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Preparation and Properties of Thyroid Cell Membranes

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Summary. Calf and human thyroids have been disrupted by nitrogen microcavitation, and the thyroid membranes prepared by repeated centrifugation in low ionic strength buffers. Two classes of membranes were prepared by centrifugation on a discontinuous gradient of ficoll. A lighter fraction was comprised of somewhat larger vesicles; they were higher in Na^+-K^+-activated ATPase, phosphodiesterase, and 5′-nucleotidase than was the heavier fraction. The heavier fraction had a higher nicotinamide adenine nucleotide dehydrogenase-diaphorase activity. Thus the lighter fraction appears to have been enriched in fragments derived from the plasma membrane.

The epithelium of the thyroid follicle with its contained colloid is enmeshed in a dense, tough connective tissue. Disruption of the thyroid in glass vessels or disruption by motor-driven homogenizers requires high shear forces which may damage cell organelles or membranes. Although tissue fractions have been prepared from thyroid homogenates which have properties consistent with plasma membranes, the origin of these fractions has been uncertain, and there has been little reason to assume any selective enrichment of a particular fraction with respect to plasma membranes [10, 13, 18].

Cell ATPase activity which is Mg^{++}-dependent, activated by Na^+ and K^+, and inhibited by ouabain is generally considered to be a property of the plasma membrane and a marker for it [1, 6, 7]. Particulate 5′-nucleotidase activity also seems to reside principally in the plasma membrane [2, 3]. Conversely, nicotinamide adenine nucleotide dehydrogenase (NADH)-diaphorase activity is thought to be a marker for membranes derived from the endoplasmic reticulum [6, 14].

In the present study, we have applied the method of Kamat and Wallach [6] for preparation of membranes from calf and human thyroid tissue. We present evidence for relative enrichment of a fraction corresponding to the plasma membranes of the cells.

Methods

Calf necks were obtained from a slaughter house and brought at once to the laboratory. Human thyroids were obtained from the Department of Pathology at Massachusetts General Hospital within a few hours after death of the patients. An afferent artery to the calf thyroids was infused with 10 to 20 ml of a solution containing 0.25 M sucrose, 5 mM Tris base, and 0.2 mM $MgSO_4$ at pH 7.4 in order to rid the glands of red cells before excising them from the tissue beds. The human glands were not perfused. Adherent fat and connective tissue were removed, and the glands were diced into pieces approximately 0.3 cm thick. Tissue (50 g) was suspended in 150 ml of a solution containing 0.25 M sucrose, 5 mM Tris, and 0.2 mM $MgSO_4$ at pH 7.4, and was subjected to 800 psi nitrogen for 20 min in a pressure chamber (Artisans, Inc., Waltham, Mass.) resting in an ice bath. The suspension was stirred constantly with a magnetic stirring bar. The tissue and suspending fluid were released suddenly through 3/16-inch tubing. Full control was obtained by a quarter-turn of the valve to avoid explosive discharge and spattering of the effluent homogenate. The homogenate was quickly pressed through cheesecloth in a kitchen potato ricer and further dispersed with one stroke in a glass-Teflon homogenizer. Abundant cell nuclei but no whole cells were present in this fluid. Ethylene diamine tetraacetate (EDTA; disodium salt) was added to a final concentration of 1 mM. From this point, the homogenate was treated as described by Kamat and Wallach [6].

