDTA in the assay reagent [26], since glucose 6-phosphate is a substrate for a number of phosphatases [55]. In connection with contaminating endoplasmic reticulum, RNA content was somewhat high in the purified membranes. This may be due to some small contamination by protein, because of ultraviolet measurement at 260 nm. However, the RNA content in our preparation was not so high as that of liver plasma membrane isolated from the microsomal fraction [17,19,20,26,39,45]. The activity of galactosyltransferase was relatively high in the crude plasma membranes but decreased in the purified plasma membranes, suggesting that the contamination of the Golgi apparatus was very small. It is, however, possible that significant quantities of this enzyme may be localized in the plasma membrane throughout the membrane fusion upon milk secretion, since the activity was detected in fresh milk fat globule membrane [53].

Morre [56] suggested that a 20- to 25-fold purification of 5′-nucleotidase relative to total homogenate was indicative of more than 90% purity for the isolated plasma membrane fraction. The relative activity of 5′-nucleotidase in the present preparations was especially high. The preparation of plasma membranes obtained from lactating mammary gland using procedure C is of reasonably high purity as determined qualitatively by electron microscopy and quantitatively by low activities of marker enzymes for contaminating organelles. A detailed study on the composition of the purified plasma membrane will be reported in a subsequent paper.

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## References

1 Patton, S. and Keenan, T.W. (1975) Biochim. Biophys. Acta 415, 273–309
2 Keenan, T.W., Morre, D.J. and Huang, C.M. (1974) in Lactation: A Comprehensive Treatise (Larson, B.L. and Smith, V.R., eds.), vol. II, pp. 193–233, Academic Press, New York
3 Bargmann, W., Fleischaur, K. and Knoop, A. (1961) Z. Zellforsch. 53, 545–568
4 Bargmann, W. and Knoop, A. (1959) Z. Zellforsch, 53, 344–388
5 Patton, S. and Tram, E.G. (1971) FEBS Lett. 14, 230–232
6 Keenan, T.W., Morre, D.J., Olson, D.E., Yunghans, W.N. and Patton, S. (1970) J. Cell Biol. 44, 80–93
7 Wooding, F.B.P. (1971) J. Ultrastruct. Res. 37, 388–400
8 Wooding, F.B.P. (1971) J. Cell Sci. 9, 805–821
9 Keenan, T.W. and Huang, C.M. (1972) J. Dairy Sci, 55, 1586–1596
10 Huang, C.M. and Keenan, T.W. (1972) J. Dairy Sci. 55, 862–864
11 Patton, S. and Jensen, R.G. (1976) Biomedical Aspects of Lactation, Pergamon Press, Oxford
12 Anderson, M.and Cawston, T.E. (1975) J. Dairy Res. 42, 459–483
13 DePierr, J.W. and Karnovsky, M.L. (1973) J. Cell Biol. 56, 275–303
14 Lauter, C.J., Solyom, A. and Tram, E.G. (1972) Biochim. Biophys. Acta 266, 511–523
15 Neville, D.M., Jr. (1960) J. Biophys. Biochem. Cytol. 8, 413–422
16 Emmelot, P., Bos, C.J., Benedetti, E.L. and Rumke, P.H. (1964) Biochim. Biophys. Acta 90, 126–145
17 Touster, O., Aronson, N.N., Jr., Dulaney, J.T. and Hendrickson, H. (1970) J. Cell Biol. 47, 604–618
18 Wallach, D.F.H. and Kamat, V.B. (1964) Proc. Natl. Acad. Sci. U.S.A. 52, 721–728
19 Ferber, E., Resch, K., Wallach, D.F.H. and Imm, W. (1972) Biochim. Biophys. Acta 266, 494–504
20 Emmelot, P., Bos, C.J., Van Hoeven, R.P. and Van Blitterswijk, W.J. (1974) Methods Enzymol. 31, 75–90
21 Bingham, E.W. and Zittle, C.A. (1963) Arch. Biochem. Biophys. 101, 471–477
22 Morton, R.K. (1955) Methods Enzymol. 2, 533–539
23 Tate, S.S. and Meister, A. (1974) J. Biol. Chem. 249, 7593–7602
24 Pennington, R.J. (1961) Biochem. J. 80, 649–654
25 Fried, R. and Fried, L.W. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), 2nd English edition, vol. 2, pp. 644–649, Verlag Chemie, Weinheim/Academic Press, New York
26 Aronson, N.N. and Touster, O. (1974) Methods Enzymol. 31, 90–102
27 Windell, C.C. and Unkeless, J.C. (1968) Proc. Natl. Acad. Sci. U.S.A. 61, 1050–1057
28 Cha, Y.N., Shin, B.C. and Lee, K.S. (1971) J. Gen. Physiol. 57, 202–215
29 Fujita, M., Kawai, K., Asano, S. and Nakao, M. (1973) Biochim. Biophys. Acta 307, 141–151

