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ADENYL CYCLASE OF OXYNTIC CELLS
ITS ASSOCIATION WITH DIFFERENT CELLULAR MEMBRANES

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SUMMARY

The adenyl cyclase of the oxyntic, or acid-secreting, cells of bullfrog gastric mucosa has been found to be a membrane-bound enzyme. A method has been developed to isolate the adenyl cyclase rich membrane fractions in a hypotonic medium containing dithiothreitol, which has been found to protect the hormonal responsivenes of the adenyl cyclase.

Highest specific activity of adenyl cyclase was localized in a light membrane fraction which also had abundant K^+-stimulated ATPase and K^+-stimulated *p*-nitrophenyl phosphatase and very low cytochrome *c* oxidase activity. The three gastric secretagogues tested, namely histamine, pentagastrin and methylcholine, significantly stimulated the adenyl cyclase activity of the light membrane fraction.

After treatment with 10 mM Mg^{2+} further subfractionation of the light membrane fraction on a sucrose density gradient yielded light membrane subfraction 1, light membrane subfraction 2 and light membrane subfraction 3 in order of increasing densities. The three subfractions had different enzymatic and chemical properties. Adenyl cyclase activity has been found to be distributed in all three subfractions. However, the hormonal responsiveness of the three fractions was quite different. Light membrane subfraction 2 could be stimulated by all three secretagogues, light membrane subfraction 1 by histamine and methylcholine, while light membrane subfraction 3 was refractory to all three secretagogues. On the basis of the cholesterol to phospholipid molar ratio, RNA content, glycoprotein content and the enzymatic data it is suggested that light membrane subfraction 1 and light membrane subfraction 2 are of general plasma-membrane type, while light membrane subfraction 3 is largely of cytoplasmic origin.

INTRODUCTION

The enzyme responsible for cyclic AMP synthesis, adenyl cyclase, has been found to be localized in the plasma membrane fractions from a number of different tissues [1]. Under normal conditions cells maintain a constant level of cyclic AMP,

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which is quickly elevated after appropriate hormonal stimulation [2]. According to the "second messenger hypothesis" [3] surface membrane located receptors respond to the effector hormones by an activation of adenyl cyclase and consequent increase in cyclic AMP synthesis. However, a few reports concerning adenyl cyclase of heart tissue have recently appeared [4–6] where the authors have presented evidence that adenyl cyclase may be localized in the sarcotubular system as well as in plasma membranes. The occurrence of adenyl cyclase in cell fractions not derived from plasma membranes does not specifically conform to the "second messenger" concept; however, such an intracellular located enzyme might provide for some additional functions or degrees of control.

Various experimental approaches have provided evidence to implicate cyclic AMP as an ultimate mediator of gastric HCl secretion [7–9]. Under non-secreting conditions gastric mucosa of rats, dogs and frogs maintain steady-state cyclic AMP levels of about $0.5 \cdot 10^{-6}$–$1.0 \cdot 10^{-6}$ mole/kg tissue [7, 9, 10]. Steady-state tissue levels of cyclic AMP are the result of the "dynamic equilibrium" of continuous formation mediated by adenyl cyclase and destruction by cyclic AMP phosphodiesterase. Gastric tissue levels of cyclic AMP have been shown to increase in response to secretagogues [9, 10] or other stimulants of HCl production [7]. The presence of an active cyclic AMP phosphodiesterase has been demonstrated as a predominantly soluble enzyme in oxyntic cells of bullfrogs [11] and rat, guinea pig and dog gastric mucosa [12]. Adenyl cyclase activity, responsive to histamine [13–15], pentagastrin [13] and prostaglandins [14] has also been found in gastric mucosa from Necturus and guinea pigs. These previous studies on gastric adenyl cyclase did not specify the cell type nor characterize the cell fraction with which the adenyl cyclase was associated. Since the control of gastric cyclic AMP levels appeared so fundamental to the process of HCl secretion we undertook a systematic investigation of the localization and characterization of adenyl cyclase. A relatively homogeneous oxyntic cell preparation from bullfrogs was used as the tissue source and subsequently fractionated into various membrane classes. Adenyl cyclase activity, stimulated by NaF and various gastric secretagogues, was found in several distinct membrane fractions.

METHODS AND MATERIALS

Preparation of membrane fractions

Adenyl cyclase is often found to be a very unstable and delicate enzyme [16, 17]. In our experience gastric adenyl cyclase activity decays very rapidly (50–70 % decrease in 24 h) without some protective procedures. We found that the incorporation of either NaF or dithiothreitol in the homogenization medium throughout the fractionation procedure did in fact preserve the enzyme activity for several days. Since membranes isolated in presence of F^- were not responsive to the hormones, a more appropriate agent was sought and dithiothreitol was found to be very effective in preserving the adenyl cyclase activity.

Oxyntic cells were prepared from bullfrog gastric mucosa according to the method developed previously in this laboratory [18]. All the operations of tissue scraping and homogenization were carried out at 0–4 °C. The general cell fractionation procedure was as follows: oxyntic cells from four to six mucosae were homogenized in 20 ml of a medium consisting of 2.0 mM Tris, pH 8.5, and containing either

10.0 mM NaF or 1.0 mM dithiothreitol. Homogenization was carried out in a Dounce loose pestle homogenizer using 10 to 12 gentle up and down strokes. For the preliminary studies with the medium containing NaF, crude subcellular fractions were prepared by differential centrifugation and further segregated by density gradient procedures as described in Table I. Various particulate bands were identified, collected by pasteur pipettes and washed free of sucrose by dilution in the NaF medium and subsequent centrifugation. The pellets were finally suspended in a NaF-free Tris (pH 8.5) medium and assayed for adenylcyclase within 1 h. The other enzymes were assayed the following day.

In the procedure using dithiothreitol in the medium, the homogenate was centrifuged at $750 \times g$ for 10 min and the supernatant was carefully drawn off by a pasteur pipette. The pellet was suspended two times in 8–10 ml of the dithiothreitol medium, centrifuged at the same speed and the supernatant was again drawn off. All the supernatants were pooled (about 40 ml) and layered over a 37 % sucrose solution prepared in the dithiothreitol medium. After centrifugation at 25 000 rev./min for 2 h in the SW 25 Spinco rotor, a band at the interface of 37 % sucrose and a pellet at the bottom of the tube were obtained. This band was designated the light membrane fraction.

Further subfractionation of the light membrane fraction was achieved by treatment with 10.0 mM $MgCl_2$ and centrifugation in a continuous gradient ranging from 27 to 45 % sucrose. The sucrose gradient also contained 2.0 mM Tris (pH 8.5), 1.0 mM dithiothreitol and 10.0 mM $MgCl_2$. After 15 h of centrifugation, the material was resolved into three bands which were designated light membrane Fraction 1, light membrane Fraction 2 and light membrane Fraction 3, respectively, starting from the lighter sucrose towards heavier density region as depicted in Fig. 6.

