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SUBCELLULAR DISTRIBUTION OF ADENYL CYCLASE AND PHOSPHODIESTERASE IN *ACANTHAMOEBA PALESTINENSIS*

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SUMMARY

1. The subcellular distributions of adenyl cyclase and phosphodiesterase were determined in *Acanthamoeba palestinensis*. The cells were homogenized and fractionated by a technique modified to preserve enzyme activity and monitored by electron microscopy.

2. The recovery of adenyl cyclase activity was highest in the microsomal fraction. Upon further fractionation of the microsomes, adenyl cyclase was concentrated by the rough endoplasmic reticulum fraction, and in only this fraction was the specific activity of the enzyme increased over the starting material.

3. The specific activity of adenyl cyclase was lower in enriched fractions of plasma membranes and mitochondria than in the homogenate, and the recoveries were very low. Further, these fractions revealed the presence of sufficient rough endoplasmic reticulum contamination to account for the adenyl cyclase activity present.

4. Most of the phosphodiesterase activity was soluble and was recovered in the post-microsomal supernatant.

INTRODUCTION

In mammalian tissues adenyl cyclase has been reported to be associated with plasma membranes[1–4] or plasma membrane-related systems, such as the T system of cardiac and skeletal muscles[5–8] and synaptic membranes of nerves[9]. In procaryotic cells the localization of adenyl cyclase has been less predictable. For example, in *Brevibacterium liquifaciens* the enzyme appears to be soluble[10], whereas in *Escherichia coli* it occurs in particulate fractions[11,12], although it can be solubilized[13].

In this communication we wish to report the results of studies on the subcellular distribution of adenyl cyclase in the soil amoeba, *Acanthamoeba palestinensis*, in which the enzyme appears to be associated primarily with an intracellular membrane system, the rough endoplasmic reticulum.

METHODS

Cell culture

A. palestinensis was cultured axenically in silicone-coated one liter Erlenmeyer flasks containing 500 ml of proteose peptone-glucose medium incubated at 29 °C with agitation (110 rev./min) on a rotary shaker[14,15]. Cells, derived from serial subcultures grown for about a year in the manner described above, were inoculated to give an initial concentration of approx. $2 \cdot 10^3$ cells/ml and grew exponentially with a 16-h generation time until about the fifth day of growth, as monitored with a Coulter Counter. On the sixth and seventh days cell growth was no longer exponential. Cultures were routinely grown for 6 or 7 days to yield a final concentration of approx. $1 \cdot 10^6$ or $2 \cdot 10^6$ cells/ml.

Cell fractionation

Cells were fractionated by the technique of Chlapowski and Band[16], modified to preserve the activity of adenyl cyclase (Fig. 1). Cultures were harvested by centrifugation at 280 $\times$ *g* for 10 min at room temperature. All subsequent steps of the cell fractionation were carried out at 0 to 4 °C. The harvested cells were resuspended in isolation medium (sucrose–TM) composed of 0.25 M sucrose containing 0.005 M Tris–HCl (pH 7.4) and 0.002 M $MgSO_4$ and harvested again by centrifugation at 700 $\times$ *g* for 10 min. The washed, packed cells were resuspended in about 2 vol. of isolation medium and homogenized with seven strokes in a 30 ml Potter-Elvehjem-type glass–Teflon grinder at 2500 rev./min. The following cell fractions were isolated in sequence in a Sorvall RC-2B centrifuge and an International B-60 ultracentrifuge.

Nuclear and plasma membrane fractions. After centrifugation of the homogenate

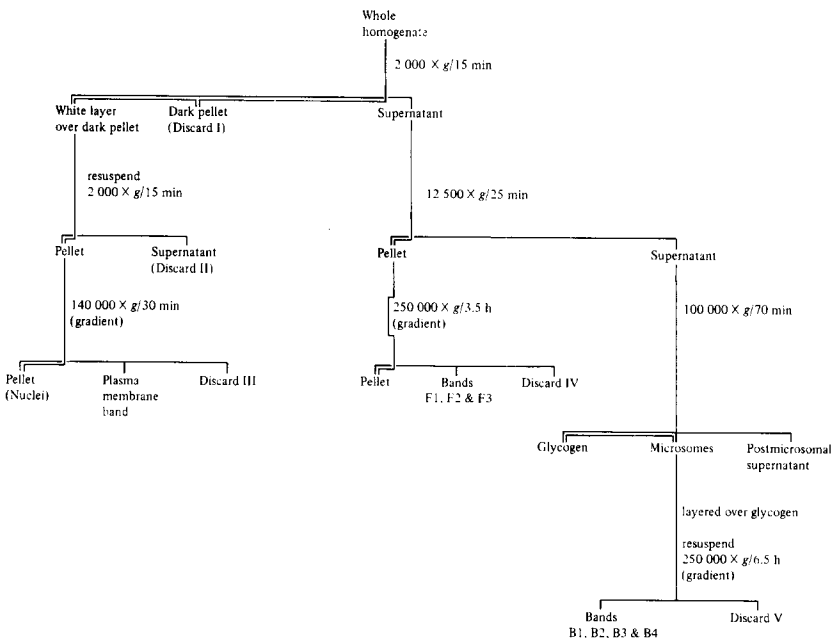

Fig. 1. A schematic representation of the cell fractionation technique.

