PRESENCE OF ADENYLATE CYCLASE ACTIVITY IN GOLGI AND OTHER FRACTIONS FROM RAT LIVER

II. Cytochemical Localization within Golgi and ER Membranes

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ABSTRACT

The presence of adenylate cyclase (AC) in liver Golgi and microsomal fractions from ethanol-treated rats was tested cytochemically using the 5'-adenylyl imidodiphosphate (AMP-PNP) lead phosphate method. Parallel biochemical assays showed that rat liver Golgi AC was only partially inhibited by lead: in the presence of 1 mM Pb^{++} 80% of the enzyme was preserved, while when 2 mM Pb^{++} was used 25% remained. No cAMP was formed when the AMP-PNP medium was incubated in the presence of 1 or 2 mM Pb^{++} but in the absence of cell fractions, indicating that at these concentrations Pb^{++} does not cause the nonenzymatic hydrolysis of AMP-PNP. Therefore, the reaction product observed by cytochemical localization is not due to the nonenzymatic hydrolysis of AMP-PNP by Pb^{++}. In Golgi subfractions, lead phosphate reaction product was widely distributed among Golgi elements: it was seen in association with the majority of the very low density lipoprotein-filled secretory droplets which predominated in the two lightest Golgi fractions (GF_1 and GF_2) as well as within the majority of the cisternae found in the heaviest Golgi fraction (GF_3). In the latter, reaction product was heaviest along the dilated peripheral rims of the cisternae. In all cases, the reaction product was localized to the outside or cytoplasmic face of the Golgi membranes. When microsomes were incubated cytochemically for AC, deposits were found on the cytoplasmic surface of smooth endoplasmic reticulum (ER) membranes, but none were observed on rough ER membranes.

The results confirm the biochemical data reported previously indicating the presence of AC in Golgi and smooth microsomal fractions from rat liver and further demonstrate that the activity is indeed indigenous to Golgi elements and not due to plasma membrane contaminants. They also indicate that AC is widely distributed among Golgi and smooth ER elements. Thus, AC is not restricted in its distribution to plasma membranes as usually assumed.

In the previous paper (2) we reported our biochemical findings indicating the presence of adenylate cyclase (AC)[1] activity in Golgi fractions from rat liver. The biochemical data provide information on the relative and specific activities of the enzyme in Golgi fractions, but they do not provide information pertaining to the localization of the enzyme within various Golgi elements. More importantly, biochemical data cannot distinguish between indigenous activity of Golgi elements and contaminant activity due to the presence of plasma membrane. As pointed out elsewhere (7), one must rely on cytochemical localization to distinguish between them.

A method for cytochemical demonstration of AC was first introduced by Reik et al. (19) in rat liver using ATP as substrate and lead as the capture agent. However, the use of ATP as substrate was strongly criticized since it also serves as the substrate for ATPases, and it is impossible to distinguish between the reaction product resulting from AC and that resulting from ATPases. After the introduction (23) of 5'-adenylyl imidodiphosphate (AMP-PNP), an adenosine triphosphate analogue containing a P-N-P linkage, and the biochemical demonstration that this analogue serves as a substrate for AC (17) but not for ATPases (16), a cytochemical method for demonstration of AC was introduced (8, 21) in which AMP-PNP was used as substrate and lead as the capture agent. It was further shown that AMP-PNP is utilized by AC to the same extent as ATP (17) but is not hydrolyzed by most nucleotide phosphohydrolases (18). Recently, Lemay and Jarett (10) questioned the validity of the AMP-PNP, lead nitrate method and claimed that this technique cannot be reliably used for the cytochemical demonstration of AC activity since 1×10^{-4} M lead completely inhibited AC activity of fat cell membranes, and very low concentrations of lead (2×10^{-3} M) induced the nonenzymatic hydrolysis of AMP-PNP to cAMP and AMP. However, our biochemical and cytochemical results (1) and those of Cutler (3, 4) indicated otherwise.

In this paper,[2] we present results on cell fractions which indicate that the AMP-PNP lead method can indeed be used successfully for the demonstration of AC in the various membranous structures of rat liver cells. Parallel biochemical data are also presented.

MATERIALS AND METHODS

Materials

AMP-PNP (>90% pure) was purchased from International Chemical and Nuclear Inc., Irvine, Calif.

Preparation of Fractions

Enzyme activity was demonstrated by cytochemical incubation of Golgi and microsomal fractions from rat liver. Both intact Golgi (in which the cisternae still remain stacked together) and Golgi subfractions were used.

PREPARATION OF GOLGI SUBFRACTIONS AND MICROSOMAL FRACTIONS: Most of the experiments were carried out on Golgi subfractions prepared by the method of Ehrenreich et al. (5), as given in the previous paper (2). The rough microsomes used were collected from the pellet and smooth microsomes from the load zone (1.17-1.15 M sucrose) of the final spin in the Golgi isolation procedure.

PREPARATION OF INTACT GOLGI APPARATUS: In a few experiments, fractions containing "intact" Golgi apparatus with stacked cisternae and associated vesicles were prepared from rat liver following the technique of Morré (13), except for the following modifications. Male Sprague-Dawley rats (200-250 g) were fed ad libitum, but were not given alcohol as in the case of the Ehrenreich procedure. The liver was removed and finely minced at room temperature in 0.25 M sucrose containing 10 mM $MgCl_2$, then homogenized (three parts sucrose to one part liver) for 40 s with a Polytron (Tekmar Co., Ohio, model no. SDT) with the rheostat set at the middle range. The homogenate was first filtered through two layers of gauze and then spun for 20 min at 3,500 g_{max}. The yellow part of the pellet was resuspended in a minimum amount of medium and the suspension was layered on top of two parts of 1.25 M sucrose and spun in a swinging-bucket type rotor at 131,000 g_{max} for 30 min. Golgi apparatus with stacked cisternae and associated vesicles was concentrated in the white band recovered at the interface.

Cytochemical Localization of AC Activity

INCUBATION MEDIUM: The medium used was essentially that of Wagner et al. (21) and consisted of 80

[1] Abbreviations used in this paper: AC, adenylate cyclase; AcPase, acid phosphatase; cAMP, cyclic adenosine 3',5'-monophosphate; AMPase, 5'-nucleotidase; AMP-PNP, 5'-adenylyl imidodiphosphate; ER, endoplasmic reticulum; G-6-Pase, glucose-6-phosphatase; TPPase, thiamine pyrophosphatase; VLDL, very low density lipoprotein.

[2] A preliminary report of these findings was published previously (1).

mM Tris maleate buffer (pH 7.2), either 1 or 2 mM $Pb(NO_3)_2$, 4 mM $MgSO_4$, 5 mM aminophylline, 0.5 mM AMP-PNP, and 3% sucrose. Lead nitrate, the last component to be introduced, was added dropwise. The final pH was then adjusted to 7.2, and the solution was passed through a Millipore filter (pore size = 0.22 μm) before use. When 20 mM NaF was used to stimulate AC activity, it was added to the complete medium, just before incubation. In the original medium of Wagner et al. (21), 6% dextran (D-25 from Pharmacia Inc., Uppsala, Sweden) was added, ostensibly to keep the solution from becoming cloudy (20). In the present work, 6% dextran was added in some early incubations but later on it was replaced by 3% sucrose (see below).