Enzyme assays

Adenyl cyclase was assayed using [α-^{32}P]ATP (New England Nuclear) as substrate. The incubation mixture unless mentioned otherwise, contained in a total volume of 0.5 ml 25.0 μmoles, Tris–HCl buffer (pH 7.4), 2.5 μmoles $MgCl_2$, 0.125 μmole [α-^{32}P]ATP, 0.05 μmole unlabeled cyclic AMP, 1.25 μmoles of theophylline and other test substances. Several sets of adenyl cyclase assays were performed in presence of an ATP regenerating system, using 10 μmoles of phosphoenol pyruvate and 2.0 μg of pyruvate kinase in the incubation mixture. No significant difference in the adenyl cyclase activity was observed whether the ATP regenerating system was used or not. Blank tubes were run in parallel without Mg^{2+} and containing 0.2 μmole of EDTA. We found that [α-^{32}P]ATP from New England Nuclear invariably contained a small amount of unknown material which had a similar mobility to cyclic AMP in our solvent system. The counts from this unidentified material disappeared, when incubated with most tissue sources in absence of Mg^{2+}. Since Mg^{2+} is required for adenyl cyclase activity the Mg^{2+}-free tubes served as very good blanks for the assay system. After 10 min of incubation the reaction was stopped by placing in a boiling water bath for 3 min. The heat-denaturated proteins were removed by centrifugation and 0.1 ml aliquot was spotted in duplicate on highly absorbent CHROM-AR-1000 paper (Mallinckrodt) for assay of cyclic AMP by paper chromatography. Chromatograms were developed in a solvent mixture of isopropanol: 30 % NH_4OH (17 : 3, v/v) for 1 h by ascending chromatography. The papers were dried in the hood.

In this solvent system cyclic AMP has a high R_F (about 0.5) leaving behind all the mono-, di- and trinucleotides at the origin. The spots were detected under ultraviolet light, cut out and then counted by liquid scintillation in Aquasol (New England Nuclear).

The cyclic AMP spot was identified and characterized by co-chromatography with authentic samples of cyclic AMP and also by digestion with cyclic AMP phosphodiesterase. When digested with cyclic AMP phosphodiesterase, about 90 % of the counts from cyclic AMP spot disappeared.

ATPase was assayed by incubation of the membranes with 2.0 μmoles ATP, 5.0 μmoles Mg^{2+} and 25.0 μmoles Tris–HCl (pH 7.5) in a total volume of 1.0 ml. The extent of stimulation of ATPase by K^+ was assessed by comparing hydrolysis rates with and without 25 μmoles KCl. The inorganic phosphorus liberated was assayed by the procedure of Sanui [19].

K^+-stimulated *p*-nitrophenyl phosphatase was assayed as described previously [20].

Cytochrome *c* oxidase was determined spectrophotometrically using 15 μmoles cytochrome *c* as substrate [21].

5-Nucleotides were assayed at pH 7.4 using 5'-AMP as substrate. The reaction mixture n a total volume of 1.0 ml contained 50 μmoles Tris buffer (pH 7.4), 5 μmoles $MgCl_2$, 20 μmoles of 5'-AMP and 100–150 μg membrane protein. The reaction was carried out for 45 min–1 h at 37 °C and stopped by adding 1.0 ml of 14 % trichloroacetic acid. The liberated phosphorus was assayed by the same procedure as for ATPase [19].

Chemical estimations

Neutral sugars were assayed by the orcinol method [22] using fucose or mannose as standard. Proteins were estimated by the procedure of Lowry et al. [23] using bovine serum albumin as standard. RNA was assayed by the method of Schneider [24].

Extraction of lipids

Lipids were extracted from the trichloroacetic acid precipitated membrane pellet by thoroughly mixing (gentle homogenization) with about 3–4 ml of freezer cold ethanol : diethyl ether (3 : 1, v/v) and left at −20 °C overnight. The extraction was continued through three changes of ethanol: diethyl ether according to the procedure of Rosenberg and Guidotti [25]. Phospholipid phosphorus was assayed in an aliquot of the lipid extract after hydrolysis in H_2SO_4 using the method of Bartlett [26]. Cholesterol was estimated in an aliquot of the extract according to the method of Zlatkis et al. [27].

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed in 1.0 % sodium dodecylsulfate following the procedure of Fairbanks et al. [28]. The gels were stained with Coomassie Blue for proteins and periodic acid Schiff's reagent for glycoproteins.

Electron microscopy

Samples of various membrane fractions (approx. 1 mg protein/ml) were

diluted with 2–4 vol. of 2 % sodium phosphotungstate (pH 7.4). A small drop of this mixture was placed onto a Formvar-carbon-coated grid, the excess fluid withdrawn with filter paper after 1 min and the grid was then immediately examined in the microscope.

Miscellaneous chemicals

ATP, cyclic AMP, 5′-AMP, cytochrome *c*, *p*-nitrophenyl phosphate and dithiothreitol were from Sigma. Histamine, acetyl-β-methylcholine bromide (methylcholine) and gastrin pentapeptide (pentagastrin) were purchased from Cal. Biochem. All the solvents used were of AR grade and either purchased from Mallinckrodt or Estman Kodak.

RESULTS

General properties of frog gastric adenyl cyclase

In order to develop a standard assay system we tested the effects of pH (Fig. 1), Mg^{2+} (Fig. 2), different enzyme protein concentrations (Fig. 3) and ATP concentrations (Fig. 4) on the adenyl cyclase activity of the oxyntic cell homogenates. The enzyme has a rather broad pH range of activity with an optimum around 7.4. Enzyme activity requires the presence of Mg^{2+}; the basal adenyl cyclase activity does not change significantly within 1–5 mM concentration of Mg^{2+}, while the F^--stimulated rate is enhanced with concentrations up to 5 mM Mg^{2+}. The diminished effect of F^- at lower Mg^{2+} concentrations may be due to limitation in the availability of free Mg^{2+} due to metallo–fluoride complex formation in the presence of 5 mM NaF. Thus our preliminary experiments suggested that functional conditions for adenyl cyclase activity occurred at pH 7.4 with ATP and Mg^{2+} concentrations of 0.25 mM and 5.0 mM, respectively. Fig. 5 shows the basal and F^- stimulated activity of adenyl cyclase in the presence and absence of an ATP regenerating system. The F^--stimulated activity remained almost the same in both cases. However, the basal activities of adenyl cyclase were somewhat lower in the presence of the ATP regenerating system. The reasons for this are not clear to us. We have actually measured the level of ATP after different times of incubation and found that the concentrations of ATP in the assay medium remain at a constant level in the presence of the ATP regenerating system. In absence of ATP regenerating system, on the other

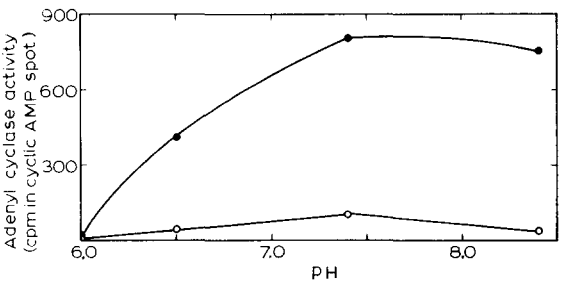


Fig. 1. Adenyl cyclase activity as a function of pH. Enzyme activity is shown in the presence (●) and absence (○) of 5.0 mM of NaF. Sodium phosphate buffer was used for pH 6.0 and 6. 5;Tris buffer was used for pH 7.4 and 8.5. About 100 μg of enzyme protein was used for each assay. Details of the assay procedure are given in Methods and Materials.

