Preparation and Characterization of Subfractions of Bovine Thyroid Plasma Membranes*

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Two subfractions of bovine thyroid plasma membranes, light membranes (L-membranes) and heavy membranes (H-membranes), were obtained by a discontinuous sucrose gradient centrifugation of plasma membranes. Electron microscopy of the plasma membrane and its subfractions showed that the H-membranes were very similar to the plasma membrane fraction, both contained junctional complexes, long membrane sheets, and vesicles. In contrast, the L-membranes consisted mainly of short membrane sheets and vesicles, and only a few junctional complexes. The H-membranes had greater adenylate cyclase activity which responded to thyroid-stimulating hormone (TSH) while this hormone had very little effect on the enzyme activity in the L-membranes. Despite the marked difference in TSH stimulation of adenylate cyclase activity in the H- and L-membrane fractions, specific binding of 125I-TSH was similar in both fractions. The L-membranes had higher specific activities of 5'-nucleotidase and (Na+ + K+)-ATPase while (Na+ + K+)-ATPase and alkaline phosphatase activities were similar in the two subfractions. Protein kinase activity of H-membranes was not significantly stimulated by exogenous cyclic adenosine 3':5'-monophosphate (cAMP). Plasma membranes and H-membranes contained a substrate capable of being phosphorylated. Such phosphorylation was slightly increased by addition of soluble protein kinase. The phosphorylation of exogenous histone by protein kinase of plasma membranes and H-membranes was augmented by cAMP. In contrast, L-membranes had very little protein kinase activity even when exogenous histone was added. They were not a very good substrate for cytosolic protein kinase.

Thyroid-stimulating hormone regulates thyroid gland function following its initial binding to specific hormone receptors on the plasma membrane and activation of the adenylate cyclase-adenosine 3':5'-monophosphate system (1-11). The increased cAMP activates protein kinase (12-15), which then presumably phosphorylates appropriate substrates mediating the intracellular effects of the hormone (14, 16, 17). TSH rapidly induces pseudopod formation in the apical thyroid plasma membrane with ingestion of colloid droplets (18-20). To define the biochemical mechanism involved in such changes, separation of thyroid plasma membranes into basal and apical fractions would be desirable. The present studies characterize two subfractions of thyroid plasma membranes in relation to TSH binding, adenylate cyclase activity, other membrane-associated enzymes, and electron microscopic appearance.

EXPERIMENTAL PROCEDURES

Cell Fractions.-- Plasma membranes were isolated from bovine thyroid glands using a slight modification of the method previously described by our laboratory (9). Batches of 6 g of minced thyroid tissue, totaling 36 to 48 g, were homogenized in 50 ml of 1 mM NaHCO3 buffer (pH 7.5) using 30 strokes of a loose-fitting Dounce homogenizer. The homogenate was diluted to 100 ml with 1 mM NaHCO3, buffer and kept on ice for 10 min. After filtering the homogenate through cheesecloth, it was centrifuged at 2900 rpm (1680 x g) for 25 min in an International RR2 centrifuge. The precipitate was reuspended in 30 ml of the 1 mM NaHCO3 buffer and centrifuged again at 2,900 rpm for 15 min. The precipitate (total 12 to 16 ml) was folded into 63% sucrose with several strokes of the Dounce homogenizer to provide a final concentration of sucrose of 45%. Sucrose solutions of 42% (10 ml) and 10% (4 to 6 ml) were layered over the membrane suspension. The tubes were centrifuged at 25,000 rpm (63,800 x g) for 120 min using a SW 25 rotor in a Beckman ultracentrifuge. The precipitates were resuspended in 10 ml of 1 mM NaHCO3, buffer and used immediately for all enzyme assays except for adenylate cyclase and protein kinase. These enzymes were assayed within 1 week, at which time there was no loss of activity as a result of storage at -20°. The remaining plasma membranes in sucrose solution were refolded into 63% sucrose to provide a final concentration of sucrose of 45%. Sucrose solutions of 41% (6.5 ml), 35% (10 ml), and 10% (2 to 3 ml) were layered over it. The tubes were centrifuged at 25,000 rpm for 120 min using a SW 25 rotor in a Beckman ultracentrifuge. After centrifugation, the materials at the interface between the layers of 10% and 35% sucrose solutions and at the interface between the layers of 35% and 41% sucrose solutions were obtained by aspiration and designated light membranes (L-membranes) and heavy membranes (H-membranes), respectively. These fractions were diluted 1:1 with 1 mM NaHCO3 buffer and then centrifuged at 18,000 rpm (33,000 x g) for 25 min in a Beckman ultracentrifuge. The precipitates were suspended in 0.2 to 0.4 ml of 1 mM NaHCO3, buffer and used immediately for enzyme assays as described above for the plasma membranes.

