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THE PURIFICATION AND CHARACTERIZATION OF PLASMA MEMBRANES AND THE SUBCELLULAR DISTRIBUTION OF ADENYLATE CYCLASE IN MOUSE PAROTID GLAND*

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SUMMARY

1. Plasma membranes have been purified 17-fold from mouse parotid gland homogenates prepared in hypertonic sucrose media using differential centrifugation. The method is fast and simple. The membranes were characterised by electron microscopy, enzyme composition and chemical composition. Further purification was achieved by isopycnic centrifugation in discontinuous sucrose gradients.

2. The purified membranes contain an adenylate cyclase activity which is stimulated by isoproterenol and fluoride. Only 50 % of the total adenylate cyclase activity sedimented in the plasma membrane fraction. The rest of the activity resided in the crude nuclear and mitochondrial pellets. However, this adenylate cyclase activity was not associated with these organelles but with membrane fragments in the pellets. Purified nuclei did not contain adenylate cyclase activity.

3. Adenylate cyclase activity was also localised by electron microscopic cytochemistry. Besides being found at the plasma membrane, large amounts of adenylate cyclase were found in a small proportion of the vesicles within the acinar cells, which appeared to be secondary lysosomes.

4. Adenylate cyclase activities, under standard assay conditions, are proportional to the time of incubation and the concentration of enzyme. The enzyme requires both Mg^{2+} and Ca^{2+} for activity. Isoproterenol increases activity 2-fold and this increase is abolished by β-adrenergic blocking agents.

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Abbreviations: cyclic AMP, adenosine 3′,5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate; EGTA, ethyleneglycol-bis(β-aminoethylether)-*N,N′*-tetraacetic acid; DCIP, dichlorophenolindophenol.

INTRODUCTION

β-Adrenergic catecholamines activate adenylate cyclase in a variety of tissues [1–3] and as a consequence cyclic adenosine 3′,5′-monophosphate accumulates within the cells of these tissues. The adenylate cyclase of many cell types is localized, at least partially and usually predominantly, at the plasma membrane [3–5]. It is thought that a specific interaction between the catecholamine and a receptor in the plasma membrane of the cell leads to activation of adenylate cyclase [6–8]. However, in some systems this enzyme is found to a greater or lesser degree in other cell organelles, such as liver and prostate nuclei [9, 10], adrenal microsomes [11] and sarcoplasmic reticulum [12] and these activities are stimulated by hormones in much the same way as is the plasma membrane-bound enzyme.

After a single injection of isoproterenol, a β-adrenergic drug, into the mouse, the levels of cyclic AMP [13–15] and cyclic GMP [13] in the parotid gland rapidly rise and the activity of adenylate cyclase is transiently increased [13, 16]. This is followed quickly by the secretion of α-amylase [17] and some 24 h later by a burst of DNA synthesis [18]. There appears to be a correlation between the former events and these latter effects [13, 17].

The following studies describe the subcellular localization of adenylate cyclase in the mouse parotid gland as determined by both subcellular fractionation and histochemical techniques. A simple method for the preparation of plasma membranes is detailed and the properties of this preparation described.

EXPERIMENTAL PROCEDURE

Materials

[α-^{32}P]ATP (0.5–2 Ci/mmol) and 8-^{3}H-labeled cyclic AMP (25 Ci/mmol) were products of the Radiochemical Centre, Amersham, England. The ATP was purified prior to use on a column of Dowex 50 (H^+ form) and stored at −80 °C. The following were generous gifts: propranolol from I.C.I. Ltd, Alderley Park, England and (±)-isoproterenol from the Sterling-Winthrop Research Institute, Renssalaer, New York.

Polyethylene-impregnated cellulose thin-layer plates were purchased from Machery-Nagel, Duren, West Germany; adenylyl imidodiphosphate from the Boehringer Corp. Ltd, London, England; Dextran T250 from Pharmacia, London, England; *p*-nitrophenylphosphate, bis-*p*-nitrophenylphosphate, sodium dodecyl sulphate (specially pure), cholesterol and *p*-dimethylaminobenzaldehyde from B.D.H. Chemicals Ltd, England; acrylamide, *N*,*N*′-methylenebisacrylamide and acetylacetone from Koch-Light Laboratories, Colnbrook, England; Dowex 50-X8 (H^+) from Biorad Laboratories, Bromley, England. Theophylline, cyclic AMP, dithiothreitol, Coomassie Blue, glucose 6-phosphate, AMP, *N*-acetyl neuraminic acid, glucosamine, pyruvate kinase, myokinase, phosphoenolpyruvate and all other fine chemicals were products of Sigma Chemical Co. Ltd, London, England.

Methods

Porton mice kept on a 12-h light and dark schedule and fed ad libitum were used when between 3 and 4 months old. Animals were starved for 2 h before killing

by cervical dislocation and removing the parotid glands which were chilled on ice. The pooled glands from 6–15 animals were minced finely with scissors and homogenized with 8 strokes at 1000 rev./min in 8 vols (w/v) cold Medium A (0.32 M sucrose in 0.05 M Tris · HCl, pH 7.4, at 20 °C, 0.025 M KCl, 0.003 M $MgCl_2$, 0.002 M $CaCl_2$) in a wide clearance (0.028 cm) Potter-Elvejhem homogenizer. Calcium was added to the homogenization medium as it has been shown to improve both the yield and structure of isolated plasma membranes [19].

Isolation of plasma membranes. Plasma membranes were obtained by the differential centrifugation of mouse parotid homogenates prepared in the hypertonic sucrose media described above. The homogenate was passed through four layers of cheesecloth, centrifuged at 650 × *g* for 10 min and the supernatant decanted off and retained. The sediment was washed twice by resuspending in Medium A and recentrifuging. The supernatants were decanted off and combined with the supernatant from the first centrifugation. The combined fractions were centrifuged at 15 000 × *g* for 10 min and the supernatant decanted off. The sediment was resuspended in Medium A and recentrifuged at 15 000 × *g* for 10 min. The supernatants were combined and centrifuged at 40 000 × *g* for 60 min. The sediment (P-1) contains plasma membranes which were washed twice by resuspending in 50 mM Tris · HCl, pH 7.4, at 20 °C and centrifuging at 40 000 × *g* for 60 min, yielding a pellet of washed membranes (P-2). Sediments were resuspended in all-glass homogenizers fitted with loose pestles, using hand homogenization. The fractionation procedure is summarized in Fig. 1. Other subcellular fractions were prepared as previously described [20].