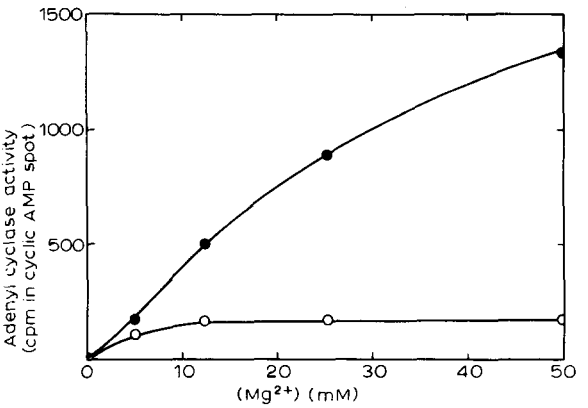


Fig. 2. The effects of Mg^{2+} concenttration on the adenyl cyclase activity. The enzyme activity was assayed in presence (●) and in absence (○) of 5.0 mM NaF. About 150 μg of enzyme protein was used for each assay.

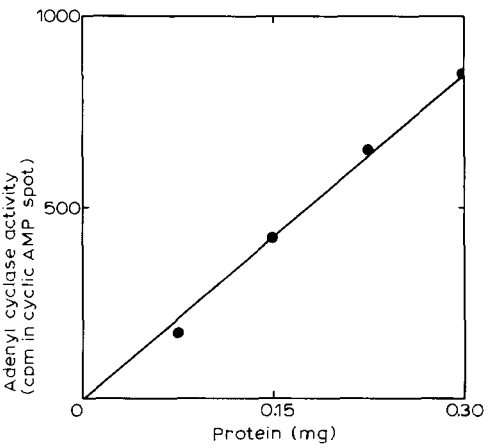


Fig. 3. The effects of different concentrations of enzyme protein on the accumulation of cyclic AMP. All assays were carried out in presence of 5.0 mM NaF. Details of the assay conditions are given in the Methods and Materials.

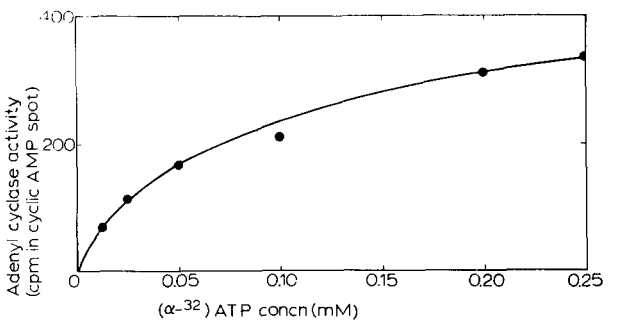


Fig. 4. The adenyl cyclase activity as a function of substrate, [α-^{32}P]ATP concentration. For each assay 5 mM NaF was included and about 80 μg of membrane protein was used.

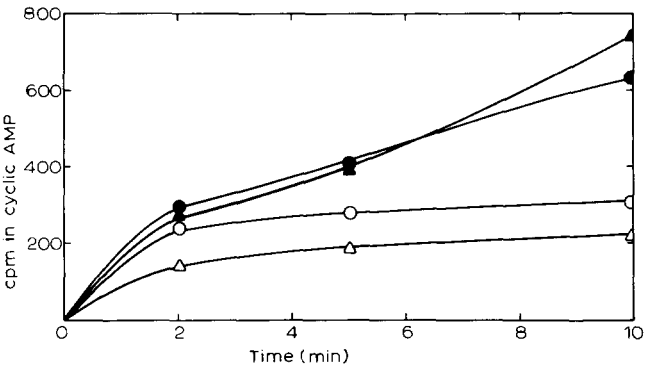


Fig. 5. Adenyl cyclase activity in presence and absence of ATP regenerating system. 10.0 mM phosphoenolpyruvate and about 2.0 μg of pyruvate kinase were added in the assay medium for regeneration of ATP. Results are compared for the non-regenerating system in the presence (●) and absence (○) of fluoride; and for the regenerating system in the presence (▲) and absence (△) of fluoride.

hand, the level of ATP fell to about 40–50 % of the original level after 10 min. Since the F^--stimulated activities were same in both cases, we ruled out the possibility that ATP was limiting under the condition of assay. The nonlinear kinetics for accumulation of cyclic AMP, as shown in Fig. 5 and suggested by the work of others [16, 29] may be due to gradual inactivation of the enzyme at 37 °C in absence of F^-.

Enzyme distribution

Our early experiments showed that when the cell fractions were obtained solely by differential centrifugation, the general pattern of distribution of various enzymes like adenyl cyclass, cytochrome *c* oxidase, Mg^{2+} ATPase and K^+-stimulated *p*-nitrophenyl phosphatase suggested a considerable cross-contamination of the various cellular organelles. In order to segregate and purify cellular organelles we subjected the crude fractions derived from differential centrifugation to equilibrium density gradient centrifugation. The distribution of the different membrane-bound enzymes after separation on a continuous sucrose gradient containing 10.0 mM NaF is shown in Table I. The lightest band from each of the fractions had the highest specific activity of adenyl cyclase and the lowest Mg^{2+}-activated ATPase and cytochrome *c* oxidase activity. Although the specific activity of adenyl cyclase was very low in the 0.75K–III pellet, the total activity of the enzyme was relatively high since the protein was so abundant in this fraction. The K^+-stimulated *p*-nitrophenyl phosphatase activity was also associated mostly with the lightest bands from all the separations; the best recovery and highest specific activity occurred in the 10K+ 100K–I band. Since adenyl cyclase was most vigorous in the bands of lowest density, regardless of the origin by differential centrifugation, we decided to use a more simple and direct approach toward harvesting the material.