INCUBATION PROCEDURE: This was essentially as described previously (7) except that the volume was scaled down to save on media since the substrate is expensive. Incubation was carried out in Beckman cellulose nitrate tubes (no. 305050, 5 ml capacity, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). 1 ml of a given fraction and 2 ml of concentrated (1.5 ×) incubation medium were added to each tube. They were allowed to equilibrate for 10 min at 30°C followed by 30 min of incubation at 30°C in a mechanical shaker bath. The reaction was stopped by filling the tubes with cold 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The tubes were kept on ice for 10 min to fix and then spun at 108,000 g_{max} for 40 min (using a SW50.1 rotor in a Beckman L5-50 or L5-65 centrifuge). The ensuing pellets were postfixed in 1% OsO_4 in acetate-Veronal buffer for 1 h at 0°C and stained in block with uranyl acetate at room temperature for 1 h. They were then cut into suitably sized pieces to be placed in flat embedding molds, and processed for electron microscopy as previously described (7).

CONTROLS: Two types of controls were routinely done. First, the incubation was carried out in medium which contained no substrate. This type will be referred to as a "no substrate" control. Secondly, tissue inactivated by heating for 3 min in a boiling water bath was used for the incubation; this will be referred to as a "boiled control." In some cases, a third control, in which incubation was carried out at 0°C ("0°C control"), was performed.

Biochemical Assays

In some experiments, aliquots were removed from tubes after incubation but before the addition of glutaraldehyde. The reaction was stopped by heating the aliquot for 3 min in a boiling water bath. The samples were stored at −20°C overnight for cAMP analysis on the following day as described in the previous paper (2).

RESULTS

Biochemical Findings

EFFECT OF LEAD ON BASAL AC ACTIVITY: In view of the fact that lead is known to exert an inhibitory effect on many enzymes, the activity of AC was determined in the presence and absence of lead. In this case, a common Golgi mixture (composed of equal volumes of GF_1, GF_2, and GF_3) was used. As shown in Table I, its basal activity was shown to be 4.3 pmol·mg protein^{-1}·min^{-1}. When 1 mM lead was used for the cytochemical incubation, over 80% of the AC activity was retained in the presence of 1 mM lead. When 2 mM lead was used, a higher portion (75%) of the enzyme activity was inhibited, but 25% of the activity was still retained.

EFFECT OF LEAD ON FLUORIDE-STIMULATED AC ACTIVITY: When 20 mM NaF was used in the absence of lead, AC activity was stimulated ~16-fold (Table I). However, in the presence of either 1 or 2 mM lead, no stimulation could be demonstrated. Instead, fluoride appeared to exert an inhibitory effect. In view of this lack of stimulation by 20 mM fluoride in the presence of lead, the effect of higher concentrations of NaF was investigated (data not shown). At concentrations higher than 20 mM, some stimulation was observed; in the presence of 80

TABLE I

Effect of Lead on Golgi AC Activity

	Specific activity		
	No Pb^{++}	1 mM Pb^{++}	2 mM Pb^{++}
	pmol·mg protein^{-1}·min^{-1}		
Basal	4.3 (4.9–3.7)*	3.9 (4.4–3.3)	1.1 (1.0–1.2)
NaF (20 mM)	69.5 (75.1–63.9)	2.7 (3.5–1.9)	0.8 (0.6–0.10)

A common Golgi fraction was incubated for 30 min in the cytochemical medium in the absence of Pb^{++}, or in the presence of 1 or 2 mM Pb^{++}. At the end of the incubation period, an aliquot was removed for biochemical assay as described in Methods. Data represent the average of two experiments with six animals per experiment.
* Range.

mM fluoride the activity was stimulated about twofold. It was not possible to test the effects of higher concentrations of fluoride because saturation of NaF is reached at ~80 mM.

EFFECT OF LEAD ON HYDROLYSIS OF AMP-PNP: In view of the recent claim (10) that lead alone caused hydrolysis of AMP-PNP, we tested the effect of 1 and 2 mM Pb^{++} on the substrate in the absence of liver fractions. As shown in Table II, none or negligible amounts of cAMP were produced in all cases tested, i.e., there was no lead-induced hydrolysis of the substrate.

Cytochemical Localization

PILOT EXPERIMENTS: In early experiments, the AMP-PNP was purchased in lots of 100 mg, stored in a deep freeze at $-180°C$, and aliquots were removed for each incubation. Accordingly, the same substrate was subjected to repeated freezing and thawing with increasing use. We noticed that when the first incubations were carried out upon receiving a new batch of substrate, the incubation medium was clear. However, media prepared with AMP-PNP which had undergone one or more freezing and thawing cycles invariably turned cloudy upon the addition of lead. Addition of 6% dextran reduced but did not prevent cloudiness. Moreover, the results of these early experiments were highly variable. AMP-PNP was subsequently purchased in smaller quantities (10 mg) so that fresh substrate could be used for each experiment. Media prepared with fresh substrate never turned cloudy. Hence, addition of dextran to the medium became unnecessary, and 3% sucrose was added instead to maintain the osmolality and to preserve the integrity of Golgi elements. Reproducible results with consistent lo-

TABLE II
Effect of Lead on AMP-PNP

	cAMP produced	
	Exp a	Exp b
	$pmol \cdot 300 \, \mu l^{-1} \cdot min^{-1}$	
Medium − lead	0	0
Medium + 1 mM lead	0	0
Medium + 2 mM lead	0	0.07

Medium alone was incubated under identical conditions as described in Materials and Methods in the absence of Golgi fractions.
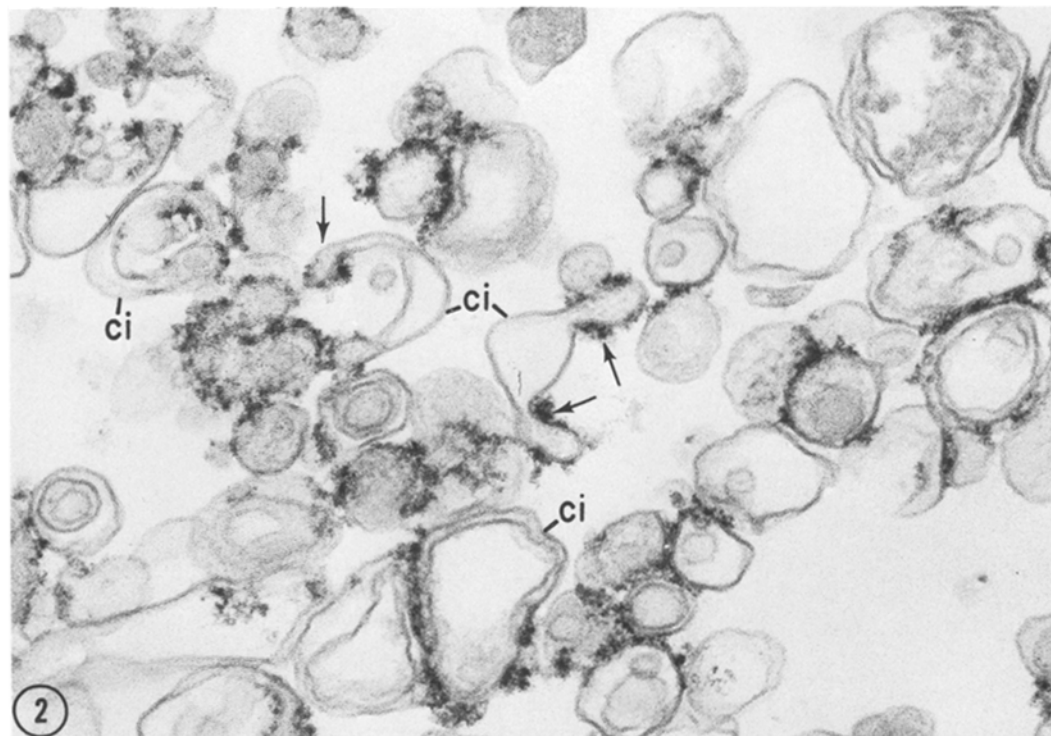
calization of AC activity was observed under these conditions.