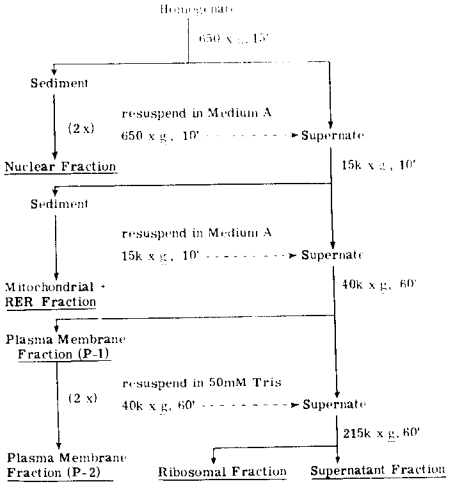


Fig. 1. Scheme of the fractionation of mouse parotid gland by differential centrifugation in sucrose media.

Analytical methods. Lipids were extracted in chloroform/methanol after the method of Colbeau et al. [21]. Nucleic acids were extracted from the residue with hot 5 % trichloroacetic acid and the DNA and RNA determined by the diphenylamine [22] and orcinol [23] reactions, respectively. Total lipids, cholesterol and phospho-

lipid phosphorus were analysed directly in the chloroform/methanol extract following the methods of Johnson [24], Clark et al. [25] and Bartlett [26]. Total phospholipid was estimated by multiplying the phospholipid phosphorus value by 25. Sialic acid and hexosamine were extracted and determined as described by Warren [27] and Gatt and Berman [28], respectively. Protein was measured by the procedure of Lowry et al. [29].

The proteins of the plasma membrane were analysed by polyacrylamide gel electrophoresis in 1 % sodium dodecyl sulphate and stained with Coomassie Blueas described by Fairbanks et al. [30].

Adenylate cyclase assay. Adenylate cyclase activity was measured by the method of Bär and Hechter [31], using the single-dimension separation on polyethyleneimine-impregnated cellulose thin-layer plates. The assay mixture contained: 40 mM Tris · HCl, pH 7.6, at 34 °C, 2.5 mM cyclic AMP, 5 mM theophylline, 10 mM phosphoenolpyruvate, 5 mM $MgCl_2$, 5 mM ATP, 10 mM NaF, 2 enzyme units pyruvate kinase, 1 enzyme unit myokinase, 40 μg bovine serum albumin and 1–3 μCi [α-^{32}P]-ATP. The reaction was started by adding the enzyme preparation (10–250 μg protein), followed by incubation at 34 °C. The total assay volume was usually 40 μl, although occasionally assays of 70 μl were performed. To terminate the reaction, 5 μl of 0.25 μCi/ml ^{3}H-labeled cyclic AMP was added and the tube placed in a boiling water bath for 3 min. After cooling on ice, the tubes were centrifuged and 20-μl aliquots of the supernatant applied to the thin-layer plates which were developed with 0.3 M LiCl. The cyclic AMP spots were cut out, eluted in liquid scintillation vials with 1 ml 0.1 M HCl/0.2 M NaCl and counted in Triton/toluene liquid scintillation fluid [32]. Blank values varied from 0.001 to 0.01 % of the added radioactivity. Normally, if the blank was higher than 0.005 %, the ATP was repurified on Dowex AG50. All estimations were performed in triplicate.

Marker enzymes. The activities of 5′-nucleotidase (EC 3.1.3.5), glucose-6-phosphatase (EC 3.1.3.9) and (Na^+, K^+)-activated ATPase (EC 3.6.1.4) were measured by determining the initial rate of release of P_i from appropriate substrates at 37 °C, using the method of Stanton [33] for estimating P_i, and the velocities expressed as nmol P_i released/min per mg protein. The incubation mixtures contained: for 5′-nucleotidase, 10 mM AMP, 5 mM $MgCl_2$, and 50 mM Tris · HCl, pH 7.5, at 37 °C; for glucose-6-phosphatase, 20 mM glucose 6-phosphate, 4 mM disodium EDTA, 1 mM NaF and 50 mM cacodylate buffer, pH 6.5; for (Na^+, K^+)-activated ATPase, 3 mM ATP (Tris form), 130 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$ and 50 mM Tris · HCl, pH 7.5, at 37 °C. Mg^{2+}-activated ATPase activity was corrected for by subtracting the activity observed in the presence of 1 mM ouabain.

Non-specific phosphomonoesterase activities, both acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1), were determined using *p*-nitrophenylphosphate as the substrate [19, 34] and phosphodiesterase activity (EC 3.1.4.1) using bis-*p*-nitrophenylphosphate as substrate [35]. Succinate-dichlorophenolindophenol (DCIP) reductase was assayed by the method of King [36]. All determinations were made in duplicate.

Electron microscopy. Histochemical localization of adenylate cyclase activity in fixed sections was carried out essentially as described by Howell and Whitfield [5], except that the time of fixation was drastically reduced. Freshly dissected parotid glands were immediately fixed in 1 % glutaraldehyde containing 4.5 % glucose in 0.05 M cacodylate/nitrate buffer, pH 7.4, at 4 °C. After fixation for 5 min, tissue

was washed with cacodylate/nitrate/glucose buffer and stored overnight at 4 °C. Blocks of parotid were then cut into 30-μm sections using a cold microtome as described by Adamstone and Taylor [37]. The sections were incubated at 30 °C for 30 min in medium containing 80 mM Tris/maleate, pH 7.4, at 30 °C, 8 % glucose, 2 mM theophylline, 2 mM $MgSO_4$, 0.5 mM 5'-adenylyl imidodiphosphate (or ATP), 10 mM NaF and 4 mM lead nitrate. Control sections were incubated in the above medium less either lead nitrate or adenylyl imidodiphosphate. Following incubation sections were briefly washed with Tris/maleate/glucose buffer and post-fixed with 1 % OsO_4 in cacodylate/nitrate/glucose buffer. The tissue was then dehydrated and embedded in araldite. Thin sections were cut with an LKB I ultratome and were examined either without staining or after brief staining with uranyl acetate (saturated solution) and lead citrate, in an AEI EM6B electron microscope.

Individual subcellular fractions were fixed in 1 % glutaraldehyde in 0.05 M cacodylate buffer, pH 7.3, at 4 °C and post-fixed in 1 % OsO_4 in the same buffer. Pellets were dehydrated, embedded, sectioned, stained and examined in the same way as described above. Negative staining was performed using a 1 % solution of phosphotungstic acid, pH 5.6.