The alternative scheme for providing the lighter membrane fraction which is rich in adenyl cyclase, using a one-step sucrose gradient in dithiothreitol medium, has been described in Methods and Materials and depicted in Fig. 6. Enzymatic characterization of various fractions is presented in Table II. The band collecting at the 37 % sucrose interface, light membrane fraction, had the highest specific activity

TABLE I

DISTRIBUTION OF ENZYMES IN FRACTIONS OF GASTRIC OXYNTIC CELLS PREPARED BY DENSITY GRADIENT CENTRIFUGATION

The crude fractions were derived from differential centrifugation according to the following force · time schedule: 750 · *g* for 10 min (0.75 K); 2000 · *g* for 10 min (2K); 10 000 · *g* for 10 min (10 K); and 100 000 · *g* for 90 min (100 K). The pellets from differential centrifugation were harvested, resuspended in about 4 ml of NaF medium and then separately layered over continuous sucrose gradient tubes. The continuous sucrose gradient was made up of 30–48 % (w/v) sucrose containing 10 mM NaF. After 15 h of centrifugation at 25 000 rev./min in a Spinco SW 25 rotor a characteristic pattern of bands appeared. The lightest bands were designated I, while the more densely sedimenting bands were II and III.

Sedimenting fraction	Gradient band	% protein recovery*	Adenyl cyclase		*p*-Nitrophenyl phosphatase (μmole · mg^{-1} · h^{-1})		ATPase μmole · mg^{-1} · h^{-1}	Cytochrome *c* oxidase** (min^{-1} · mg^{-1})
			Specific activity (nmoles · mg^{-1} · h^{-1})	Total activity nmoles · h^{-1}	− K$^+$	+ K$^+$		
0.75 K	I	2.5	6.6	2.2	0	1.2	50.0	n.d.
	II	4.8	1.0	0.6	0	0.8	74.1	14.5
	III	71.8	0.3	2.9	0.02	0.08	105.3	n.d.
2K	I	16.3	4.3	2.1	0.2	4.8	43.5	n.d.
	II	53.4	0.9	1.5	0	0	115.0	13.8
10 K + 100 K	I	32.5	5.9	16.5	2.1	10.4	67.9	0.1
	II	25.1	2.2	4.7	1.4	3.3	73.6	7.6
	III	25.3	0.8	1.8	0.7	0.7	135.0	17.0

* Values represent the percentage of the protein applied to the gradient that was collected in the respective band.

** n.d., not determined.

TABLE II

ISOLATION OF THE ADENYL CYCLASE RICH FRACTION FROM OXYNTIC CELL HOMOGENATES USING A ONE-STEP SUCROSE GRADIENT

The nuclear pellet was prepared by centrifuging the total homogenate at 750 · *g* for 10 min, followed by three washings at the same speed for 5 min. The combined supernatant and washings were taken as the post-nuclear supernatant and applied to a step gradient of 37 % sucrose and centrifuged in an SW-25 rotor for 2 h at maximum speed. A band of particulate material which layered at the top of the 37 % sucrose was taken as the light membrane fraction. Some particulate material was dispersed between the light membrane fraction and the pellet, and a large amount of the post-nuclear supernate protein did not sediment during the gradient centrifugation step. This final supernatant material was not assayed further since earlier studies showed little or no adenyl cyclase activity in the soluble supernatant. n.d., not determined.

Fraction	Protein (mg)	Adenyl cyclase (nmoles · mg^{-1} · h^{-1})		*p*-Nitrophenyl phosphatase (μmoles · mg^{-1} · h^{-1})		ATPase (μmoles · mg^{-1} · h^{-1})		cytochrome *c* oxidase (min^{-1} · mg^{-1})
		$-F^-$	$+F^-$	$-K^+$	$+K^+$	$-K^+$	$+K^+$	
Total homogenate	202	0.33	1.02	0.56	1.91	30	31	15
Nuclear pellet	18.4	0.38	0.79	0.08	0.10	58	58	n.d.
Post-nuclear supernatant	179	0.38	1.10	0.58	2.28	36	36	16
Density gradient band light membrane fraction	17.6	2.29	5.63	1.49	12.58	42	47	1.9
Density gradient pellet	35.4	0.33	0.45	0.32	0.67	165	165	29.1

TABLE III

DISTRIBUTION OF MARKER ENZYMES IN OXYNTIC CELL MEMBRANE SUBFRACTIONS PRODUCED FROM THE LIGHT MEMBRANE FRACTION BY DENSITY GRADIENT CENTRIFUGATION AFTER Mg^{2+} TREATMENT

Values are given as the mean of experimental values from six separate preparations ±S.E. 5′-Nucleotidase was measured for four of the preparations and cytochrome *c* oxidase on two of the preparations. Protein data are expressed as the percentage of the total protein applied to the sucrose density gradient which was harvested as the designated subfraction.

	Protein (%)	Adenyl cyclase (nmoles · mg^{-1} · h^{-1})		*p*-Nitrophenyl phosphatase (μmoles · mg^{-1} · h^{-1})		ATPase (μmoles · mg^{-1} · h^{-1})		5′-Nucleotidase (μmoles · mg^{-1} · h^{-1})	Cytochrome *c* oxidase (min^{-1} · mg^{-1})
		$-F^-$	$+F^-$	$-K^+$	$+K^+$	$-K^+$	$+K^+$		
Total light membrane fraction band	100	4.8±1.2	15.6±4.4	1.3±0.3	8.5±1.4	31.3±3.8	35.6±3.9	0.32±0.02	1.4±0.4
Light membrane subfraction 1	13.2±0.7	9.3±2.6	21.7±6.7	1.9±0.6	7.9±1.3	7.9±1.2	12.7±1.7	0.90±0.11	0.2±0.06
Light membrane subfraction 2	39.8±2.3	3.3±1.2	8.6±3.2	1.2±0.3	10.0±1.7	21.7±2.7	32.8±4.5	0.28±0.05	0.6±0.05
Light membrane subfraction 3	38.1±2.2	4.5±1.6	9.4±3.0	0.8±0.2	2.0±0.3	43.4±4.9	46.4±5.4	0.10±0.01	3.5±0.5

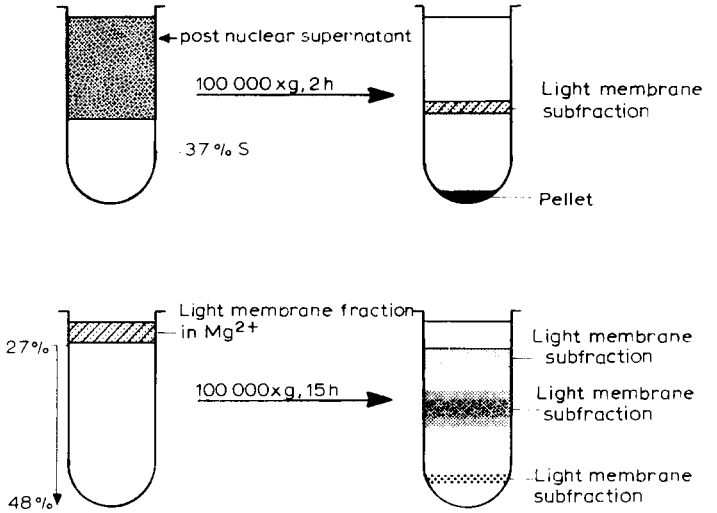


Fig. 6. Schematic representation of procedure for isolation of light membrane fraction (Mb) from the post-nuclear supernatant (pns) and subsequent subfractionation after Mg^{2+} treatment in the sucrose gradient. In the one-step gradient 10 ml of 37 % sucrose in Tris–dithiothreitol medium (37 %S) was used. After isolation the crude light membrane fraction was treated with 10 mM $MgCl_2$ (light membrane fraction in Mg^{2+}) and applied to a continuous gradient consisting of 27–48 % sucrose in Tris–dithiothreitol medium containing 10 mM $MgCl_2$. After 15 h of centrifugation at 100.000 × *g* three membranous bands were separated. Light membrane subfraction 1 (Mb_1) had equilibrium density < 1.12; light membrane subfraction 2 (Mb_2) = 1.14 (average); and light membrane subfraction 3 (Mb_3) = 1.16. See Methods and Materials for further details.