Enzyme Assays-- Enzyme activities were determined in duplicate in fresh preparations except for adenylate cyclase and protein kinase as described above. Adenylyl cyclase activity was assayed as previously described (21). Peroxidase was assayed by the method of Hossay and Ui (22). Standard methods were used for assay of 5'-nucleotidase (23), (Na+ + K+)-ATPase (24), Mg2+ATPase (25), alka-
line phosphatase, DPNH cytochrome c reductase, and cytochrome c oxidase. Inorganic phosphate liberated was analyzed by the Fiske-Subbarow method (28) or by the method of Chen et al. (29). Intrinsic protein kinase activity of plasma membranes and the subfractions was assayed by a slight modification of a previously described method (30). The assay mixture contained 50 mM glycero-

phosphate (pH 6.5), 2 mM theophylline, 10 mM MgCl2, 0.3 mM EGTA, 20 mM γ-32P]ATP, 0.5 μg of membrane protein with or without 1 μM cAMP, and 10 mM NaF in a final volume of 0.27 ml. In some experiments 100 μg of cytosolic protein kinase of thyroid were added to examine its effect on phosphorylation of the membrane fractions. Cytosolic protein kinase was prepared from bovine thyroid by the method of Miyamoto et al. (31). The purification procedure was carried out through the (NH4)2SO4 precipitation and the dialyzed enzyme solution was stored at -20°. Intrinsic protein kinase activity of the membrane fractions was also assayed in some experiments by adding exogenous substrate (100 μg of histone). Phosphorylation of exogenous substrate was not corrected for endogenous membrane phosphorylation. Protein was measured by the method of Lowry et al. (32).

Other Procedures - Specific binding of 125I-TSH to plasma mem-

branes and subfractions was measured by the method previously described (8). The electron microscopic analysis of the membrane fractions was carried out by techniques described elsewhere (33).

Materials - ATP, cAMP, and histone II A mixture were obtained from Sigma. α-32P]ATP (15 to 20 Ci/mmol) and γ-32P]ATP (1 to 3 Ci/mmol) were purchased from New England Nuclear and Amersham/ Searle Corp., respectively. TSH (NIH-B6) was kindly provided by the National Institute of Arthritis, Metabolism, and Digestive Diseases. All other reagents were of analytical grade.

RESULTS

The data in Table I summarize the recoveries of protein and several enzyme activities in plasma membranes, its subfrac-

tions, and the second band. The yield of plasma membrane was about 0.15 mg of protein/g of wet weight of thyroid tissue, representing approximately an 0.08% recovery from the initial whole homogenates. Wolff and Jones reported that the recovery of plasma membrane protein was 0.01 to 0.02% from a 1000 g supernatant of bovine thyroid gland homogenate (10). Approximately 70% of the protein in the plasma membranes was recovered in the L- and H-membrane fractions. The amount of protein of L-membranes was about one-third of that of the H-membranes.

Specific activities of membrane marker enzymes in the plasma membranes were all significantly greater than those of the homogenate and second band. In the plasma membranes the recovery of 5′-nucleotidase was the lowest, while that of (Na+ + K+)-ATPase was the highest. Although these recoveries are much lower than those reported with other tissues (34), they are very similar to that obtained for thyroid tissue by Wolff and Jones (10) and Roques et al. (35). L-membranes had significantly greater 5′-nucleotidase and Mg2+ATPase specific activities than H-membranes and plasma membranes, and the recovery of 5′-nucleotidase activity was also greater in the L-membranes than in the H-membranes. Specific activities of (Na+ + K+)-ATPase and alkaline phosphatase were similar in plasma membranes and the two subfractions. The recovery of these two enzymes was similar in both the H- and L-mem-

branes. Since iodination of thyroglobulin takes place at or near the apical end of the thyroid cell (36, 37), peroxidase activity was measured to determine if it could be a marker for the apical plasma membranes. Although peroxidase activity was found in plasma membrane and its subfractions, the highest specific activity of peroxidase was found in the second band. This fraction also had the highest specific activity of DPNH-cytochrome c reductase, an enzyme characteristic of microsomes (38). Based on the specific activity of this enzyme, H- and plasma membranes had similar contamination with microsomes. The mitochondrial contamination was similar in the plasma membranes and the two membrane subfractions as indicated by cytochrome c oxidase specific activity.