For unfixed sections, parotid glands were quick frozen using solid CO_2. 10-μm sections were cut using a Zeiss cryostat. Sections were incubated as above with the addition of 7 % Dextran (average mol. wt 250 000). Sections were then fixed in 1 % glutaraldehyde/12 % Dextran and post-fixed in 1 % OsO_4 in cacodylate nitrate buffer containing 6 % Dextran.

RESULTS

Membrane preparation

Membranes prepared from mouse parotid by differential centrifugation of homogenates made in hypertonic sucrose as described under Methods represent 2.3 % of the protein from the crude homogenate (Table I). The purity of the preparations was checked by electron microscopy and the assay of marker enzymes.

Electron microscopy. Electron micrographs of the P-1 preparation revealed a predominance of smooth membrane structures mainly in the form of vesicles (Fig. 2A). There was a small contamination with rough endoplasmic reticulum and Golgi apparatus when it is remembered that the parotid as a secretory tissue contains very large amounts of these structures. The mild homogenization procedure employed results in a minimum disruption of the rough endoplasmic reticulum, which remains as large fragments which sediment predominantly in the mitochondrial fraction. There is no contamination with nuclei and only the occasional mitochondrion is observed. However, there is a significant amount of amorphous material and storage granules present and a variable number of free ribosomes. Washing the membranes twice removes most of the amorphous material and storage granules and many of the ribosomes (Figs 2B and 2C). A further third washing removes more of these materials but the improvement is only marginal. The ribosomal contamination could be eliminated by the insertion of a 2 M sucrose layer below the aqueous suspension during the first wash of the P-1 pellet. Only the ribosomes are dense enough to enter the sucrose layer, while the membranes collect at the interface between the two layers. This does, however, lead to decreased yields of membranes.

TABLE I

DISTRIBUTION OF MARKER ENZYMES BETWEEN SUBCELLULAR FRACTIONS OF MOUSE PAROTID

The specific activity of the homogenate is shown in parenthesis and expressed as nmol product formed/min per mg protein. Conditions were as described under Experimental Procedure. n.d., not determined.

Fraction	Protein, percent recovery in fractions	5′-Nucleotidase	Relative specific activity					
			(Na^+, K^+)-ATPase	Alkaline phosphatase	Phospho-diesterase	Glucose-6-phosphatase	Acid phosphatase	Succinate DCIP reductase
Homogenate	100	1 (50.7)	1 (32.8)	1 (13.1)	1 (6.4)	1 (4.3)	1 (24.5)	1 (6.4)
Nuclei	26.2±2.9	0.4	0.7	0.4	0.7	2.4	0.5	2.1
Mitochondria+ rough endoplasmic reticulum	3.1±0.3	4.1	6.4	0.2	6·0	4.7	2.0	12.2
P-1 fraction	3.2±0.3	13.2	14.5	12.9	11.1	0.9	0.5	<0.1
P-2 fraction	2.3±0.3	17.5	19.0	17.1	14.8	0.3	0.3	<0.1
Supernatant	58.8±5.9	0.7	0.2	0.7	0.4	0.2	n.d.	<0.1
Total recovery in fractions (%)		103.4	92.2	90.1	81.8	89.5	—	92.1

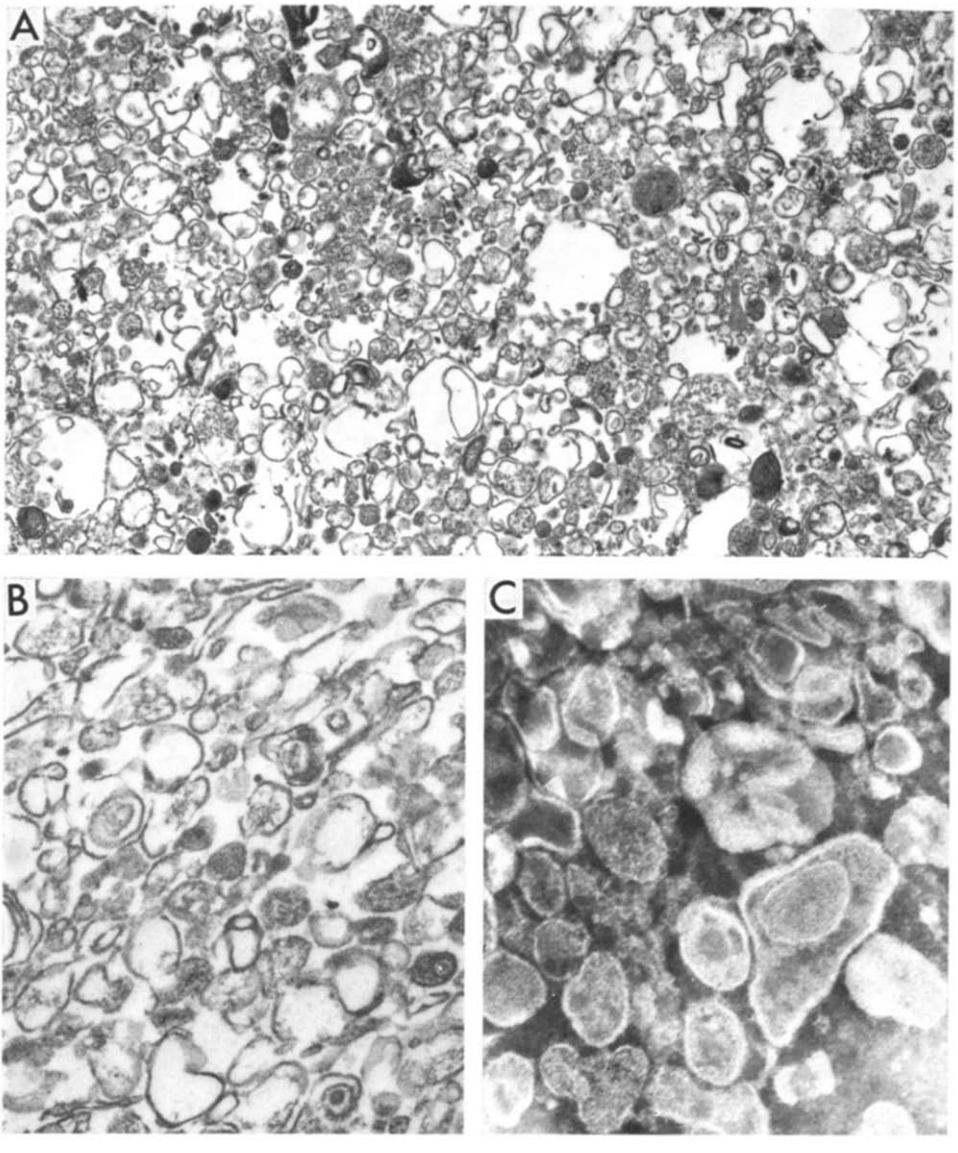


Fig. 2. Typical thin section surveys of the plasma membrane fractions. (A) P-1 fraction, 21 000. (B) P-2 fraction, 44 100. (C) P-2 fraction, negative staining, 72 800.