LOCALIZATION IN GOLGI SUBFRACTIONS IN THE PRESENCE OF 2 MM LEAD: When the individual Golgi fraction GF_1, GF_2, or GF_3 (Figs. 1-2) or a common fraction consisting of equal volumes of the three subfractions (Figs. 3-5) is incubated in the presence of 2 mM lead, reaction product is seen in association with the majority of the Golgi elements. In GF_1 and GF_2 it is associated with both the very low density lipoprotein (VLDL)-filled vacuoles (Figs. 1 and 3) and cisternae (Fig. 5). In GF_3 it is found on the flattened cisternal elements (Figs. 2 and 4) which predominate in the fraction. In all cases, reaction product was located primarily on the outer (cytoplasmic) surface of the Golgi membranes (Figs. 1-5). In the case of the cisternal elements, it was more concentrated on the dilated ends of the cisternae than in their centers (Figs. 2 and 4) (see also Fig. 13).

LOCALIZATION OF GOLGI SUBFRACTIONS IN THE PRESENCE OF 1 MM LEAD: When 1 mM lead was used, the amount of reaction product present was less than in the case of 2 mM lead, but the general localization of reaction product remained unchanged: it was associated with VLDL-filled vacuoles (Fig. 6) and the dilated ends of cisternal elements (Fig. 7), and was largely restricted to the cytoplasmic face of Golgi membranes.

LOCALIZATION IN ISOLATED GOLGI WITH INTACT CISTERNAE AND VESICLES: With isolated Golgi prepared by the method of Morré (15) in which the characteristic association of Golgi elements is preserved, reaction product was also associated primarily with the cytoplasmic face of Golgi membranes, mostly localized to the outside of the secretory droplets and to the outside of the peripheral dilated rims of the Golgi cisternae which are packed with VLDL particles (Figs. 11 and 12). Only occasionally was reaction product observed over the central, flattened region of cisternal elements. Also reactive were some small vesicles situated mostly on the cis side (cf. reference 5) or forming face of the Golgi apparatus. They are presumably of smooth microsomal origin.

LOCALIZATION IN THE PRESENCE OF NAF: When 10 or 20 mM NaF was added to the cytochemical incubation in the presence of 1 or 2 mM lead, no difference was observed in the
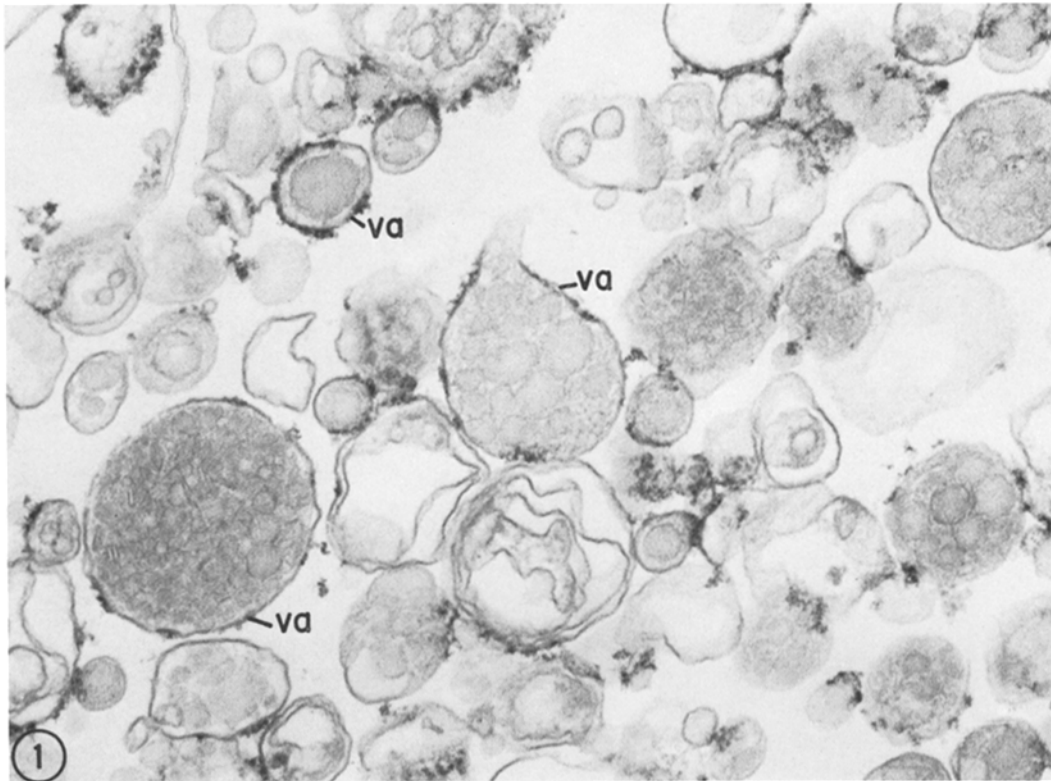
FIGURE 1   Mixture of the lightest (GF₁) and intermediate (GF₂) Golgi subfractions incubated for adenylate cyclase activity in the presence of 2 mM Pb⁺⁺. Lead phosphate reaction product is seen in association with the membranes of many VLDL-filled secretory vacuoles (*va*) of various sizes which characterize these fractions. In places where the membrane is cut in normal section, it can be seen that the deposits are present primarily along the outer or cytoplasmic surface of the membrane. × 62,500.