To characterize the subfractions further, adenylate cyclase activities were assayed in plasma membranes and the two subfractions (Table II). TSH (10 milliunits/tube) and NaF (10 mM) significantly increased adenylate cyclase activity in plasma membranes. Stimulation was considerably greater when the ATP concentration was increased from 0.4 to 2 mM confirming the previous report of Wolff and Jones (10). H-membrane fractions also contained adenylate cyclase activity which was markedly increased by both TSH and NaF. The effect of TSH was similar with both concentrations of ATP, but recovery of enzyme activity was significantly lower when the assays were done with 2 mM ATP. L-membranes contained significantly less adenylate cyclase activity which was not increased by TSH (10 milliunits/tube). This amount of TSH did not increase the enzyme activity with 2 mM ATP even when the incubation was continued for 30 min. However, increasing the TSH to 100 milliunits/tube did cause some increase in adenylate cyclase activity at both 10 and 30 min, but it was considerably less than that observed in the H-membranes (results not shown). NaF significantly increased the enzyme activity in L-membranes with 0.4 mM but not 2 mM ATP. Despite this marked difference in stimulation of adenylate cyclase by TSH in the two membrane subfractions, specific binding of 125I-TSH was similar in the plasma mem-

branes and the H- and L-subfractions (Table III). The total binding was higher in the H-membranes since a larger amount of that fraction than L-membranes was recovered from the initial plasma membrane preparation.

The morphology of the plasma membranes and the H- and L-subfractions is illustrated in Figs. 1 to 4. The plasma mem-

brane fraction contained numerous junctional complexes, single membrane sheets, and vesicles (Fig. 1). Occasionally, profiles compatible with the apical plasmalemma of the thyroid cell with its adjacent vesicles were observed (Fig. 2). The H-

membrane fraction was very similar in appearance to the plasma membrane fraction (Fig. 3), whereas the L-membrane fraction appeared as short membrane sheets or as vesicles (Fig. 4). An unusual feature of this fraction, which was observed frequently, was the apparent continuity of the mem-

brane profiles associated with junctional complexes (Fig. 4).

An attempt was made to identify the structures which contain 5′-nucleotidase using a cytochemical procedure for visu-
alizing the enzyme in isolated cell fractions (33). In all three fractions, lead precipitate was distributed very sparsely, and concentrated on the vesicles (results not shown).

The intrinsic protein kinase activities of plasma membranes and the L- and H-subfractions and their responses to cAMP, TSH, and NaF are shown in Table IV. Basal activity was significantly higher in H-membranes and lower in L-membranes compared to plasma membranes. In none of the four prepara-

tions was there any significant stimulation by cAMP (1 μM) or TSH (100 milliunits/ml). In contrast, NaF, an inhibi-

tor of ATPase (39) and phosphoprotein phosphatase (40), significantly augmented protein kinase activity in plasma mem-

branes and H-membranes but not in L-membranes. Addition of histone significantly increased the cAMP-independent pro-

tein kinase activity in both the plasma membranes and the H-

membranes but not in the L-membranes (Table V). NaF (10 mM) was present in all assays. In contrast to the results obtained using endogenous substrate, when histone was added cAMP significantly increased protein kinase activity in
Table I

Recoveries of protein and enzyme activities in plasma membrane, its subfractions, and second band

Specific activities of 5'-nucleotidase, (Na⁺ + K⁺)-ATPase, Mg²⁺ ATPase, and alkaline phosphatase are expressed as micromoles/min/mg of protein and activity is expressed on basis of wet weight of tissue. The amount of protein is indicated as milligrams of protein/g of wet weight of thyroid tissue. Recovery is the amount of protein or the activity of the enzyme expressed as percentage of that in initial homogenate. Values are means ± S.E. for the number of preparations given in parentheses.