The homogenate was centrifuged for 15 min at 10,000 rpm in the Sorvall SS-34 rotor (approximately $12{,}100 \times g$) at 4 °C. The pellet was resuspended in 0.25 M sucrose, 5 mM Tris, 0.2 mM $MgSO_4$, pH 7.4, and centrifuged again. The supernatants were pooled and centrifuged at 40,000 rpm for 45 min in the Spinco Ti 50 rotor at 4 °C. The pellet from this centrifugation was resuspended in 0.01 M Tris base adjusted to pH 8.6 with HCl, stirred at 4 °C for 30 min, and centrifuged at 40,000 rpm for 45 min. The pellet was resuspended with a glass-Teflon homogenizer in 1 mM Tris, pH 8.6, stirred at 4 °C for 30 min, and centrifuged at 40,000 rpm for 45 min. The pellet was suspended in 1 mM Tris, 1 mM $MgSO_4$, pH 8.6, and dialyzed against the same buffer for 2 hr at 4 °C. The homogenate was layered over ficoll (density 1.096 at 4 °C) (Pharmacia, > 100,000 mol wt) containing 1 mM Tris, 1 mM $MgSO_4$, pH 8.6, (approximately 1:2, membrane suspension/ficoll) at 24,000 rpm for 17 to 22 hr at 4 °C in the SW 25.3 rotor. Two bands formed, one at and just below the barrier and another at the bottom of the tube as a pellet. At times a third somewhat diffuse band was seen below the barrier. The band at the barrier and the pellet were collected, resuspended in five volumes of 1 mM Tris, 0.01 M EDTA, pH 8.6, and spun again at 40,000 rpm for 45 min. The pellets were then suspended once again in 1 mM Tris, 0.01 M EDTA at pH 8.6, and dialyzed for 30 min against the same buffer, then for 1 hr against 1 mM Tris at pH 8.6, and then frozen. Storage in the presence of Mg^{++} tended to cause aggregation of membranes and interfered with separation on the ficoll gradient.

Protein was determined by the method of Lowry, Rosebrough, Farr and Randall [8]. ATPase activity was determined by incubation of approximately 100 μg of membrane protein in a total volume of 1.0 ml containing 5 mM ATP, 5 mM $MgCl_2$, 0.05 M Tris, pH 7.4, 1 mM EDTA, pH 7.4, and, when appropriate, 30 mM NaCl and 15 mM KCl. Incubation was at 37 °C for 1 hr, and the reaction was stopped by addition of 2 ml of 10% TCA. Phosphate in the supernatant was measured by the method of Fiske and Subbarow [5]. Sialic acid was measured by Warren's method [17] using neuraminidase (Calbiochem, Los Angeles, Calif.) or digestion with sulfuric acid. Adenyl cyclase was assayed by a modification of the method of Streeto and Reddy [12]. RNA was measured by the orcinol method [9].

NADH-diaphorase was measured by a modification of the method of Kamat and Wallach [6], using potassium ferricyanide as donor. The reaction was followed in the

Gilford recording spectrophotometer. 5′-Nucleotidase activity was determined by incubating approximately 100 μg of membrane protein for 30 min at 37 °C with 8-^{14}C-AMP [(Schwarz Bio Research, Inc., Orangeburg, N.Y.) (AMP final concentration, 17 μM in 0.06 M Tris, pH 7.8)], 16 mM glycyl glycine buffer, pH 7.8, and 2 mM $MgSO_4$, in a total volume of 3.25 ml. After addition of appropriate carriers, a 0.1-ml fraction was chromatographed on Whatman #40 paper in an ethanol −0.1 M boric acid (3.5:1) solvent system, pH 4.0. Spots were located under ultraviolet light. The entire chromatogram from origin to solvent front was cut into 1-cm lengths and measured for ^{14}C in a liquid scintillation counter; the 20-ml vials contained toluene with 7 g of isopropyl-phenylbiphenylyloxadiazole-1,3,4 (Pilot Chemicals, Inc., Watertown, Mass.) per liter. The fraction of substrate converted was estimated from the counts recorded from the 5′-AMP spot and the adenosine zone. Phosphodiesterase activity was measured similarly, using ^{3}H-cyclic AMP (Schwarz Bio Research) and cyclic AMP, in the same buffer system at pH 7.8.