of adenyl cyclase and K^+-stimulated *p*-nitrophenyl phosphatase, and the lowest cytochrome *c* oxidase activity. The K^+-stimulated component of the ATPase, an enzymatic activity shown to be characteristic of oxyntic cell microsomes [30], was present in the light membrane fraction band. The pellet, on the other hand, had very low adenyl cyclase and K^+-stimulated *p*-nitrophenyl phosphatase, but had the highest specific activity for cytochrome *c* oxidase. While the highest activity of basal, Mg^{2+} activated, ATPase occurred in the pellet, there was no K^+-stimulated ATPase. These results suggest the pellet to be predominantly of mitochondrial origin and thus it is clearly distinguishable from the light membrane fraction band.

In order to subfractionate the constituents membranes, the light membrane fraction band from the one-step density gradient was treated with 10 mM $MgCl_2$ and subsequently applied to a continuous sucrose density gradient. This procedure, as represented in Fig. 6, produced three distinct bands or subfractions designated light membrane subfraction 1, light membrane subfraction 2 and light membrane subfraction 3 and having the following densities: light membrane subfraction 1 had a density less than 1.12; light membrane subfraction 2 had an average density of 1.14; and light membrane subfraction 3 had a density of 1.16. Enzyme activities of the gradient subfractions are given in Table III. All three subfractions exhibited a significant quantity of F^--stimulated adenyl cyclase with highest specific activity occurring in light membrane subfraction 1. The specific activity of K^+-stimulated *p*-nitrophenyl phosphatase was highest and nearly equal for the light membrane subfraction 1 and light membrane

subfraction 2 (in fact, somewhat higher in light membrane subfraction 2). On the other hand, K^+-stimulated *p*-nitrophenyl phosphatase was very low in light membrane subfraction 3. The basal activity of Mg^{2+}-activated ATPase was lowest in light membrane subfraction 1 and highest in light membrane subfraction 3, but, conversely, the degree of stimulation of ATPase by K^+ was highest in light membrane subfraction 1 and lowest in light membrane subfraction 3. Cytochrome *c* oxidase activity was highest in light membrane subfraction 3; based on an evaluation of specific activity of the mitochondrial fraction, it was estimated that light membrane subfraction 3 contained about 15–20 % mitochondrial membranes.

5′-Nucleotidase, which is known to be a good marker enzyme for the plasma membrane of a number of different cells [1] has very low activity in oxyntic cell membranes. However, using higher protein concentration and longer time of incubation one can get a measurable amount of phosphorus release. Such low values could be attributed to some nonspecific phosphatase. In any case, assuming that it is a true 5′-nucleotidase, the highest specific activity was found in light membrane subfraction 1 and decreased in the order light membrane subfraction 1 > light membrane subfraction 2 > light membrane subfraction 3.

Hormone responsiveness of various membrane fractions

Table IV shows the effect of F^- and different gastric secretagogues on the adenyl cyclase activity of the subfraction band. Although the activity of adenyl cyclase showed considerable variability from preparation to preparation, there was a consistent and significant stimulatory effect by F^- and the three secretagogues tested on the adenyl cyclase activity. Normalization of the data, by setting basal adenyl cyclase activity equal to 100 %, provides a means for statistical evaluation of the percent stimulation.

It was shown in Table III that all light membrane subfractions contained F^--stimulated adenyl cyclase activity. However, as demonstrated in Table V, only light membrane subfraction 1 and light membrane subfraction 2 subfractions were significantly stimulated by addition of gastric secretagogues. Light membrane subfraction 2 showed

TABLE IV

EFFECTS OF SECRETAGOGUES AND FLUORIDE ON ADENYL CYCLASE ACTIVITY OF FOUR DIFFERENT PREPARATIONS OF LIGHT MEMBRANE FRACTION FROM BULLFROG OXYNTIC CELLS

Application of Student's *t*-test showed that stimulation by all agents was significant to the 5 % level.

Agent tested	Adenyl cyclase activity (nmoles · mg^{-1} · h^{-1})				Normalized mean ±S.E.*
	Prepn 1	Prepn 2	Prepn 3	Prepn 4	
None	5.6	15.7	7.4	1.5	100
F^- (5 mM)	26.5	113.9	29.4	6.9	513±72
Histamine (10^{-4} M)	8.8	16.4	10.6	2.0	134±11
Methylcholine (10^{-4} M)	11.6	25.5	10.7	1.7	157±20
Pentagastrin ($5 \cdot 10^{-5}$ M)	9.8	18.4	9.7	1.8	136±13

* Values are normalized for variation in individual preparations by setting the basal value (no additions) for each set of data equal to 100.

TABLE V

EFFECT OF SECRETAGOGUES ON ADENYL CYCLASE ACTIVITY OF OXYNTIC CELL MEMBRANE SUBFRACTIONS PRODUCED FROM LIGHT MEMBRANE FRACTION

All activities expressed as a percentage of the basal rate, i.e. rate with secretagogue/basal rate × 100. The number of different preparations is given in parentheses; the calculated ± S.E. is also shown.

	Light membrane subfraction		
	1	2	3
Basal	100	100	100
Histamine (10^{-4} M)	134.2 (4) ± 8.3*	233. (4) ± 36.8*	113.0 (2) ± 13.0
Histamine (10^{-4} M) plus burimamide (10^{-3} M)	100.5 (2) ± 0.5	109.5 (2) ± 13.5	98.5 (2) ± 0.5
Methylcholine (10^{-4} N)	117.7 (3) ± 5.6*	198.0 (4) ± 15.4*	88.5 (2) ± 10.5
Pentagastrin ($5 \cdot 10^{-5}$ M)	89.3 (3) ± 11.7	161.7 (4) ± 13.7*	85.5 (2) ± 16.5

* Denotes significance of $P < 0.05$.

the most vigorous and consistent stimulation to all three secretagogues tested; histamine and methylcholine were about equally effective in doubling the basal rate, while pentagastrin stimulated basal activity by about 60 %. The light membrane subfraction 1 was not stimulated by pentagastrin. On the other hand, the response to histamine and methylcholine was significant ($P < 0.5$) but much less than in light membrane subfraction 2. For both light membrane subfraction 1 and light membrane subfraction 2 the addition of 10^{-3} M burimamide, a histamine type H_2 receptor antagonist [31], abolished the stimulatory effects of 10^{-4} M histamine.