FIGURE 2   Heaviest Golgi fraction (GF₃) incubated for adenylate cyclase activity in the presence of 2 mM Pb⁺⁺. Reaction product is seen to be associated with the majority of the cisternal elements (*ci*) that predominate in this fraction. The lead phosphate deposits are located along the outer or cytoplasmic surfaces of the membranes and are heaviest along the dilated peripheral rims of the cisternae (arrows). × 85,000.
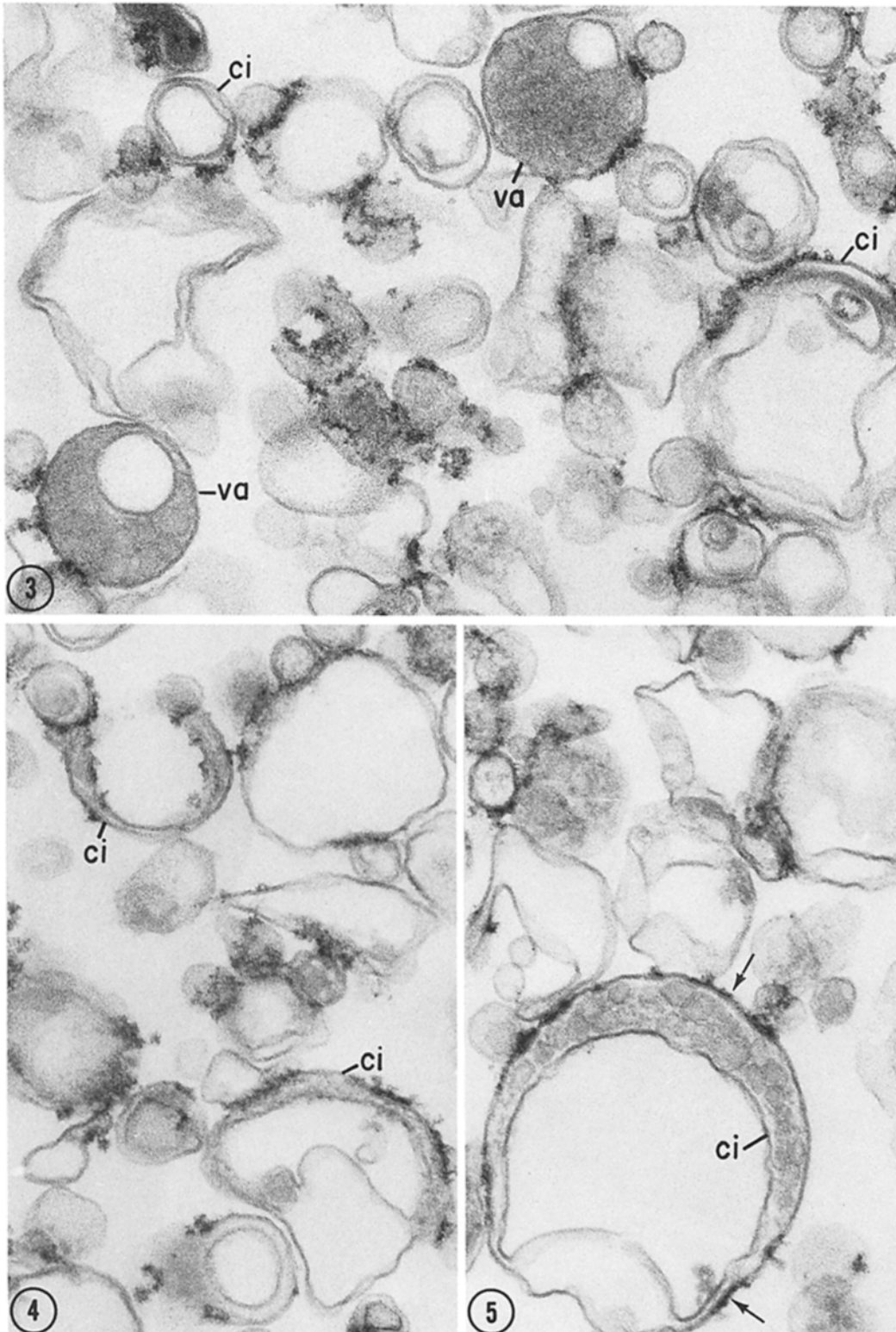
FIGURES 3-5   Golgi subfractions (consisting of equal volumes of GF_1, GF_2, and GF_3) incubated for adenylate cyclase activity in the presence of 2 mM Pb^{++}. Fig. 3 shows reactive VLDL-filled vacuoles (*va*) of the type found in the two lightest Golgi fractions as well as reactive cisternae (*ci*). Fig. 4 shows several reactive cisternal elements (*ci*) of the type which predominate in the heaviest Golgi fraction (GF_3). Fig. 5 shows a large reactive Golgi cisterna half filled with VLDL's. In all these elements it can be seen that the reaction product is found predominantly along the outer or cytoplasmic face of the Golgi membranes. The location of the lead phosphate deposits is especially well shown in Fig. 5 (arrows). (See also Fig. 13). (Figs. 3 and 4) × 85,000; (Fig. 5) × 90,000.
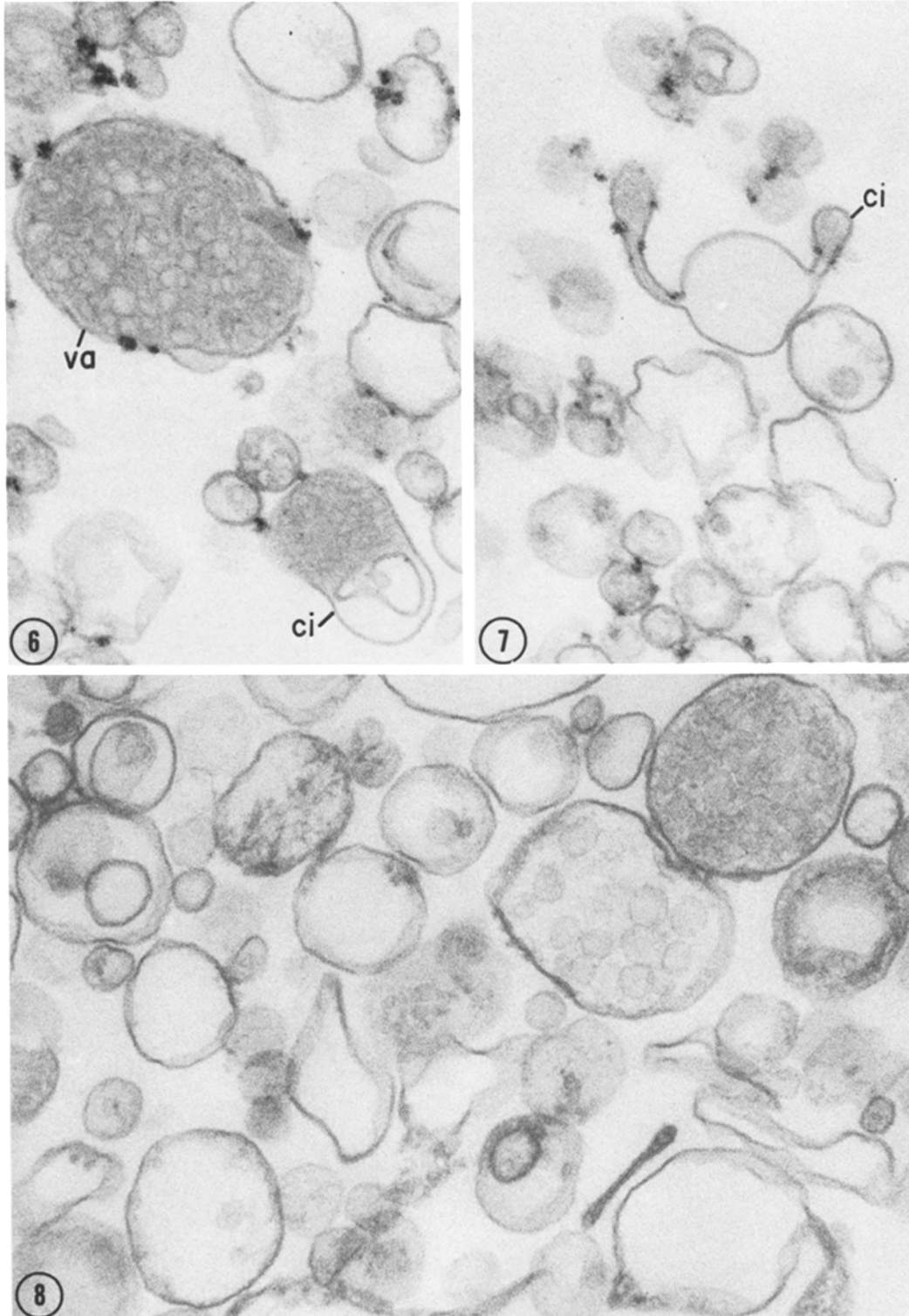
FIGURES 6–7   Golgi preparation similar to that in Figs. 3–5 except that the AC reaction was carried out in the presence of 1 mM Pb^{++}. The general localization of reaction product is the same as with 2 mM Pb^{++}, but the amount of reaction product is less. Fig. 6 shows a reactive VLDL-filled vacuole (va) and a dilated cisterna (ci) filled with VLDL's. Fig. 7 shows another reactive flattened cisternal element (ci). × 96,000.

FIGURE 8   "No Substrate" control for the adenylate cyclase procedure. A common Golgi mixture (consisting of equal volumes of GF$_1$, GF$_2$, and GF$_3$) was incubated without substrate in the presence of 2 mM Pb^{++}. No reaction product is seen on any of the Golgi elements. × 96,000.

amount or distribution of reaction product (Fig. 13). This is in keeping with the biochemical data obtained indicating that there is no stimulation of AC by 20 mM fluoride in the presence of lead.