Phosphodiesterase and alkaline phosphatase activities were measured by the methods of Bosmann, Hagopian and Eylar [2], using bis (p-nitrophenyl)-phosphate and p-nitrophenyl-phosphate as substrates, respectively. In each case, final readings were made at 400 mμ. Phosphodiesterase was also measured using cyclic AMP and ^{3}H-cyclic AMP as substrate, under the same conditions employed for measurement of 5′-nucleotidase as described above. UDPase activity was measured as described above for ATPase.

Results

Discontinuous ficoll gradients separated the membranes into two and sometimes three fractions. Approximately 80 to 90% of the protein appeared in the pellet at the bottom of the ficoll. Most of the remaining protein was in a sharp band just at the barrier between the buffer and the ficoll. In some gradients, a faint wider band was visible below the barrier, but its protein content was too low for consistent harvesting and measurement.

Electron micrographs of the two fractions appear in Fig. 1. The species at the barrier were approximately 1,500 A in diameter; those in the pellet were about 1,100 A. These appeared to be vesicular membranous structures with only a minimum amount of accompanying debris. Highly diluted membranes were dried on glass and observed in the scanning electron microscope. Vesicular structures which varied moderately in size were again seen.

Table 1 shows assays of several enzyme activities of the two membrane fractions from a single experiment. NADH-diaphorase had an activity with respect to protein in the pellet which was approximately three times that in the light fraction. On the other hand, Na^{+}-K^{+}-activated ATPase activity in the light fraction was about twenty times and 5′-nucleotidase activity about nine times the activity in the pellet. Phosphodiesterase and alkaline phosphatase activities were three to four times higher in the light fraction. Mixing experiments in which the light fraction was mixed with