| Homogenate | Plasma membranes | L-membranes | H-membranes | Second band |
|------------|------------------|-------------|-------------|-------------|
| Specific activity | Activity | Specific activity | Activity | Recovery | Specific activity | Activity | Recovery | Specific activity | Activity | Recovery |
| 5'-Nucleotidase | 0.507±0.037 (6) | 94.4±7.0 (6) | 2.91±0.27 (6) | 0.418±0.041 (6) | 0.446±0.041 (6) | 10.1±1.3 (5) | 0.25±0.026 (5) | 0.270±0.034 (5) | 1.33±0.18 (5) | 0.094±0.015 (5) | 0.099±0.006 (5) | 0.802±0.070 (5) | 0.063±0.000 (5) | 0.072±0.000 (5) |
| Mg²⁺ ATPase | 0.893±0.080 (4) | 166±16 (4) | 13.8±1.1 (4) | 1.98±0.16 (4) | 1.23±0.16 (4) | 33.4±1.8 (4) | 0.030±0.004 (5) | 0.518±0.006 (5) | 12.1±0.7 (4) | 0.862±0.018 (4) | 0.526±0.060 (4) | 4.38±0.12 (4) | 0.345±0.016 (4) | 0.817±0.044 (4) |
| (Na⁺ + K⁺)-ATPase | 0.039±0.000 (4) | 7.25±1.1 (4) | 6.15±0.38 (4) | 0.886±0.054 (4) | 12.8±1.4 (4) | 7.96±1.27 (4) | 0.199±0.032 (4) | 2.98±0.78 (4) | 5.93±0.76 (4) | 0.421±0.054 (4) | 6.28±1.48 (4) | 0.650±0.33 (4) | 0.051±0.025 (4) | 0.660±0.230 (4) |
| Alkaline phosphatase | 0.067±0.014 (5) | 12.4±2.6 (5) | 2.70±0.53 (5) | 0.389±0.075 (5) | 3.33±0.59 (5) | 4.35±0.63 (5) | 0.109±0.015 (5) | 0.956±0.136 (5) | 2.64±0.60 (5) | 0.187±0.041 (5) | 1.53±0.26 (5) | 0.564±0.075 (5) | 0.044±0.000 (5) | 0.422±0.142 (5) |
| Peroxidase | 0.206±0.23 (3) | 38.4±2.3 (3) | 321±63 (3) | 0.046±0.000 (3) | 0.125±0.000 (3) | 197±55 (5) | 0.015±0.000 (5) | 0.013±0.000 (5) | 351±48 (5) | 0.025±0.000 (5) | 0.068±0.000 (5) | 1547±192 (5) | 0.120±0.000 (5) | 0.311±0.030 (5) |
| DPNH-cytochrome c reductase | 6.14±1.35 (3) | 1192±256 (3) | 19.6±6.2 (3) | 0.51±0.99 (3) | 0.252±0.000 (3) | 11.9±0.1 (4) | 0.297±0.022 (4) | 0.022±0.000 (4) | 18.6±4.6 (4) | 1.32±0.33 (4) | 0.095±0.030 (4) | 81.7±3.2 (4) | 6.45±0.65 (4) | 0.557±0.133 (4) |
| Cytochrome c oxidase | 0.015±0.009 (2) | 2.79±0.19 (2) | 0.72±0.25 (2) | 0.010±0.000 (2) | 0.039±0.053 (2) | 0.023±0.022 (2) | 0.001±0.000 (2) | 0.109±0.019 (2) | 0.039±0.000 (2) | 0.005±0.000 (2) | 0.160±0.006 (2) | 0.005±0.000 (2) | 0.005±0.000 (2) | 0.005±0.000 (2) |
| Protein | 185±4 (7) | 0.14±0.01 (9) | 0.07±0.006 (7) | 0.025±0.002 (7) | 0.014±0.001 (7) | 0.071±0.002 (7) | 0.035±0.001 (7) | 0.079±0.003 (5) | 0.042±0.001 (5) |

* p < 0.001 as compared to those of second band.
* p < 0.001 as compared to those of 5'-nucleotidase, Mg²⁺ ATPase, and alkaline phosphatase.
* p < 0.01 as compared to that of H-membrane.
* p < 0.01 as compared to that of second band.
* p < 0.01 as compared to that of 5'-nucleotidase.
binding of \( ^{125}\text{I}-\text{TSH} \) to plasma membranes and its subfractions

Membranes (30 \( \mu \text{g} \) of protein) were incubated at 22° for 60 min in 20 \( \text{mm} \) Tris/\( \text{HCl} \) buffer (pH 7.5), 2 \( \text{mm} \) \( \text{MgSO}_4 \), 0.6 \( \text{mm} \) EDTA, 10 \( \text{mm} \) theophylline, 1.3% albumin, 37 \( \text{pm} \) of ATP, and 150 microunits of \( ^{125}\text{I}-\text{TSH} \) (4.4 \( \times \) 10\(^4\) cpm) in a final volume of 0.16 ml. The values are mean \( \pm \) S.E. of six experiments.