LOCALIZATION IN ROUGH AND SMOOTH ENDOPLASMIC RETICULUM (ER) MEMBRANES: AC reaction product could also be demonstrated on membranes of the smooth ER (Fig. 14), but was not present on rough ER membranes (Figs. 15 and 16). This confirmed our biochemical observations reported in the previous paper (2) that AC activity was associated with smooth microsomes, but not with the rough microsomes. The majority of the small, smooth-surfaced vesicles present in these fractions were reactive. As in the case of Golgi elements, the lead phosphate deposits were found on the outside or cytoplasmic face of the smooth ER membranes.

CONTROL EXPERIMENTS: At 1 or 2 mM lead, no reaction product was observed in "no substrate" controls (Fig. 8), boiled controls (Fig. 9), or 0°C controls (Fig. 10).

DISCUSSION

The cytochemical results presented here confirm the biochemical data reported in the previous paper (2) indicating that AC is present in Golgi and smooth microsomal fractions from rat liver. The cytochemical findings further indicate that the enzyme is indigenous to the Golgi and smooth ER membranes and is not due to the presence of plasma membrane contaminants in the fractions. The cytochemical results not only confirm but also extend the biochemical findings: they demonstrate that among Golgi elements the enzyme is widely distributed, since reaction product was seen to be associated with the majority of the VLDL-filled secretory droplets of GF_1 and GF_2 as well as with the majority of cisternal elements in GF_3. In addition, they show that enzyme activity is not localized in rough ER membranes but is widely distributed among smooth ER membranes since the majority of the vesicles in the smooth microsome fraction possessed some reaction product, whereas

FIGURE 9   Boiled control. Enzyme activity was destroyed by placing a common Golgi mixture in a boiling water bath for 3 min before incubation. The contents of the cisternae appear condensed, but no reaction product was observed in any of the Golgi elements. × 93,000.
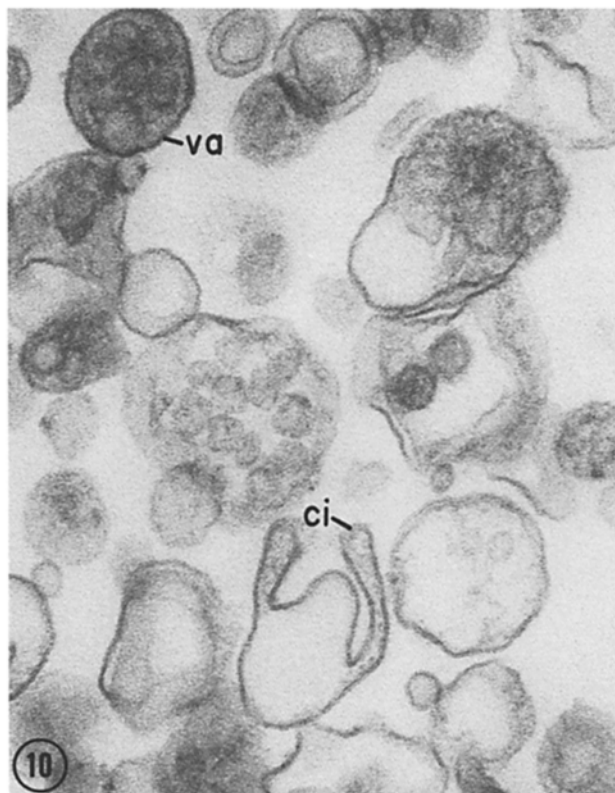
FIGURE 10   0°C control. The incubation of a common Golgi mixture for adenylate cyclase was carried out on ice. No reaction product was observed on any of the Golgi vacuoles (*va*) or cisternal (*ci*) structures. × 96,000.

the rough ER membranes lacked lead deposits. In areas in which the plane of the section was normal to that of the membranes it could be seen that reaction product was localized primarily or exclusively to the outer or cytoplasmic face of the membranes in question.

To the best of our knowledge, cytochemical demonstration of AC on Golgi and ER membranes has not been reported before. There have, however, been a few reports describing the cytochemical localization of AC on plasma membranes from several different tissues, including hepatocytes (19), pancreatic β-cells (8), capillary endothelium (21), proximal kidney tubule (9), and the slime mold, *Dictyostelium* (3, 6). In all of this work except the last, the reaction product was found to be associated with the outside of the plasma membrane whether demonstrated in fixed or fresh tissue. This is puzzling since, according to the commonly accepted model for the AC system

based on biochemical studies (14), the enzyme itself is situated on the inside of the plasma membrane whereas the hormone receptors are on the outside. Thus, if the model is correct, cytochemical reaction product should be found on the inside of the plasma membrane. We have recently demonstrated AC reaction product on the inside of isolated liver plasma membranes (Cheng and Farquhar, unpublished data).[3] In the present study, AC reaction product was localized to the outside or cytoplasmic face of Golgi and ER membranes of rat liver. The findings are therefore in agreement with the model for the AC system since when Golgi membranes fuse with the plasma