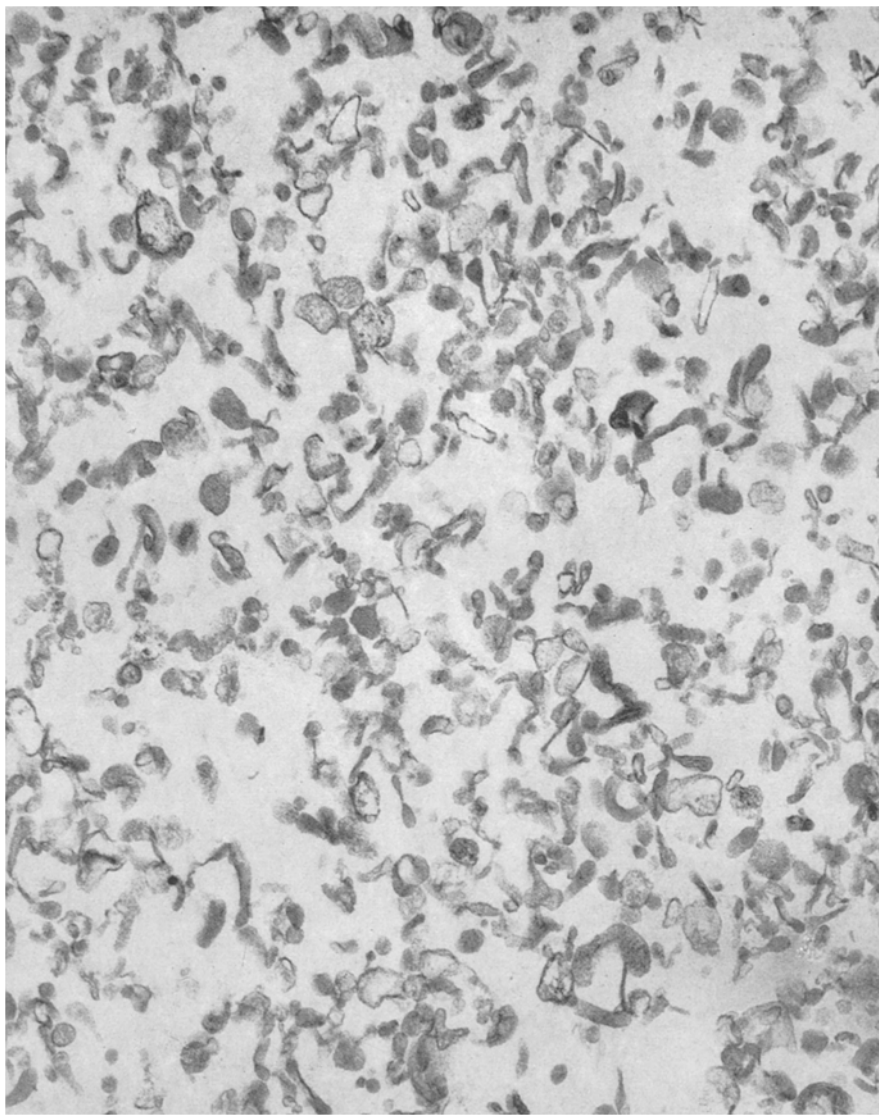

Fig. 1 A

Fig. 1. Vesicular membranous structures prepared from human thyroids. *A*, membranes from barrier after discontinuous ficoll gradient centrifugation; *B*, membranes from pellet. × 54,000 (by reproduction reduced to 3/5)

the pellet indicated that there was no inhibition of light-fraction enzyme activity by the pellet: activities were additive. UDPase activities were approximately the same. Phosphodiesterase activity was much higher when bis-(p-nitrophenyl)-phosphate was the substrate rather than cyclic AMP.