Marker enzymes. The marker enzyme data, summarized in Table I, demonstrate a substantial increase in the specific activity of 5'-nucleotidase, (Na^+, K^+)-activated ATPase, alkaline phosphatase and phosphodiesterase in fractions P-1 and P-2, all of these enzymes having been shown to be plasma membrane markers in a variety of tissues [19, 38]. The average increase in the specific activity of these enzymes in the P-2 fraction would indicate a 17-fold purification of plasma membranes. The succinate-DCIP reductase and acid phosphatase activities in the P-2 fraction suggest that there is little contamination with mitochondria or lysosomes, while the glucose-6-

phosphatase activity indicates a removal of at least a large part of the microsomal contamination. The rough endoplasmic reticulum is found predominantly in the 15 000 $\times g$ sediment [20] and this distribution is confirmed by the glucose-6-phosphatase activity being highest in this fraction.

Chemical composition. The chemical composition of the plasma membrane preparations is shown in Table II. The preparations contain the high cholesterol and sialic acid content and cholesterol : phospholipid molar ratio that are characteristic of surface membranes [38, 39]. Although the amount of DNA present was very low, the membranes contained a variable amount of RNA which was higher than that normally found in plasma membrane preparations. This appears to result from the variable ribosomal contamination which has already been alluded to. A large part of the RNA was removed by including a 2 M sucrose layer in the first wash.

TABLE II

CHEMICAL COMPOSITION OF PLASMA MEMBRANES OF MOUSE PAROTID

Duplicate determinations were made on plasma membranes prepared from the pooled parotid glands of two preparations each of 50 mice. Other conditions were as described under Experimental Procedure.

Compound	Membrane preparation (μg/mg protein)	
	P-1	P-2
Sialic acid	4.9	5.8
Hexosamine	17.8	18.6
RNA	72	41 (19)**
DNA	6.0	1.8
Cholesterol	119	163
Phospholipid	381	435
Cholesterol : phospholipid*	0.68	0.83
Total lipid	564	648

* Molar ratio

** With 2 M sucrose layer included in wash.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Samples at various stages of membrane purification were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Profiles of gel scans of the various fractions are shown in Fig. 3. Comparison of these profiles and of the gel scans themselves show the following features. The nuclear and mitochondrial fractions contain mostly proteins of lower molecular weight. The plasma membrane fraction, in contrast, shows a predominance of high molecular weight proteins which appear as only very minor bands in the total homogenate. There is essentially only one component with a molecular weight of less than 30 000.

Adenylate cyclase activity

Distribution. Table III summarizes the distribution of basal, isoproterenol-, and fluoride-stimulated adenylate cyclase activities among the subfractions of mouse parotid gland homogenates. All three activities were obtained in greatest yield and

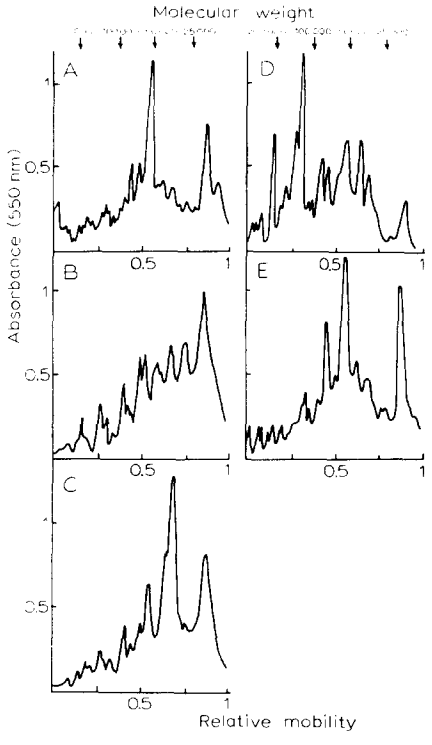


Fig. 3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis profiles of fractions from mouse parotid. Conditions were as described under Experimental Procedure. A, homogenate; B, nuclei; C, mitochondria; D, P-2 membranes; E, supernatant.

TABLE III

DISTRIBUTION OF ADENYLATE CYCLASE ACTIVITY AMONG THE SUBCELLULAR FRACTIONS OF MOUSE PAROTID

Determinations were made on three preparations each with the pooled glands from 10 animals. The incubation time was 10 min where isoproterenol (1 mM) was present or there was no addition and 15 min with NaF added. Other conditions were as described under Experimental Procedure except that the homogenate was prepared in Medium A lacking 0.002 M $CaCl_2$. After aliquots of the homogenate were taken for assay of adenylate cyclase activity, the calcium was added. Figures in parenthesis are the percentage of initial homogenate activity present in each fraction. n.d., not determined.

Fraction	Adenylate cyclase activity (nmol/cyclic AMP formed/per min per mg protein)		
	Basal	Isoproterenol	NaF
Homogenate	0.062	0.104	0.273
Nuclei	0.059 (25)	0.087 (22)	0.168 (16)
Mitochondria + rough endoplasmic reticulum	0.422 (21)	0.698 (21)	1.77 (20)
P-1 fraction	0.696 (36)	1.44 (44)	3.67 (43)
P-2 fraction	0.560	1.22	4.68 (40)
Ribosomes	n.d.	n.d.	0.505 (4.4)
Supernatant	0.005	0.005	0.005

specific activity in the plasma membrane fractions. However, while the fluoride-stimulated adenylate cyclase activity was maximal in the purified P-2 membrane fraction, both basal and isoproterenol-stimulated activities were highest in the partially purified P-1 fraction. Considerable amounts of activity were also present in both the nuclear and the mitochondrial fractions. Since purified nuclei have in some cases been found to contain adenylate cyclase activity [10, 11], pure nuclei were prepared as has been previously described [20]. These nuclei contained no measurable adenylate cyclase activity, indicating that the total activity of this enzyme in the nuclei is less than 0.5 % of the whole gland activity. However, the recovery of nuclei in this procedure is very low, as most parotid nuclei have rough endoplasmic reticulum tags reducing their density. Part of the activity in the mitochondrial fraction appears to reside in plasma membrane fragments in this fraction, as re-homogenization and centrifugation gives further material which sediments at 40 000 $\times g$ and is enriched in plasma membrane marker enzymes. However, a considerable proportion of the activity still sediments at 15 000 $\times g$.