Chemical composition of the different membrane fractions

Analytical data for the various membrane fractions is shown in Table VI. On a mg protein basis the amounts of neutral sugar, cholesterol and phospholipid were highest in the light membrane subfraction 1 and gradually decreased in the order light membrane subfraction 1 > light membrane subfraction 2 > light membrane subfraction 3 > mitochondria. The lipid content was consistent with the relative densities of the fractions. The molar ratio of cholesterol to phospholipid were similar for light membrane subfraction 1 and light membrane subfraction 2, while this ratio was considerably reduced for the light membrane subfraction 3 and mitochondrial fractions. The amount of RNA originally present in the total light membrane fraction band appeared almost exclusively in the light membrane subfraction 3; only a small portion was recovered in the lower density fractions.

Polyacrylamide gel electrophoresis and electron microscopy

Various membrane fractions were solubilized with 1 % sodium dodecylsulfate and the macromolecular subunits separated by polyacrylamide gel electrophoresis. The protein and glycoprotein profiles of the different fractions are shown in Fig. 7.

TABLE VI

CHEMICAL COMPOSITION OF MEMBRANE SUBFRACTIONS DERIVED FROM GASTRIC OXYNTIC CELLS

n.d., not determined

	Neutral sugar (μg/mg protein)	RNA (μg/mg protein)	Phospholipid (μg/mg protein)	Cholesterol (μg/mg protein)	Cholesterol / Phospholipid (M/M)
Total light membrane fraction	211±19 (4)	108±3 (3)	587±24 (3)	n.d.	–
Light membrane subfraction 1	388±13 (4)	13±2 (3)	1210±74 (3)	457±10 (3)	0.75±0.05
Light membrane subfraction 2	294±12 (4)	42±8 (3)	595±39 (3)	258±28 (3)	0.83±0.04
Light membrane subfraction 3	178±16 (4)	223±23 (3)	557±39 (3)	100±12 (3)	0.35±0.03
Mitochondria	n.d.	5.0 (1)	300±60 (2)	32±2 (2)	0.21±0.03

The mitochondrial fraction has a protein profile quite distinct from the other fractions (Fig. 7) and, in accord with an earlier report [30], did not have a visible glycoprotein band (not shown). The protein and glycoprotein profiles are generally similar for light membrane subfraction 1 and light membrane subfraction 2. On the other hand, the protein banding pattern of the light membrane subfraction 3 subfraction is significantly different from that of light membrane subfraction 1 and light membrane subfraction 2, the former having a greater predominance of lower molecular weight proteins and reduced glycoprotein (Fig. 7).

Figs 8a, 8b and 8c show the morphology of the different subfractions, when

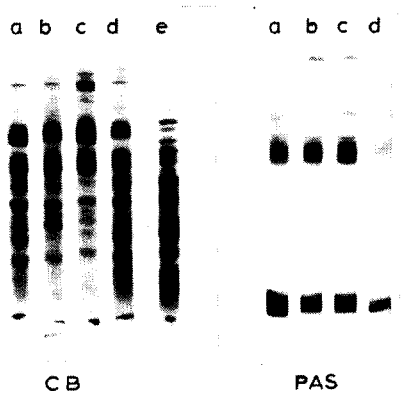
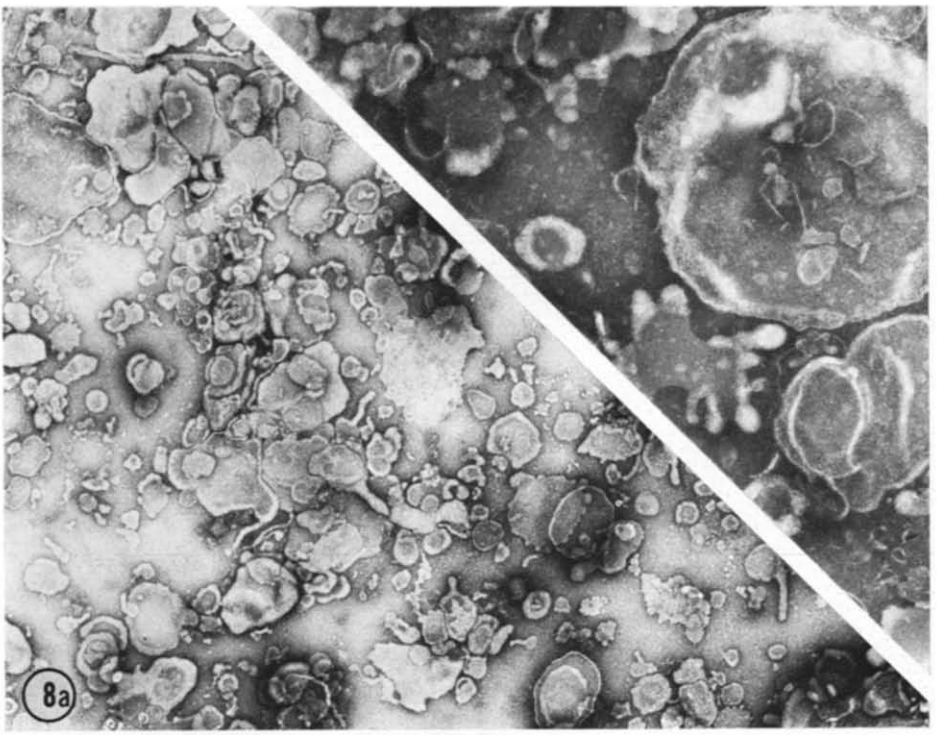
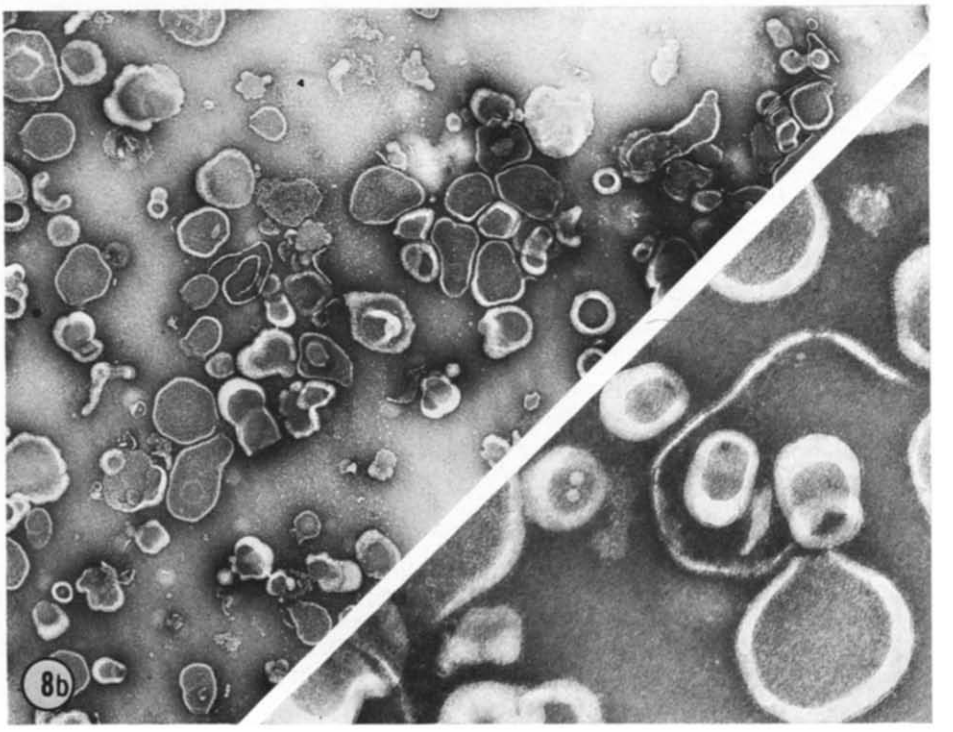