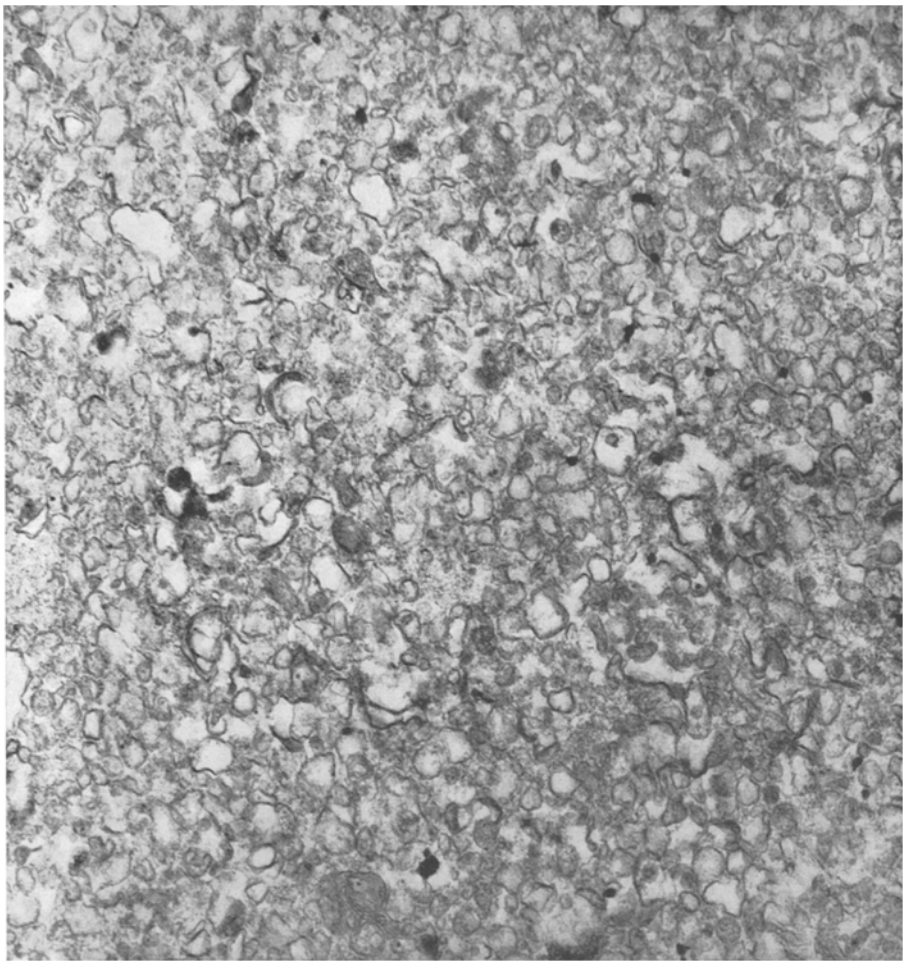

Fig. 1B

Table 1. *Enzyme activities in calf thyroid membranes*

Enzyme	Activity (μmoles/mg protein/hr)	
	at barrier	in pellet
Na^{+}-K^{+}-activated ATPase	3.95	0.19
Mg^{++}-dependent ATPase	9.46	3.09
NADH-diaphorase[a]	0.12	0.41
Phosphodiesterase [bis-(p-nitrophenyl)-phosphate]	2.22	0.89
Phosphodiesterase (cyclic AMP)	0.085	0.021
Alkaline phosphatase	2.04	0.66
UDPase	2.89	2.02
5′-Nucleotidase	1.37	0.16

[a] mmoles per min

Table 2. *Na^+-K^+-activated ATPase and NADH-diaphorase activity in thyroid membranes*

Experiment no.	Na^+-K^+-activated ATPase (μmoles P_i/mg protein/hr)		NADH-diaphorase (mmoles/mg protein/min)	
	at barrier	in pellet	at barrier	in pellet
1	2.1	0.26	0.13	0.40
2	4.3	1.9	0.23	0.90
3[a]	7.1	1.8	0.29	0.67
4	4.8	3.6	0.22	0.99
5	3.5	1.3	0.25	1.1
6	2.4	0.94	0.22	0.72
7	2.3	0.48	0.13	0.27
8	3.6	0.23	0.14	1.0
9	0.71	0	0.16	0.52
10	1.7	0	0.46	1.0

[a] Human thyroid

Some of the 5′-nucleotidase activity was presumably due to nonspecific phosphatase activity, but the ratio of specific activity between the light fraction and the pellet for 5′-nucleotidase activity was always substantially greater than for alkaline phosphatase (at pH 10) or for phosphatase activity measured at pH 7.8. This finding suggests that there is a phosphatase in these membranes which is specific for 5′-AMP.

Data on Na^+-K^+-activated ATPase and NADH-diaphorase activity appear in Table 2. Although there was considerable variation in activity among experiments, the ATPase activity was usually three or more times higher in the light membrane fraction than in the ficoll pellet fraction. Mg^{++}-dependent ATPase activity was also generally higher in the light membrane fraction, but the differential was usually much less than with Na^+-K^+-dependent activity. The diaphorase activity was three to six times higher in the pellet fraction. Sialic acid was measured on four experiments. Values ranged from 0.013 to 0.026 μmoles per mg protein in the pellet and from 0.023 to 0.085 μmoles in the light fraction, being two to seven times more concentrated in the light fraction.

Four sets of membrane preparations were solubilized in 0.5% sodium dodecyl sulfate and measured for optical density at 260 and 280 mμ. The ratios varied from 1.01 to 1.17 for the fraction from the barrier and from 1.24 to 1.26 in the pellet. These findings are consistent with a larger concentration of RNA relative to protein in the pellet. Treatment of the pelleted material for 1 hr at 20 °C with RNAase followed by pelleting and solubilizing in SDS failed to change the 260:280 ratio. RNA was

measured on one occasion by the orcinol method. The concentration in the light fraction was 5.2 μg/mg protein, and in the pellet, 30.3 μg.

No adenyl cyclase activity was demonstrable in the membranes. No experiments were done with membranes prepared from thyroid stimulating hormone (TSH)-stimulated thyroid tissue. Phosphodiesterase activity, using bis-(p-nitrophenyl)-phosphate as substrate, was present in both classes of membranes but had a higher specific activity in the barrier material than in the pellet. The specific activity of phosphodiesterase, using cyclic AMP as substrate (without diesterase inhibitors added), was always far lower. Addition of bis-(p-nitrophenyl)-phosphate at a molar concentration 10 to 100 times that of cyclic AMP failed to inhibit cyclic AMP-diesterase activity.