**Table II**

| ATP | Specific activity | Activity | Specific activity | Activity | Recovery | Specific activity | Activity | Recovery |
|-----|------------------|----------|------------------|----------|----------|------------------|----------|----------|
| mM | pmol/10 min/mg protein | pmol/10 min/g tissue | pmol/10 min/mg protein | pmol/10 min/g tissue | % | pmol/10 min/mg protein | pmol/10 min/g tissue | % |
| Control | 0.4 | 206 \( \pm \) 66\(^a\) | 29.7 \( \pm \) 9.6 | 14.2 \( \pm \) 1.6 | 0.96 \( \pm \) 0.04 | 1.9 \( \pm \) 0.8 | 187 \( \pm \) 61\(^a\) | 13.3 \( \pm \) 7.5 | 45 \( \pm \) 3 |
| TSH (10 milli-units/tube) | 0.4 | 1129 \( \pm \) 287\(^b\) | 161 \( \pm \) 37 | 56.7 \( \pm \) 22.7 | 1.42 \( \pm \) 0.57 | 1.1 \( \pm \) 0.6 | 1155 \( \pm \) 282\(^b\) | 81.9 \( \pm \) 19.4 | 51 \( \pm \) 10 |
| NaF (10 mM) | 0.4 | 2054 \( \pm \) 548\(^c\) | 296 \( \pm \) 79 | 71 \( \pm \) 41 | 4.30 \( \pm \) 1.01 | 1.7 \( \pm \) 0.6 | 2491 \( \pm \) 525\(^b\) | 177 \( \pm \) 36 | 62 \( \pm \) 10 |
| Control | 2 | 493 \( \pm \) 76\(^d\) | 71.0 \( \pm \) 10.9 | 307 \( \pm \) 96 | 7.68 \( \pm \) 2.40 | 10 \( \pm \) 2 | 483 \( \pm \) 133 | 34.3 \( \pm \) 9 | 46 \( \pm \) 9 |
| TSH (10 milli-units/tube) | 2 | 4414 \( \pm \) 169\(^h\) | 637 \( \pm \) 25 | 557 \( \pm \) 200 | 13.9 \( \pm \) 5.0 | 2.2 \( \pm \) 0.7 | 2850 \( \pm \) 410\(^c\) | 210 \( \pm \) 29 | 33 \( \pm \) 4 |
| NaF (10 mM) | 0.4 | 5553 \( \pm \) 823\(^h\) | 800 \( \pm \) 118 | 900 \( \pm \) 363 | 22.5 \( \pm \) 9.1 | 2.7 \( \pm \) 0.9 | 4391 \( \pm \) 1024\(^g\) | 312 \( \pm \) 73 | 38 \( \pm \) 6 |
| Recovery of protein (%) | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

\(^a\) \( p < 0.05 \) as compared to that of L-membrane.
\(^b\) \( p < 0.05 \) as compared to control.
\(^c\) \( p < 0.05 \) as compared to that of 0.4 mM ATP.
\(^d\) \( p < 0.02 \) as compared to control.
\(^e\) \( p < 0.02 \) as compared to that of 0.4 mM ATP.

**Table III**

*Binding of \( ^{125}\text{I}-\text{TSH} \) to plasma membranes and its subfractions*

Membranes (30 \( \mu \text{g} \) of protein) were incubated at 22° for 60 min in 20 \( \text{mm} \) Tris/\( \text{HCl} \) buffer (pH 7.5), 2 \( \text{mm} \) \( \text{MgSO}_4 \), 0.6 \( \text{mm} \) EDTA, 10 \( \text{mm} \) theophylline, 1.3% albumin, 37 \( \mu \text{m} \) of ATP with an ATP-regenerating system, and 150 microunits of \( ^{125}\text{I}-\text{TSH} \) (4.4 \( \times \) 10\(^4\) cpm) in a final volume of 0.16 ml. The values are mean \( \pm \) S.E. of six experiments.

| Specific binding | Binding | Recovery |
|-----------------|---------|----------|
| microtissues | microtissues | TSH/g tissue | % |
| Plasma membrane (6) | \( 62 \pm 20 \) | 11.7 \( \pm \) 2.8 | 100 |
| L-membrane (6) | \( 110 \pm 32 \) | 2.74 \( \pm \) 0.80 | 22 \( \pm \) 1 |
| H-membrane (6) | \( 79 \pm 22 \) | 5.60 \( \pm \) 1.60 | 46 \( \pm \) 3 |

\(^p < 0.001 \) as compared to L-membrane.

all of the membrane fractions except the L-membranes. Addition of protein kinase prepared from the cytosol of bovine thyroid increased significantly phosphorylation of endogenous substrate in plasma and H-membranes but not in L-membranes. However, such phosphorylation was not significantly augmented by cAMP. The greatest phosphorylation of all the fractions was obtained when both cytosolic protein kinase and histone were added. As would be expected from the experiment in which histone was added, cAMP increased phosphorylation when both protein kinase and histone were added.