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[3] The difference between our findings on AC localization in plasma membrane and those of others could be due to differences in material used (cell fractions instead of intact cells) or methods of preparation (fixed vs. unfixed specimens).
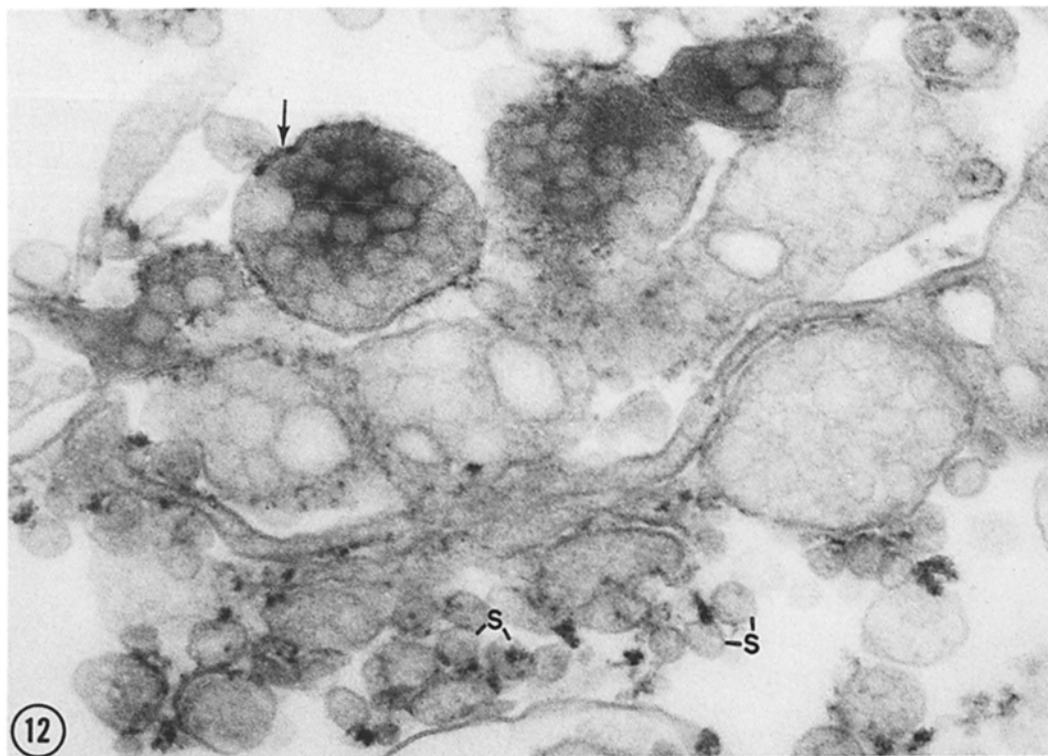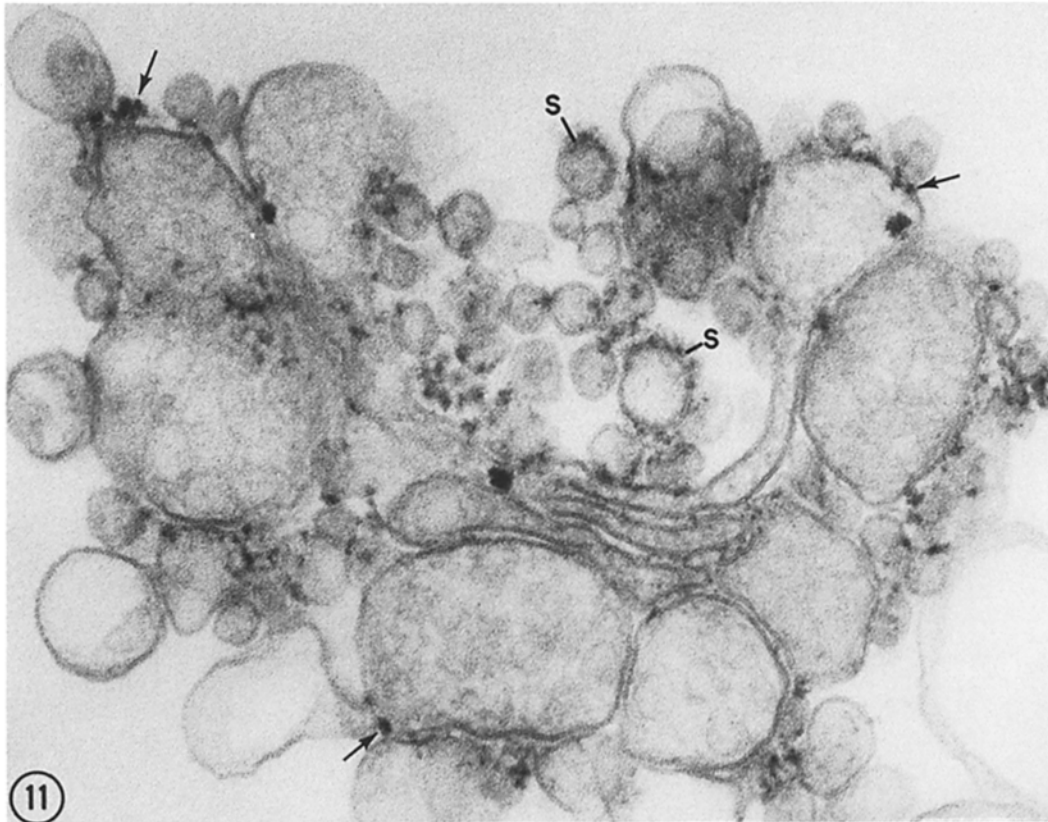
FIGURES 11-12   Golgi apparatus with intact cisternae and vesicles (prepared by the technique of Morré [13]), incubated for adenylate cyclase activity in the presence of 1 mM Pb++. Reaction product is seen primarily in association with the outside of the membrane of the VLDL-filled secretory droplets and dilated rims of cisternae (arrows). It is also seen in association with the membranes of many small, smooth vesicles (s) located along the cis side of the Golgi apparatus. (Fig. 11) × 120,000; (Fig. 12) × 90,000.

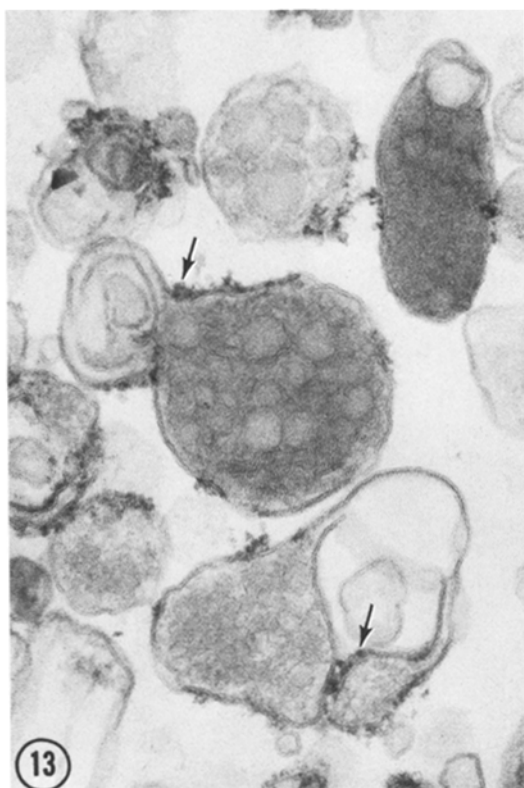FIGURE 13 Mixture consisting of equal parts of GF_1 and GF_2 was incubated in the presence of 10 mM NaF and 2 mM Pb^{++}. The amount and localization of reaction product is identical to that found in preparations incubated without NaF (Figs. 1-5): it is associated with VLDL-filled vacuoles and the dilated ends of cisternal elements (arrows) and is confined primarily to the cytoplasmic surface of the membranes. × 120,000.

membrane at the time of discharge (by exocytosis) of the VLDL-filled secretory droplets (cf. reference 5), the cytoplasmic face (outside) of the Golgi membrane becomes the inside of the plasma membrane.

Previously, 5'-nucleotidase (AMPase), acid phosphatase (AcPase), and thiamine pyrophosphatase (TPPase) have also been shown by cytochemical methods to be indigenous to Golgi elements (7). In each case, the pattern of distribution of a given enzyme is characteristic and reproducible in regard to both the elements which are reactive and the side of the membrane on which lead deposits are located (see Table III). In the case of AcPase and TPPase, lead deposits were associated with some of the cisternae and VLDL-filled secretory droplets in GF_1 and GF_2, but were absent

from the cisternal elements which predominate GF_3.[4] Moreover, the reaction product was typically localized to the contents of secretory droplets. By contrast, AMPase reaction product was found associated with the membranes of Golgi elements in all three fractions, but its localization varied. In GF_1 and GF_2 it was found along the inside of the secretory droplet membranes, and in GF_3 it was found on the outside of the cisternal elements where it was concentrated along the dilated cisternal rims. Glucose-6-phosphatase (G-6-Pase) was absent from all Golgi elements. Thus, the pattern of deposition of reaction product among Golgi elements incubated for AC was different from that of any phosphatase examined so far, in that lead deposits were localized primarily or exclusively to the outside surfaces of the Golgi membranes in all three fractions.

The distribution of cytochemical reaction product within ER elements also appears to be characteristic for a given enzyme. Reaction product for G-6-Pase is widely distributed in all the rough and smooth ER elements where it is always located on the inside of the ER membranes (7, 11). AMPase is also widely distributed among ER elements of both the rough and smooth variety, but lead deposits are typically found on the outside of the ER membranes (22). Reaction product for AC was found in this study to be absent from the rough ER and to be restricted to smooth ER membranes where it was found along the outer membrane surfaces. The specificity and reproducibility of the localizations described lend support to the assumption that within Golgi and ER elements reaction product is deposited on the side of the membrane where the enzyme or its active site is located.