30 Mackler, B. (1967) Methods Enzymol. 10, 551–553
31 Kato, K., Yoshida, K., Tsukamoto, H., Nobunaga, M., Masuya, T. and Sawada, T. (1960) Chem. Pharm. Bull. 8, 239–242
32 Morre, D.J., Merlin, L.M. and Keenan, T.W. (1969) Biochem. Biophys. Res. Commun. 37, 813–819
33 Fleischer, B. (1974) Methods Enzymol. 31, 180–191
34 Snyder, S.H. and Hendley, E.D. (1971) Methods Enzymol. 17B, 741–746
35 Chen, P.S., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756–1758
36 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
37 Fleck, A. and Munro, H.N. (1962) Biochim. Biophys. Acta 55, 571–583
38 Burton, K. (1956) Biochem. J. 62, 315–323
39 Brown, A.E., Lok, M.P. and Elovson, J. (1976) Biochim. Biophys. Acta 426, 418–432
40 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
41 Laemmli, U.K. (1970) Nature (London) 227, 680–685
42 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
43 Neville, D.M., Jr. and Glossmann, H. (1974) Methods Enzymol. 32, 92–102
44 Wallach, D.F.H. and Lin, P.S. (1973) Biochim. Biophys. Acta 300, 211–254
45 House, D.R., Poulis, P. and Weidemann, M.J. (1972) Eur. J. Biochem. 24, 429–437
46 Shin, B.C., Ebner, K.E., Hudson, B.G. and Carraway, K.L. (1975) Cancer Res. 35, 1135–1140
47 Huggins, J.W., Trenbeath, T.P., Chesnut, R.W., Carraway, C.A.C. and Carraway, K.L. (1980) Exp. Cell Res. 126, 279–288
48 Carraway, K.L., Fogle, D.D., Cesnut, R.W., Huggins, J.W. and Carraway, C.A.C. (1975) J. Biol. Chem. 251, 6173–6178
49 Keenan, T.W., Sasaki, M., Eigel, W.N., Morre, D.J., Franke, W.W., Zulak, I.M. and Bushway, A.A. (1979) Exp. Cell Res. 124, 47–61
50 Diaz-Maurino, T. and Nieto, M. (1976) Biochim. Biophys. Acta 448, 234–244
51 Kitchen, B.J. (1974) Biochim. Biophys. Acta 356, 257–269
52 Baumrucker, C.R. and Keenan, T.W. (1975) J. Dairy Sci. 58, 1282–1287
53 Powell, J.T., Jarlfors, U. and Brew, K. (1977) J. Cell Biol. 72, 617–627
54 Kanno, C. and Yamauchi, K. (1979) J. Biochem. 85, 529–534
55 Tsai, C.-M., Chen, K.-Y. and Canellakis, E.S. (1979) Biochim. Biophys. Acta 401, 196–212
56 Morre, D.J. (1973) in Molecular Techniques and Approaches in Developmental Biology (Chrispeel, M.J., ed.), pp. 1–27, Wiley Interscience, New York