**Discussion**

Two distinct subfractions of thyroid plasma membranes were obtained by discontinuous sucrose gradient centrifugation of plasma membranes. These subfractions, designated H- and L-membranes, had significant differences in some of their enzyme activities indicating that they represent different parts of the plasma membrane and not just different size fragments of the same membrane. The electron microscopic examination showed that the plasma membrane fraction contained junctional complexes, which must have originated from the lateral plasmalemma (41), together with membranes which probably originated from the apical plasmalemma. At present, it is much harder to identify membranes from the basal plasmalemma morphologically; the conclusion that these membranes are present in the plasma membrane fraction is based solely on the biochemical results. The origin of the vesicles which are enriched in 5'-nucleotidase is also not certain, even though the enzyme is concentrated in the plasma membrane of a variety of cells (23). The identification of the region of the plasma membrane from which the vesicles are derived must await the results of cytochemical studies which are in progress on intact cells. The results do, however, indicate that the fragmentation and subsequent fractionation of the plasma membrane need not be the same in different cells. Wisher and Evans prepared six different subfractions of hepatic plasma membranes and tentatively identified them as being from different areas of the cell (43). The blood-sinusoidal face was identified by its high concentration of adenylate cyclase activity and its responsiveness to glucagon. Such membranes were composed mostly of vesicles and were of lower density than those fractions which were considered representative of the contiguous faces of the hepatocytes. Another light membrane fraction, designated Z-L, and also composed primarily of vesicles, was felt to represent bile-canicular face membranes since they were very rich in 5'-nucleotidase, alkaline phosphatase, and Mg\(^{2+}\)ATPase.

The results obtained with the H- and L-membrane fractions from the thyroid do not permit such definitive identification. The H-membranes might represent basal membranes because of the marked TSH stimulation of adenylate cyclase. Although Wisher and Evans identified a light membrane fraction as the blood-sinusoidal membrane based on high adenylate cyclase activity and its response to glucagon, five of their six subfractions contained some adenylate cyclase activity which was
FIG. 1 to 4. Electron micrographs of thin sections of bovine thyroid membrane fractions obtained as described in the text.  
FIG. 1 (top). Representative field of the plasma membrane fraction. Junctional complexes (long arrows), large and small vesicular profiles (short arrow and arrowhead, respectively) are clearly evident. × 22,500.  
FIG. 2 (center). Plasma membrane fraction field selected to illustrate a profile which resembles the apical plasmalemma of an intact thyroid cell. × 15,000.  
FIG. 3 (bottom left). Representative field of the heavy membrane fraction. The appearance is similar to that of the plasma membrane fraction. × 22,500.  
FIG. 4 (bottom right). Representative field of the light membrane fraction. Many of the profiles are apparently vesicular (arrowheads) including those associated with junctional complexes (arrow). × 22,500.
most of the hepatic adenylate cyclase activity of plasma thyroid hormone bound to both the contraluminal and luminal membranes. Kinne et al. reported that parathyroid hormone bound to both the contraluminal and luminal membranes does not exclude the possibility that the H-membranes represent basal membranes. Kinne et al. reported that parathyroid hormone bound to both the contraluminal and luminal membranes.