The finding that lead deposits are larger in the presence of 2 mM lead than 1 mM lead has been a consistent finding with this technique on Golgi and ER membranes as well as on plasma membranes (Cheng and Farquhar, unpublished data). At first glance, this may seem surprising in view of the greater enzyme activity (80% vs. 25%) which was measured by biochemical assay in the presence of 1 mM lead. However, the fact that the size of the lead deposits does not necessarily correspond to the amount of enzyme activity present is well

[4] Evidence was previously given (7) indicating that GF_1 and GF_2 represent primarily trans-Golgi elements from the secretory Golgi face, whereas GF_3 consists largely of cis-Golgi components from the opposite face.
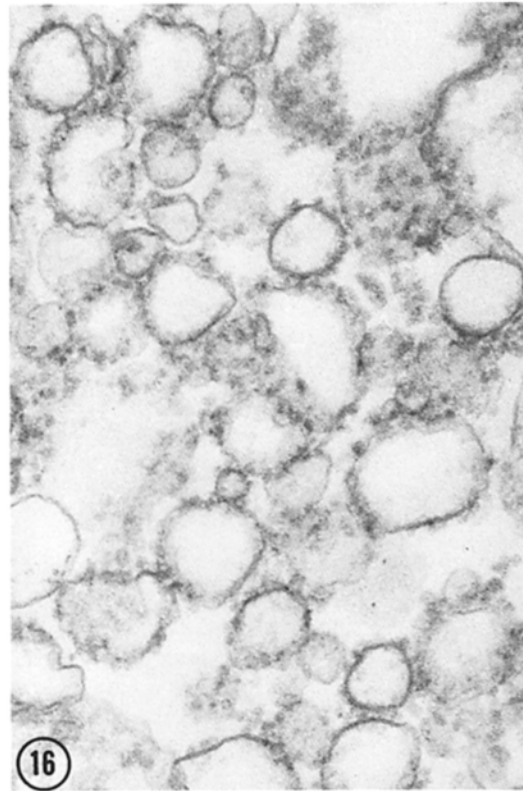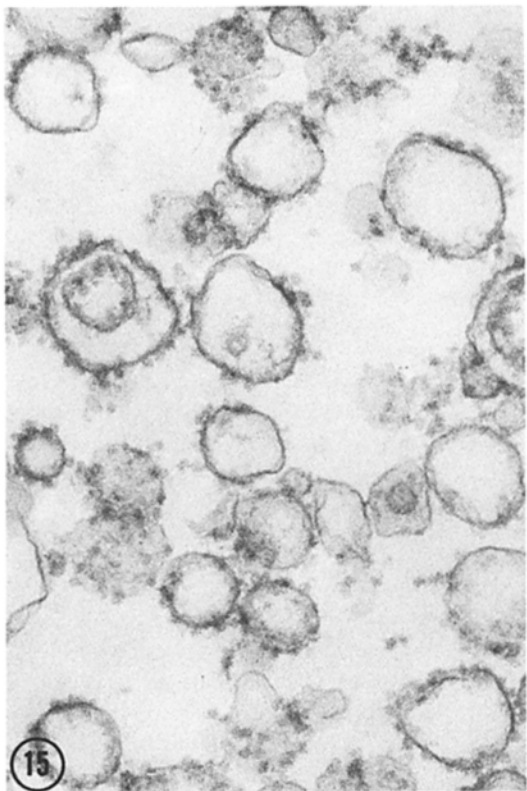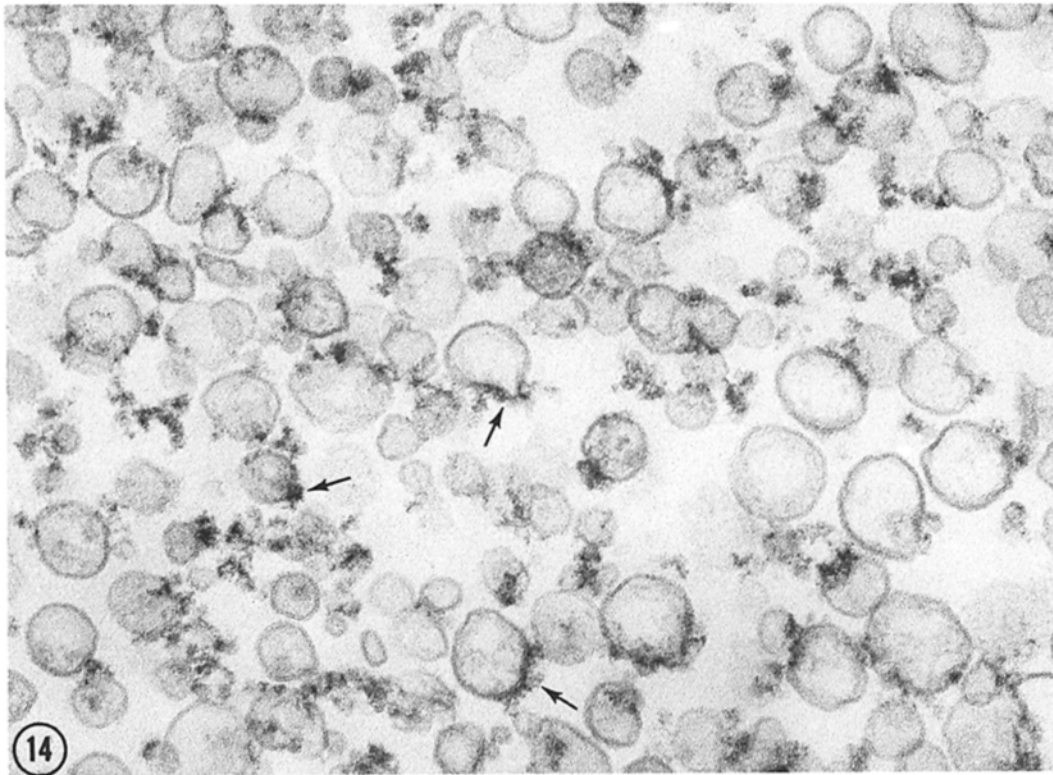
FIGURE 14   Smooth microsomes obtained from the 1.15–1.17 M region of the sucrose gradient in the Golgi isolation procedure and incubated for adenylate cyclase activity in the presence of 2 mM Pb^{++}. The majority of the vesicles present show reaction product located primarily along the cytoplasmic surface of their membranes (arrows). × 88,400.

FIGURES 15–16   Rough microsomes obtained from the pellet of the final spin in the Golgi isolation procedure and incubated for adenylate cyclase activity in the presence of 2 mM Pb^{++}. In Fig. 14 the ribosomes are more intensely stained and show up to better advantage, but no lead deposits are seen in either field. × 65,000.

TABLE III

Site of Cytochemical Localization of Reaction Product for Various Enzymes in Golgi Subfractions*

Enzyme	GF₁ and GF₂		GF₃	
	Membrane	Content	Membrane	Content
AMPase	+ (inside)	+	+ (outside)	–
AcPase	–	+	–	–
TPPase	+ (inside)	+	–	–‡
G-6-Pase	–	–	–	–§
AC	+ (outside)	–	+ (outside)	–

* Cytochemical incubations were carried out at 25-30°C for 30 min on unfixed Golgi fractions. Data on all enzymes except AC are taken from reference 7.

‡ A few (rare) reactive elements were seen which consisted of small VLDL-filled vacuoles of the type which predominate in GF₁ and GF₂.

§ Reaction product was not seen in morphologically identifiable Golgi elements. However, deposits were seen occasionally in GF₂ and slightly more frequently in GF₃ within small smooth vesicles which were presumed to represent contaminating smooth ER elements.

known in enzyme cytochemistry (cf. reference 12), because lead phosphate deposition depends on the efficiency of trapping of the released phosphate. The fact that the deposits are larger in the presence of 2 mM lead simply indicates that with this technique the higher lead concentration favors the capture and precipitation of released phosphate before it can diffuse away from its site of production.