Fig. 7. Acrylamide gel electrophorograms of sodium dodecylsulfate-solubilized membrane fractions of bullfrog oxyntic cells. (a) Total light membrane fraction, (b) light membrane subfraction 1, (c) light membrane subfraction 2, (d) light membrane subfraction 3 and (e) mitochondrial fraction. About 80 μg of the various fractions were applied to gels for protein (CB), while 160 μg was applied to those stained for carbohydrate (PAS). The dark line at the lower end of the gels shows the position of the tracking dye.


8a


8b

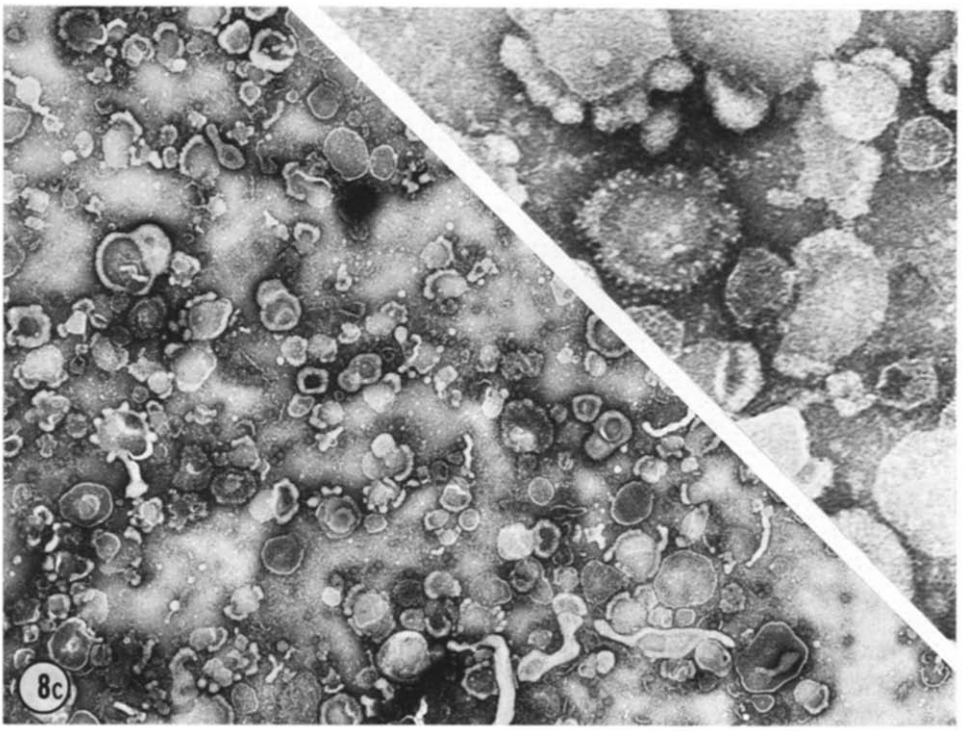


Fig. 8. Electron micrographs of the various membrane fractions from bullfrog oxyntic cells seen in negative contrast after sodium phosphotungstate staining. a, light membrane subfraction 1 (× 28 000, inset × 70 000); b, light membrane subfraction 2 (× 28 000, inset × 112 000); c, light subfraction 3 (× 28 000, inset × 112 000). A variety of membrane fragments and vesicular elements are apparent and are described in the text.

viewed by electron microscopy after negative staining. Light membrane subfraction 1 (Fig. 8a and inset) contained various sized fragments, but the largest irregular membrane structures (some 3–4 μm in diameter) occurred in this fraction. There was an abundance of membranous structures with numerous knobs or clubby projections extending over the surface. A few strands and tubular structures were also evident in light membrane Fraction 1.

The most morphologically homogeneous fraction was light membrane subfraction 2 (Fig. 8b and inset). The vast majority of structrues appeared vesicular in form, either as rounded, sealed units or as flask-shaped, open-ended vesicles. There were also some strands and a very few sub-mitochondrial fragments evident.

Light membrane subfraction 3 (Fig. 8c and inset) was considerably heterogeneous. Numerous sub-mitochondrial fragments occurred as well as various tubular and vesicular membranous forms. As observed in light membrane subfraction 1, knobbed membranous structures were also readily apparent.

DISCUSSION

Oxyntic cells are very delicate and fragile cells, which break up very easily even with the mildest possible technique for homogenization. Furthermore, the plasma

membranes also fragment extensively during the homogenization procedures. Our early efforts to keep the plasma membranes intact by varying the tonicity, pH and divalent cation (Ca^{2+} and Mg^{2+}) content of the homogenizing medium met with limited success [32]. Lowering the pH of the homogenization medium to about 6.8 somewhat stabilized the plasma membranes, a large portion (about 50–60 %) of which sedimented with the low speed pellet. But this low speed pellet was highly contaminated with heavy mitochondria, and we found it impossible to purify the plasma membranes without extensive particle fragmentation occurring. Thus, we directed our efforts toward allowing the plasma membranes to fragment as extensively as possible; in this state, the plasma membrane fragments do not sediment with the nuclear pellet and they may thereby be recovered from the post-nuclear supernatant. Difficulties exacerbated by this approach included the separation of the fragmented plasma membranes from mitochondrial and intracellular microsomal membranes.

An additional problem was finding a suitable marker for the oxyntic cell plasma membranes. Most of the well known marker enzymes for plasma membranes of other tissues like 5'-nucleotidase, adenosine triphosphate pyrophosphohydrolase, phosphodiesterase, (Na^+–K^+)-ATPase, etc. [1] are virtually absent in amphibian oxyntic cells. From the bulk of evidence in the literature adenyl cyclase appeared to be a useful marker for plasma membranes and we decided to use this enzyme for our oxyntic cell studies.