5′-Nucleotidase activity generally gave better discrimination between the two membrane fractions than any other modality tested. In some experiments showing good activity in the membranes from the barrier, no 5′-nucleotidase activity was found in the pellet. In most experiments, ratios between the activity in the barrier and in the pellet were found to be from 10 to 20.

Discussion

The method of cell preparation described here was adapted from that of Kamat and Wallach [6] for the Ehrlich ascites carcinoma cells. It takes advantage of the fact that in the presence of a divalent cation, and at low ionic strength, the vesicles which are formed from the plasma membrane shrink less than those from the endoplasmic reticulum. Accordingly, they have a different net density, enabling the two species of membranes to be separated. Extensive centrifugations and dialysis serve to rid the preparation of much of the adherent and entrapped protein. Differentiation of the lighter fraction as enriched in plasma membranes was based on higher relative concentration of cation-activated ATPase activity in the light membranes and higher NADH-diaphorase activity in the heavy fraction. In addition, the light membranes were more effective in binding agglutinating antibodies [14]. Bosmann, Hagopian and Eylar [2], employing a different method of cell fractionation of HeLa cells, have shown that 5′-nucleotidase is found almost exclusively in the plasma membrane fraction.

Cation-activated, ouabain-sensitive ATPase activity has been demonstrated repeatedly in thyroid preparations. Turkington [13] prepared a fraction from calf thyroid homogenate according to the method of Emmelot and Bos [4] and found ATPase activity which was ouabain sensitive, was activated by Na^+ and K^+, and was stimulated by thyrotropin. Using a

similar method of preparation, we obtained analogous results, except that when a highly purified preparation of TSH was used there was no significant stimulation [10]. Our tissue preparation also effected a transfer of phosphate from ATP to receptor protein (dephosphophosvitin) in the presence of phosphate buffer [11]. Although it has been assumed, in none of these experiments has clear evidence been presented for selective enrichment of the membrane preparations with plasma membranes.

Tissue disruption by nitrogen microcavitation has the advantage that large amounts of thyroid tissue can be quickly and easily homogenized without the risk of excessive heating which may accompany homogenizing methods requiring high mechanical shear. The membrane fractions are widely separated on the discontinuous ficoll gradients, and harvesting is simple.

The membrane fractions at the top of the ficoll gradient differed from those at the bottom in several respects. Membranes from the barrier had a Na^+-K^+-ATPase activity several times higher than that from membranes from the pellet. NADH-diaphorase activity, however, was higher in the membranes from the pellet. Sialic acid was in higher concentration in the membranes from the barrier. The best discriminant between the two membrane fractions was 5′-nucleotidase which was much more active in the barrier membranes than in those from the pellet. Phosphodiesterase activity was also higher in the barrier membranes when bis-(p-nitrophenyl)-phosphate was the substrate rather than when cyclic AMP was used; activity with the bis-(p-nitrophenyl)-phosphate was much higher and failed to inhibit cyclic AMP-diesterase activity, even when present in large excess.

The findings reported here are consistent with those reported by Wallach and his colleagues [6, 15, 16] for membranes prepared from ascites tumor cells and with those of Bosmann et al. [2] for membranes prepared from HeLa cells. However, we found UDPase activity in the light membranes approximately equal to that in the pellet, whereas Bosmann et al. found the activity largely excluded from the fraction which they identified with the plasma membranes.

Dissociation of Na^+-K^+-activated ATPase activity, phosphodiesterase activity, and 5′-nucleotidase activity from NADH-diaphorase activity suggests a different anatomical origin for the two fractions and is consistent with one fraction being relatively enriched with plasma membrane. This dissociation of membrane enzyme activity has been coupled with consistent electron micrographic evidence by Bosmann et al. [2] and with evidence of Kamat and Wallach [6] linking cell surface antigens with the fraction thought on other grounds to be relatively enriched in plasma membranes.

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