Validity of the AMP-PNP, Lead Method

The validity of the lead nitrate method for the cytochemical demonstration of AC has recently been questioned by Lemay and Jarett (10) who reported: (a) that the AC activity of fat cell membranes was completely inhibited by 1×10^{-4} M lead and (b) that very low concentrations of lead $(2 \times 10^{-3}$ M) induced the nonenzymatic hydrolysis of AMP-PNP (to cAMP and AMP). However, our biochemical data on unfixed liver fractions indicate that considerable basal AC activity is retained in the presence of 1 and 2 mM lead (>80% and 25%, respectively) (Table I). Moreover, no evidence was found for the nonenzymatic hydrolysis of AMP-PNP by lead since little or no cAMP was detected by biochemical assay in the absence of fractions (Table II), and no reaction product was seen in any of the cytochemical controls. Cutler (4) has also presented biochemical data on fixed tissues indicating survival of considerable

AC activity in the presence of lead in three different systems—the slime mold, parotid gland, and blood platelets. The negative data of Lemay and Jarett in the face of the positive data obtained by Cutler and ourselves could be due to tissue differences, or to differences in the substrate (purity, method of storage, etc.), or to the fact that in the case of Lemay and Jarett the biochemical assays were not done under the same conditions as the cytochemical localizations. In the present work and in that of Cutler (4), an aliquot was removed directly from the incubation tube at the end of each cytochemical incubation and subjected to cAMP analysis.

Nature of Golgi and ER Adenylate Cyclase

The cytochemical results together with the data obtained by biochemical assay (2) have demonstrated the presence of AC in Golgi and ER membranes, as well as in the plasma membrane. The presence of AC in all these compartments raises the question of what it is doing there. The data do not give any indication as to the relationship between these activities i.e., whether they represent the same or different enzymes. It is conceivable that we are dealing with different enzymes with distinct functions, or, alternatively, that there is only one AC, the plasma membrane enzyme, which is synthesized in the ER and passes through the Golgi apparatus before being delivered to the plasma membrane. The location of AC along the cytoplasmic face of Golgi membranes, the increasing activity gradient from GF₃ to GF₁ (2) as well as the similar pattern of fluoride response between the activity in GF₁ (2) and that in the plasma membrane (15) are consistent with the possibility that there may be a biogenetic relationship between Golgi AC and plasma membrane AC. However, kinetic data are needed to resolve this question.

In conclusion, AC has been localized by EM cytochemistry to Golgi membranes and smooth ER membranes of rat liver. Reaction product is associated with the VLDL-filled secretory droplets as well as the dilated rims of the cisternal elements, and with the majority of the smooth ER vesicles. In all cases, reaction product is localized primarily or exclusively to the cytoplasmic face of the membrane.

REFERENCES

1. CHENG, H., and M. G. FARQUHAR. 1974. Presence of adenylate cyclase in Golgi fractions from rat liver. *J. Cell Biol.* **63:**58*a* (Abstr.).

2. CHENG, H., and M. G. FARQUHAR. 1976. Presence of adenylate cyclase activity in Golgi and other fractions from rat liver. I. Biochemical determination. *J. Cell Biol.* **70:**660-670.

3. CUTLER, L. S. 1974. Cytochemical localization of adenylate cyclase on the inner aspect of the plasma membrane of eucaryotic cells. *J. Cell Biol.* **63:**74*a* (Abstr.).

4. CUTLER, L. S. 1975. Comments on the validity of the use of lead nitrate for the cytochemical study of adenylate cyclase. *J. Histochem. Cytochem.* **23:**786-787.

5. EHRENREICH, J. H., J. J. M. BERGERON, P. SIEKEVITZ, and G. E. PALADE. 1973. Golgi fractions prepared from rat liver homogenates. I. Isolation procedure and morphological characterization. *J. Cell Biol.* **59:**45-72.

6. FARNHAM, C. J. M. 1975. Cytochemical localization of adenylate cyclase and 3′,5′-nucleotide phosphodiesterase in *Dictyostelium. Exp. Cell Res.* **91:**36-46.

7. FARQUHAR, M. G., J. J. M. BERGERON, and G. E. PALADE. 1974. Cytochemistry of Golgi fractions prepared from rat liver. *J. Cell Biol.* **60:**8-25.

8. HOWELL, S. L., and M. WHITFIELD. 1972. Cytochemical localization of adenyl cyclase activity in rat islets of Langerhans. *J. Histochem. Cytochem.* **20:**873-879.

9. JANDE, S. S., and P. ROBERT. 1974. Cytochemical localization of parathyroid hormone activated adenyl cyclase in rat kidney. *Histochemistry.* **40:**323-327.

10. LEMAY, A., and L. JARETT. 1975. Pitfalls in the use of lead nitrate for the histochemical demonstration of adenylate cyclase activity. *J. Cell Biol.* **65:**39-50.

11. LESKES, A., P. SIEKEVITZ, and G. E. PALADE. 1970. Differentiation of endoplasmic reticulum in hepatocytes. I. Glucose-6-phosphatase distribution *in situ. J. Cell Biol.* **49:**264-287.

12. MARCHESI, V. T., and G. E. PALADE. 1967. The localization of Mg-Na-K-activated adenosine triphosphatase on red cell ghost membranes. *J. Cell Biol.* **35:**385-404.

13. MORRÉ, D. J. 1971. Isolation of Golgi apparatus. *In* Methods in Enzymology. Vol. XXII. W. B. Jakoby, editor. Academic Press, Inc., New York. 130-148.

14. PERKINS, J. P. 1973. Adenyl cyclase. *In* Advances in Cyclic Nucleotide Research. Vol. 3. P. Greengard and G. A. Robison, editors. Raven Press, New York. 1-64.

15. POHL, S. L., L. BIRNBAUMER, and M. RODBELL. 1971. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. I. Properties. *J. Biol. Chem.* **246:**1849-1856.

16. RODBELL, M. 1971. In vitro assays of adenyl cyclase. *In* In Vitro Methods in Reproductive Cell Biology. E. Diczfalusy, editor. 3rd Karolinska Symposia on Research Methods in Reproductive Endocrinology. *Acta Endocrinol.* **67**(Suppl. 153):337-347.

17. RODBELL, M., L. BIRNBAUMER, S. L. POHL, and H. M. J. KRANS. 1971. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. V. An obligatory role of guanyl nucleotides in glucagon action. *J. Biol. Chem.* **246:**1877-1882.

18. RODBELL, M., and G. KRISHNA. 1974. Preparation of isolated fat cells and fat cell "ghosts"; Methods for assaying adenylate cyclase activity and levels of cyclic AMP. *In* Methods in Enzymology. Vol. XXXI. S. Fleischer and L. Packer, editors. Academic Press, Inc., New York. 103-114.

19. REIK, L., G. L. PETZOLD, J. A. HIGGINS, P. GREENGARD, and R. J. BARRNETT. 1970. Hormone-sensitive adenyl cyclase: cytochemical localization in rat liver. *Science (Wash. D. C.).* **168:**382-384.

20. SZMIGIELSKI, S. 1971. The use of dextran in phosphatase techniques employing lead salts. *J. Histochem.* **19:**505-507.

21. WAGNER, R. C., P. KREINER, R. J. BARRNETT, and M. W. BITENSKY. 1972. Biochemical characterization and cytochemical localization of a catecholamine-sensitive adenylate cyclase in isolated capillary endothelium. *Proc. Natl. Acad. Sci. U. S. A.* **69:**3175-3179.

22. WIDNELL, C. C. 1972. Cytochemical localization of 5′-nucleotidase in subcellular fractions isolated from rat liver. I. The origin of 5′-nucleotidase activity in microsomes. *J. Cell Biol.* **52:**542-558.

23. YOUNT, R. G., D. BABCOCK, W. BALLANTYNE, and D. OJALA. 1971. Adenylyl imidodiphosphate, an adenosine triphosphate analog containing a P-N-P linkage. *Biochemistry.* **10:**2484-2489.