When the fractions obtained by differential centrifugation were purified by equilibrium density gradient centrifugation, the adenyl cyclase was found to be predominantly associated with the low density membrane fractions (Table I). Thus the one-step sucrose gradient was developed as a rapid harvesting procedure, which produced a good yield of membranes, collectively designated the light membrane fraction band (Table II). Maximum recovery of adenyl cyclase, responsive to the three gastric secretagogues, was achieved in this membrane band. The light membrane fraction also contained highest K^+-stimulated *p*-nitrophenyl phosphatase, K^+-stimulated ATPase and very low cytochrome *c* oxidase. All these data clearly demonstrate the membranous nature of the adenyl cyclase and its association with membranes of lighter density, clearly distinguishable from the heavier pellet rich in mitochondria.

Treatment of light membrane fraction with 10 mM Mg^{2+} agglutinates some of the membranes, which is immediately apparent by the cloudiness after treatment with the divalent cation. Subsequent centrifugation of the Mg^{2+}-treated membranes on a continuous equilibrium density gradient resulted into three bands designated as light membrane subfraction 1, light membrane subfraction 2 and light membrane subfraction 3 with an approximate protein distribution of 1 : 3 : 3, respectively. Adenyl cyclase was distributed in all the fractions with highest specific activity in light membrane Fraction 1; light membrane subfraction 2 and light membrane subfraction 3 had about similar activities.

Kamat and Wallach [33] noted that surface membranes of ascites tumor cells were relatively insensitive to divalent cations compared to intracellular membranes, which tended to shrink and aggregate to a far greater degree. A similar differential membrane sensitivity to divalent cations has also been demonstrated in other cell types [34, 35].

Intracellular membranes from oxyntic cells may also be more susceptible to

divalent cation aggregation. This view is supported by the fact that most of the mitochondrial membranes (as judged from cytochrome *c* oxidase activity) and RNA originally present in the light membrane fraction appear in the heaviest light membrane subfraction 3 after Mg^{2+} contraction and subfractionation. Additional support is provided by electron microscopic examination (Fig. 8c) where mitochondrial particles are frequentlyo bservable in light membrane subfraction 3; while they are very rare in light membrane subfraction 1 and light membrane subfraction 2.

Membranes in light membrane subfraction 1 had an unusually high content of lipid, consistent with the very low density of this subfraction. Both light membrane subfraction 1 and light membrane subfraction 2 had high activity of K^+-stimulated ATPase, K^+-stimulated *p*-nitrophenyl phosphatase and very low activity of cytochrome *c* oxidase. In addition to the similarities with respect to the enzymatic make up, light membrane subfraction 1 and light membrane subfraction 2 bands are also very similar with respect to their protein and glycoprotein profiles on polyacrylamide gels (Fig. 7) and high cholesterol to phospholipid molar ratio (Table VI). High phospholipid to cholesterol molar ratio is a general characteristic of plasma membranes [36, 37]. In spite of the overall general similarities between light membrane subfraction 1 and light membrane subfraction 2 in several parameters, the absolute values for phospholipid, cholesterol, neutral sugars and RNA on a mg protein basis are significantly different.

The responsiveness of the adenyl cyclase associated with the three light membrane subfractions to the secretagogues has been found to be quite different (Table V). Adenyl cyclase activity of light membrane subfraction 2 was significantly stimulated by histamine, methylcholine and pentagastrin. On the other hand, the enzyme activity of light membrane subfraction 1 was stimulated to some extent by histamine and methylcholine but not by pentagastrin, while adenyl cyclase of light membrane subfraction 3 was found to be refractory to all the secretagogues. Burimamide, which is an antagonist to H_2 type histamine receptors and inhibits amphibian gastric HCl secretion [38], completely abolished the response to histamine in both light membrane subfraction 1 and light membrane subfraction 2. This burimamide effect confirms the observation made by Sung et al. [39], and suggests receptor specificity for histamine-stimulated adenyl cyclase.

As pointed out earlier maximum sensitivity of the adenyl cyclase to secretagogues required the use of dithiothreitol in the isolation procedure. It is of interest that reducing agents, such as dithiothreitol, have important influence on the acetylcholine receptors [40] in post-junctional membranes of electroplax. Thus it is possible that the failure of other investigators to demonstrate the acetylcholine responsive adenyl cyclase in oxyntic cells may be related to the absence of reducing agents in their homogenization and subsequent isolation procedure.

The differential effects of gastric secretagogues on the adenyl cyclase activity on the three membrane subfractions, together with their other chemical and enzymatic characterization, strongly suggest the structural and functional specialization of the three fractions. Thus there is support for the notion that the light membrane subfraction 1, light membrane subfraction 2 and light membrane subfraction 3 are principally derived from different regions of the cells. However, there is a certain degree of cross-contamination among the three subfractions, and some comment on the possibilities and limitations as to their cellular origin is justified here.

The major sources of membrane from the gastric oxyntic cell are the apical

plasma membrane, the basal (and lateral) plasma membrane, the abundant and specialized tubulovesicular membrane system, Golgi membrane, mitochondrial membrane, rough surfaced endoplasmic reticular membrane and nuclear membrane. One must also consider the possibility that some of the membranes may be derived from non-oxyntic cell sources since the method of cell harvesting would also include a limited number of glandular endocrine and basal cells. Nuclei were eliminated early in the preparative procedures and marker studies showed that mitochondria and rough endoplasmic reticulum were present in significant amounts in light membrane 3 subfraction only. The evidence of this paper suggests that light membrane subfraction 3 is largely of intracellular origin, thus the association of adenyl cyclase activity, non-responsive to gastric secretagogues, is a curious finding. Although, we cannot with certainty ascribe the adenyl cyclase activity of light membrane subfraction 3 to an intracellular organelle, the possibility remains and will be a focus of future investigation. As pointed out earlier, adenyl cyclase has been localized in certain purified intracellular membrane fractions (sarcoplasmic reticulum) [4, 6].

According to the widely held "second messenger" hypothesis for cyclic AMP we would expect that hormone sensitive receptor sites for stimulating adenyl cyclase would be present on the basal or nutrient plasma membrane surface of gastric secretory cells. Following this reasoning, together with the chemical composition and enzyme data, the basal plasma membrane fragments would likely be present in light membrane subfraction 1 and light membrane subfraction 2.

It is still possible, even though gastric secretagogues may ultimately operate through cyclic AMP, that specific receptor sites may be at loci other than the basal plasma membrane, e.g. apical membrane and tubulovesicular system. This would, of course, require that the secretagogues permeate through the basal plasma membrane to gain access to the receptor. There is evidence that at least one gastric secretagogue, histamine, can enter cells of the gastric mucosa [41, 42].

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