The total activity recovered in all the fractions is 80 % of the initial homogenate activity. It is believed that this loss of activity is accounted for primarily, if not entirely, not by the partial inactivation of the adenylate cyclase during fractionation but through the discarding of two fractions which contain some adenylate cyclase activity: (a) the residual fat material which floats to the surface at the first centrifugation and

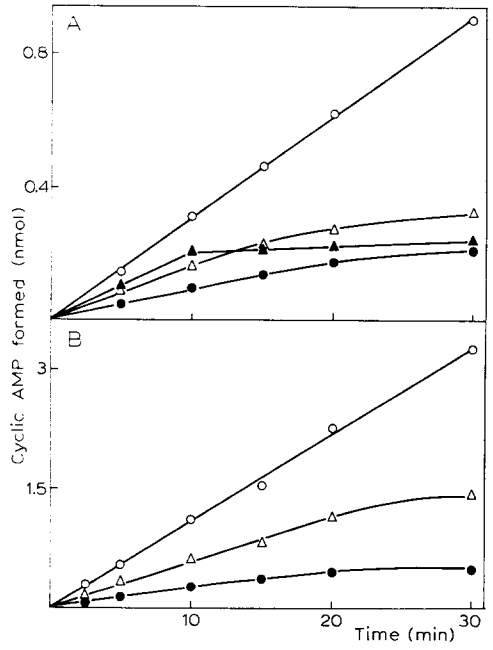


Fig. 4. Time course of the adenylate cyclase reaction. (A) Crude homogenate, 119 μg protein (plus NaF) and 160 μg protein (others) was used as the source of enzyme. (B) P-2 plasma membranes. 24 μg protein (plus NaF) and 48 μg protein (others) was used as the source of enzyme. Additions: ○—○, 10 mM NaF; ●—●, control; △—△, 1 mM isoproterenol; ▲—▲, 3 mg isoproterenol injected 2.5 min prior to killing the animal.

is removed and (b) erythrocytes which contaminate the nuclear fraction but can be removed to a great extent because they pack tightly at the bottom of the centrifuge tube and can be left behind when resuspending the nuclei.

Assay conditions for adenylate cyclase activity. Fig. 4 presents the time course of the adenylate cyclase reaction with the homogenate and P-1 membrane fraction with varying additions. In the presence of fluoride, the reaction was linear for at least 30 min with both enzyme preparations, but in the presence of isoproterenol or with no addition the reaction was linear for only 10 min. A stimulation of adenylate cyclase activity in the homogenate could also be seen when isoproterenol was administered in vivo, but in this case the reaction appeared to cease completely after 10 min. Without the ATP-regenerating system the reaction was linear for only a very short period even in the presence of fluoride, presumably because of the potent ATPase that is present and which is not completely inhibited by fluoride. Assays were never carried out with more protein than was used in these determinations and up to these concentrations activity was proportional to the amount of protein used (not shown).

The effects of varying ATP, Mg^{2+} and F^- concentrations on adenylate cyclase activity are shown in Fig. 5. 10 mM F^- was found to give a maximal stimulation. In agreement with other workers (see ref. 39) there was not a simple 1 : 1 stoichiometry in the concentrations of ATP and magnesium required but this ratio gave very nearly maximum activity and so was employed. The stimulation of adenylate cyclase activity

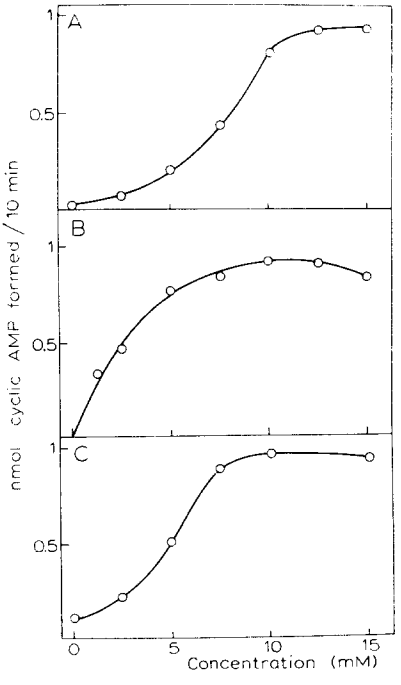


Fig. 5. Effect of varying the concentration of individual components of the reaction mixture upon adenylate cyclase activity. P-2 plasma membranes were used as the source of enzyme. Other conditions were as described under Experimental Procedure except that in A, the Mg^{2+} concentration was varied; B, the ATP concentration was varied; C, the NaF concentration was varied.

by isoproterenol was prevented by propranolol but not affected by phentolamine.

Electron microscopic cytochemistry. The results of the subcellular fractionation of mouse parotid and the distribution of adenylate cyclase activity suggest that, although only approx. 50 % of the recovered enzyme activity was situated in the plasma membrane fraction, most of the activity in the crude nuclear and mitochondrial fractions is not associated with these organelles. However, it was not possible to completely rule out the possibility of small amounts of adenylate cyclase activity residing in the nuclei and mitochondria. In an attempt to resolve this problem and to confirm that most of the adenylate cyclase activity did indeed reside at the plasma membrane, the subcellular location of the enzyme was determined using electron microscopic cytochemistry.

Using this technique, hormone-responsive adenylate cyclase activity has been localized at the plasma membrane of liver cells [40], the cells of the rat nephron [41] and the cells of the islets of Langerhans [5]. The method can also localize adenylate cyclase activity within cells as shown by Schultze et al. [42] using cardiac muscle.