**Table IV**

*Intrinsic protein kinase activities of plasma membranes and its subfractions*

Membranes (50 µg of protein) were incubated at 37° for 5 min in 50 mM glycerophosphate, pH 6.5, 2 mM theophylline, 10 mM MgCl₂, 0.3 mM EGTA, and 20 µM [γ-32P]ATP with or without 1 µM cAMP. TSH (100 milliunits/ml), and NaF (10 mM). The final volume was 0.27 ml. Specific activity is expressed as picomoles of [32P] incorporated into protein/5 min/mg of protein and activity is based on grams of wet weight of tissue. Recovery is expressed as the percentage of that in plasma membrane. The values are the mean ± S.E. of four experiments.

| Plasma membrane | L-membrane | H-membrane | Recovery | Specific activity | Activity | Recovery | Specific activity | Activity | Recovery |
|-----------------|------------|------------|----------|-------------------|---------|----------|-------------------|---------|----------|
| Control         | 139 ± 8    | 20 ± 1     | 139 ± 8  | 44.7 ± 10.2       | 1.11 ± 0.26 | 5.5 ± 1.0 | 199 ± 2          | 14.1 ± 0.1 | 74 ± 4    |
| cAMP (1 µM)     | 157 ± 9    | 23 ± 1     | 157 ± 9  | 53.8 ± 10.8       | 1.35 ± 0.27 | 5.8 ± 0.9 | 216 ± 3          | 15.3 ± 0.2 | 68 ± 3    |
| TSH (100 milli- | 136 ± 8    | 20 ± 1     | 136 ± 8  | 41.9 ± 9.4        | 1.05 ± 0.23 | 5.2 ± 0.9 | 198 ± 3          | 14.1 ± 0.2 | 73 ± 3    |
| units/ml)       |            |            |          |                   |          |          |                   |          |          |
| NaF (10 mM)     | 237 ± 19   | 34 ± 3     | 237 ± 19 | 64.4 ± 13.0       | 1.61 ± 0.33 | 4.6 ± 0.6 | 336 ± 11         | 23.8 ± 0.8 | 73 ± 5    |

* p < 0.001 as compared to those of L-membrane.
* p < 0.01 as compared to histone (L-membrane).
* p < 0.01 as compared to control (L-membrane).
* p < 0.001 as compared to control.
* p < 0.001 as compared to control (H-membrane).
* p < 0.01 as compared to control.

**Table V**

*Intrinsic protein kinase activities of plasma membranes and its subfractions and the effect of addition of cAMP, histone, and cytosolic protein kinase*

Membranes (50 µg of protein) were incubated at 37° for 5 min in 50 mM glycerophosphate, pH 6.5, 2 mM theophylline, 10 mM MgCl₂, 0.3 mM EGTA, 10 mM NaF, and 20 µM [γ-32P]ATP with or without 1 µM cAMP, 100 µg of histone, and 150 µg of cytosolic protein kinase of thyroid. The results are the mean ± S.E. of the number of experiments in parentheses.

| Addition | cAMP | Plasma membrane | L-membrane | H-membrane | Second band |
|----------|------|-----------------|------------|------------|-------------|
| Control  | -    | 287 ± 14 (7)a   | 47.1 ± 7.1 (5) | 443 ± 30 (12) | 196 ± 13 (5) |
| Histone  | +    | 310 ± 13 (7)    | 60.3 ± 6.8 (5) | 468 ± 30 (12) | 216 ± 18 (5) |
| Protein  | -    | 514 ± 54 (5)a,b | 105 ± 39 (4) | 743 ± 63 (10)e | 271 ± 23 (5)e |
| kinase   | +    | 750 ± 62 (6)e   | 156 ± 49 (4) | 990 ± 85 (10)e | 379 ± 34 (5) |
| Protein  | -    | 364 ± 21 (6)e   | 67.4 ± 16.3 (4) | 566 ± 29 (5) | 206 ± 2 (2) |
| kinase   | +    | 401 ± 19 (6)    | 57.0 ± 16.5 (4) | 607 ± 35 (5) | 252 ± 7 (2) |
| + histone| +    | 944 ± 98 (4)f   | 407 ± 141 (2)f | 1379 ± 100 (3) | 414 ± 30 (3) |
|          | +    | 1517 ± 81 (4)f  | 1374 ± 173 (2) | 2062 ± 188 (3) | 1067 ± 75 (3) |

* p < 0.01 as compared to those of second band.
* p < 0.001 as compared to cAMP.
* p < 0.05 as compared to control (cAMP).
* p < 0.02 as compared to control.
* p < 0.001 as compared to histone (cAMP).
* p < 0.01 as compared to histone (L-membrane).
* p < 0.001 as compared to cAMP.