Figs 6–8 are representative electron micrographs of the lead staining obtained in the parotid. Considerable involution of the plasma membrane occurs in parotid cells, so that sections do not continuously follow contiguous parts of this membrane. Some areas of the plasma membrane between adjoining acinar cells show heavy lead deposits (Figs 6A and 7A), however, enzyme activity is not spread throughout this membrane but confined to certain areas. Staining is observed fairly uniformly at the basal cell membrane of the acinar cells (Fig. 8A), but by far the heaviest staining is observed around the duct lumens into which acinar cells discharge their secretory vesicles (Figs 7A, 7B and 8A). Within the parotid cells there are no lead deposits at the nuclear, mitochondrial or rough endoplasmic reticulum membranes. However, a small proportion of the vacuoles within the cells exhibit an extremely strong lead staining (Figs 6A, 6B and 7A). In any section only a very few cells show staining vacuoles, indicating that they are not numerous within the cells. The presence of staining within these vacuoles and also on the membrane of fat droplets which accumulate within acinar cells after isoproterenol administration (Revis, N. W. and Durham, J. P., unpublished) indicates that substrate is penetrating the cells so that the absence of lead deposits associated with the nuclei, mitochondria and rough endoplasmic reticulum is unlikely to result from a lack of accessibility to substrate. To confirm this the nuclear and mitochondrial fractions were also studied and, again, no staining was associated with these organelles. However, the mitochondrial fraction was enriched in the vesicles which appeared to contain adenylate cyclase activity. When this fraction was subfractionated by isopycnic centrifugation on discontinuous sucrose gradients, the vesicles were concentrated in the density 1.167 layer (Fig. 8B).

Glutaraldehyde treatment of the parotid results in a partial inactivation of adenylate cyclase activity. If there was adenylate cyclase situated at the membrane of either the nucleus or the mitochondrion which was differentially sensitive to glutaraldehyde, one might fail to detect activity because of preferential inactivation. To allow for this possibility, the distribution of adenylate cyclase was also studied in unfixed sections. No difference in the distribution was observed using this technique.

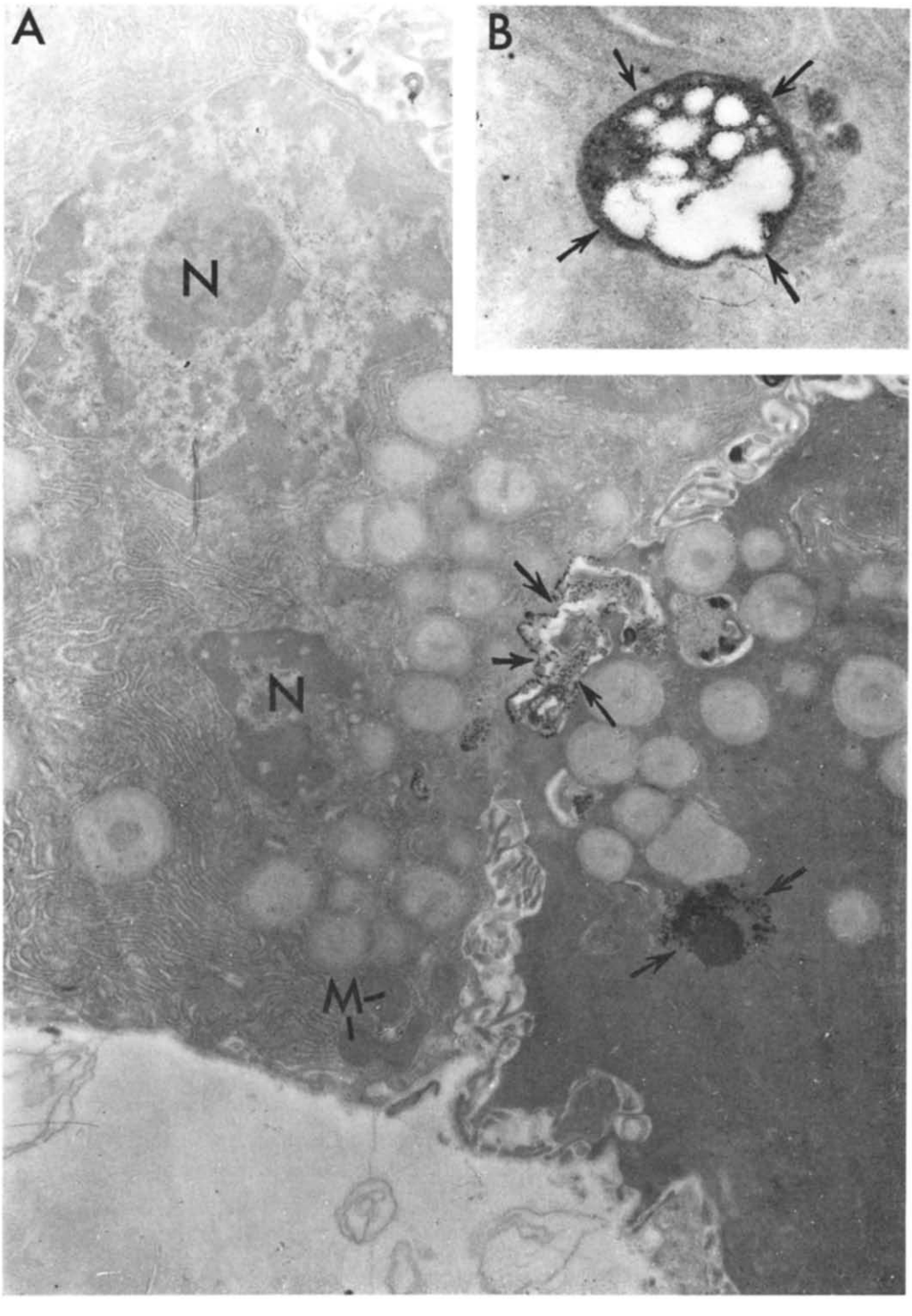


Fig. 6. The distribution of adenylate cyclase in a uranyl acetate-stained section of the mouse parotid. (A) Reaction product is localized on the plasma membrane and in the extracellular space (arrows). Inside the acinar cells lead deposits were located in occasional vesicles, while the mitochondria (M) and nucleus (N) remained free of lead (×15300). (B) A higher magnification of a vesicle (arrows) from an acinar cell which contains lead deposits (×33300)

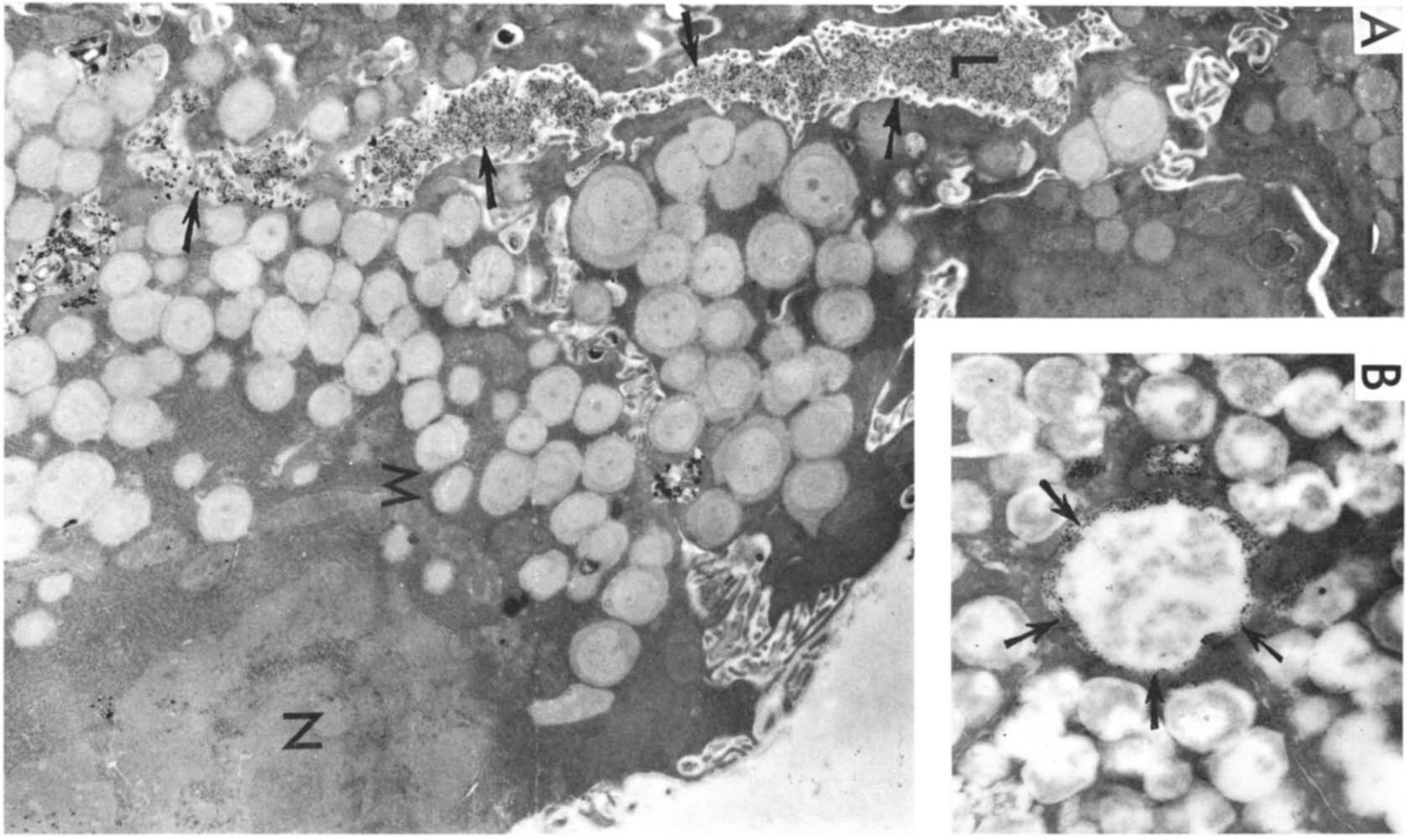
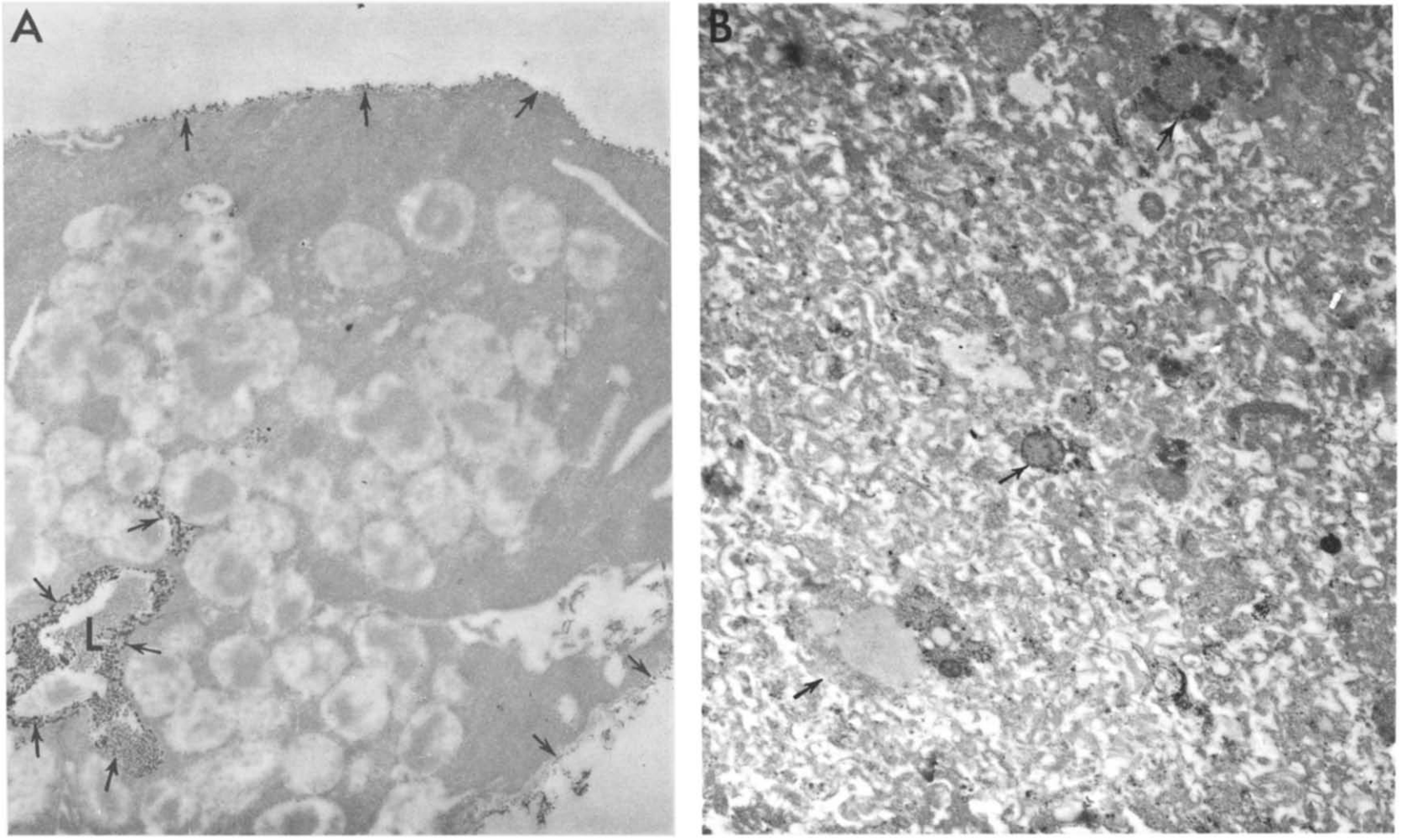