stimulated by glucagon (42). In contrast, Toda et al. reported that most of the hepatic adenylate cyclase activity of plasma membranes was in a heavy membrane fraction but the percentage of increase induced by glucagon was similar in both the heavy and light subfractions (34). The procedure which they used was similar to that which they utilized except which they employed isotonic solutions. Toda et al. observed a similar distribution of 5'-nucleotidase, Mg²⁺ ATPase, and alkaline phosphatase as did Wisher and Evans (42); that is these activities were considerably higher in the light membrane fractions than in the heavy ones. Although the distribution of these enzyme activities in the light membranes of thyroid plasma membranes is similar to that reported in the liver, it does not help to localize such fractions in the thyroid. The equivalent binding of [125I]-TSH to the L- and H-membranes does not exclude the possibility that the H-membranes represent basal membranes. Kinne et al. reported that parathyroid hormone bound to both the contraluminal and luminal fractions of rat renal cortical epithelial cells (42). In their experiments adenylate cyclase activity stimulated by parathyroid hormone was localized to the contraluminal plasma membrane.

The increased vesicles in the L-membrane fraction might be indicative of microvilli in the intact membranes as suggested by Wisher and Evans (42). The apical plasma membrane of the thyroid contains numerous microvilli which are of great importance in colloid droplet ingestion (20). The identification of specific regions of the hepatic plasma membrane (42) is facilitated by cytochemical localization of individual enzymes (44); similar studies have yet to be carried out on the thyroid. Since iodination of thyroglobulin occurs at or near the apical end of the thyroid cell (36, 37), it was hoped that assay of peroxidase in the H- and L-membranes might assist in their identification. However, peroxidase activity was very low in both the H- and L-membranes. Most of the peroxidase activity was in the material referred to as the second band. This material also
contained the highest specific activity of DPNH-cytochrome c
reductase, an enzyme characteristic of microsomes.

The intracellular function of cAMP involves activation of
protein kinase and phosphorylation of appropriate substrates
(12-17). It has been speculated that colloid droplet injection
may involve phosphorylation of the apical membranes by pro-
tein kinase (45) similar to the mechanism proposed by
Schwartz et al. for vasopressin action on the renal medulla
(46). They found that vasopressin activates adenylate cyclase
in the contraluminal membrane and the cAMP which is gener-
atived then activates a protein kinase in the luminal plasma
membrane. The subsequent phosphorylation alters the perme-
ability to water. Although the present results do not exclude
this formulation, they do not provide unequivocal support for
such a mechanism. Although protein kinase activity was de-
monstrable in plasma membranes and L- and H-membrane
subfractions, it was relatively independent of cAMP. These
results are different from those reported by Roques et al. (35).

These investigators demonstrated increased phosphorylation
of thyroid plasma membranes when the incubation was done
in phosphate buffer but not when acetate buffer was used.
However, when we used phosphate buffer with membrane
fractions, cAMP was still apparently without effect. It is
possible that a small amount of cAMP-dependent protein ki-

nase activity was present in the plasma membranes, but it was ob-
sured by the much larger amount of the cAMP-independent
activity (47).

The low protein kinase activity in the L-membranes does
not reflect the absence of an appropriate substrate since when
addition of histone did not significantly increase phosphorylation.
Phosphorylation of added histone by H-membranes was fur-
ther augmented by cAMP. Thus with exogenous substrate, in
contrast to endogenous substrate, the protein kinase activity of
the H-membranes demonstrated some cAMP dependency.
The apparent cAMP independence of phosphorylation of en-
dogenous substrate in the H-membranes is further supported
by results obtained when cytosolic protein kinase was added.

Addition of cytosolic protein kinase to H-membranes caused a
small, but significant, increase in phosphorylation of endoge-

nous substrate, which was relatively cAMP-independent. Cy-
tosolic protein kinase from thyroid is activated by CAMP when
histone is the substrate (30). Cytosolic protein kinase did not
significantly augment phosphorylation of endogenous sub-
strate of L-membranes. Addition of both cytosolic protein ki-
nase and histone was associated with increased phosphoryla-

tion in both L- and H-membranes, and as would be expected,
this was augmented by cAMP (30). Although the basal phos-
phylation varied when both histone and protein kinase from
the cytosol were added, the additional increment in the presence
of cAMP was the same in all the subfractions suggesting
that it was independent of the membrane subfraction.

Since the protein kinase of the H-membranes was cAMP-depen-
dent when histone was added to the incubation, it is pos-
sible that this enzyme phosphorylates a substrate in the
cytosol which then induces the membrane changes seen in
response to TSH. Another possibility is that the endogenous
substrate of the protein kinase of the H-membranes was lost
during preparation of this fraction. Either of these explana-
tions would preserve the concept of a TSH-induced membrane
change as a result of phosphorylation.

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