Fig. 7. Adenylate cyclase of mouse parotid in a section treated with uranyl acetate. (A) The reaction product appears around duct lumens (L) and between the acinar cells. The mitochondria (M) and nucleus (N) are free of lead deposits (× 25 000). (B) Transverse section of a duct showing strong lead deposits (× 20 000).



g. 8. (A) Adenylate cyclase of mouse parotid in a section not treated with uranyl acetate. Uniform staining is observed around the basal plasma membrane (arrows) and heavy ining at a duct lumen (L) (11 250). (B) Adenylate cyclase in a mitochondrial subfraction obtained by isopycnic centrifugation in a discontinuous sucrose gradient of mitochondrial sediment and collection of the density 1.167 layer. There is a preponderance of staining in vesicles of the type shown in Fig. 6 (11 250).

DISCUSSION

Procedures for the purification of plasma membranes most often utilize the fact that, in most cases, membrane fragments co-sediment with the nuclei [38]. It is unusual for the greater part of the plasma membrane to sediment after 150 000 $g \cdot$ min, especially as a very mild homogenization procedure was used, resulting in most of the rough endoplasmic reticulum sedimenting under these conditions. Although purifications depending upon simple differential centrifugation have been tried in other tissues [38], usually it has been necessary to include one or more isopycnic centrifugation steps in the procedure to obtain a good purification. However, in the parotid, differential centrifugation provides a simple and quick method of preparation. The yield of membranes is very good and although the membranes are clearly not completely pure, the method provides a preparation which is suitable for many purposes. Further purification has been found to be possible if sucrose density gradients are employed. Both chemical composition and the presence of a number of enzymes known to be associated with the plasma membrane in other tissues indicate that the purified membranes are derived from the cell surface.

Adenylate cyclase activity is considerably enriched, relative to parotid homogenates, in the purified plasma membranes. The increase in the specific activity of adenylate cyclase is identical to the increase in the specific activity of plasma membrane marker enzymes, indicating that there has been no loss of activity during purification, as is often observed. The membrane preparations exhibit the same activation by fluoride and isoproterenol as in the crude homogenate. However, only approx. 50 % of the total adenylate cyclase activity is recovered in the plasma membrane fraction. Although it appears probable that in all cells with adenylate cyclase at least part of the enzyme activity is located at the plasma membrane, the demonstration of the enzyme at this site has sometimes been taken as indicating that this is its sole position within the cell. But in a number of systems adenylate cyclase activity has been found in other localities within the cell [9–12]. In the parotid, adenylate cyclase did not appear to be associated with the nuclei or mitochondria, although activity was found in these subcellular fractions. Therefore, electron microscopic cytochemistry was used to determine where this enzyme is present in parotid cells.

Evidence has previously been presented by other workers [5, 40–43] that the lead pyrophosphate method is capable of demonstrating and localising adenylate cyclase. The method used for the visualisation of adenylate cyclase is moderately specific, as only the few enzymes which split pyrophosphate from ATP can utilise adenylyl imidodiphosphate as a substrate. However, the reaction is not unique. There are several lines of evidence that suggest that adenylate cyclase activity is being studied. Firstly, lead staining was only observed when either ATP or adenylyl imidodiphosphate was added to the incubations. Secondly, both isoproterenol and fluoride greatly enhanced the lead staining. Thirdly, propranolol inhibited the isoproterenol effect.

Biochemical studies on the organisation of adenylate cyclase within the plasma membrane have shown that the catalytic subunit of the enzyme is situated on the inside of the membrane [2]. In agreement with previous cytochemical studies on adenylate cyclase [5, 40–43], we found lead deposits localised outside of the membrane. This is not necessarily contradictory, for whilst cyclic AMP is released into

the cytoplasm it is the pyrophosphate also formed in the reaction which is being localised and it has been suggested that this might be immediately transferred to the outside of the membrane [43].

In our experiments on adenylate cyclase, lead phosphate deposits were localised over the whole of the plasma membrane of the acinar cells, but the deposits were most prominent at the luminal membrane. This may simply represent the ready access of components of the incubation medium to this surface or could represent a true concentration of the enzyme. Cyclic AMP has been implicated in protein secretion from the rodent parotid gland [14, 17]. Previously, this has been believed to be related to the transmission of sympathetic nerve stimulation into the cell through the basal cell membrane with cyclic AMP acting as a second messenger. The distribution of adenylate cyclase may now indicate an additional role for cyclic AMP at the luminal membrane. Adenylate cyclase has also been localised at the luminal membrane in the cervicovaginal epithelium of mice [43].

Besides being situated at the plasma membrane, adenylate cyclase was found in a small number of vesicles within the cytoplasm. These vesicles appear to be identical to structures characterised as secondary lysosomes in the rat parotid [44]. The presence of electron-dense granules and smaller membrane-bound vacuoles within the vesicles gives them an appearance making it most unlikely that they could represent sections through invaginations of the plasma membrane.

The observation of adenylate cyclase in vesicles within the cytoplasm could provide the answer to the observation that isoproterenol administration causes a marked decrease in measurable adenylate cyclase activity in the rat parotid [45], if these vesicles were secreted upon isoproterenol stimulation. Heavily staining vesicles have been observed outside of cells but these could have come from damaged cells. What happens to the vesicles upon isoproterenol administration is at present under study. The function of adenylate cyclase in these vesicles is unknown. The parotid gland does, however, accumulate unmetabolised isoproterenol within the cells [46]. Thus, the hormone may well be available to activate the enzyme.

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