at 2000 × *g* for 15 min in a Sorvall HB-4 swinging-bucket rotor, a two-layered pellet was observed. A dark brown pellet containing mainly unbroken cells was overlain by a loosely packed white layer containing nuclei and plasma membranes. This white layer was contaminated with microsomes and mitochondria. The 2000 × *g* supernatant was decanted for further subfractionation and the white layer (plasma membranes and nuclei) was carefully resuspended in isolation medium so as not to disturb the underlying dark pellet (Discard I). The resuspended white layer was washed by centrifugation at 2000 × *g* for 15 min in the Sorvall HB-4 rotor. Most of the mitochondrial and microsomal contaminants were removed by this step (Discard II). The washed pellet was resuspended in 1 vol. of 1.3 M sucrose, and 0.25 ml was layered over a 3.5 ml continuous sucrose–TM gradient extending from 1.3 to 2.0 M. The gradient was centrifuged at 140 000 × *g* for 30 min in an International SB-405 rotor.

At the end of this centrifugation, microsomal contaminants were floating near the top of the tube, the plasma membranes lay in two bands closely spaced about one-fourth of the way down the gradient, and the nuclei were in a translucent pellet at the bottom of the tube. The two bands were removed with a pipette and pooled. The remaining supernatant (Discard III) was decanted to expose the nuclear pellet.

12 500 × g fraction. The 2000 × *g* supernatant was centrifuged at 12 500 × *g* for 30 min in a Sorvall SS-34 fixed-angle rotor to sediment the so-called mitochondrial fraction. The supernatant was decanted and reserved. In experiments involving further fractionation, the 12 500 × *g* pellet was resuspended in 1 vol. of 1.04 M sucrose and 0.2 ml was layered over a 3.5 ml continuous sucrose gradient extending from 1.04 to 2.0 M. The gradient was centrifuged at 250 000 × *g* for 3.5 h, resulting in 3 bands floating in the middle of the tube and a pellet. The bands were removed by pipetting and the remaining solution was decanted (Discard IV) to expose the pellet.

Microsomal fraction. The 12 500 × *g* supernatant was placed in a 55 ml screw cap polycarbonate centrifuge tube and was centrifuged at 100 000 × *g* for 70 min in an International A-192 fixed-angle rotor. After centrifugation, the microsomal elements were loosely packed over a large, clear glycogen pellet. The postmicrosomal supernatant, along with the floating lipid layer, was carefully decanted and the loose microsomes aspirated off the glycogen pellet with a pipette.

About 0.1 ml of the microsomal fraction was layered over a 3.5 ml continuous sucrose gradient extending from 1.04 to 2.0 M and centrifuged for 6.5 h at 250 000 × *g*. This separated the fraction into four bands, and as much as possible of each was removed with a Pasteur pipette. The remaining material was decanted (Discard V).

Isolated fractions were either fixed for electron microscopy or used immediately for enzyme assays.

Microscopy

Cell fractions were resuspended and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pelleted by centrifugation at 30 000 × *g* for 30 min, post-fixed in 2% osmium tetroxide in 0.1 M phosphate buffer, dehydrated and embedded in Epon[17], as previously described[16]. Pellets were oriented so that the plane of thin sections included the entire thickness of the pellet.

Sections were cut on a Porter-Blum MT-1 ultramicrotome, placed on grids and stained with 4% aqueous uranyl acetate and lead citrate[18]. Specimens were examined with a Philips-300 electron microscope at 60 kV.

Adenyl cyclase assay. Carefully resuspended cell fractions were made to appropriate dilutions in a medium containing 1 mM $MgSO_4$, 2 mM glycylglycine (pH 7.4) and 1 mM dithiothreitol. Aliquots (0.5 ml) of the diluted cell fractions were added to an equal volume of a reaction mixture containing 48 μmoles Tris buffer, pH 7.4, 8 μmoles caffeine, 1.2 μmoles dithiothreitol and 4 μg bovine serum albumin. The reactions were started by adding 3.6 μmoles $MgSO_4$ and 2.4 μmoles ATP (pH 7.2), in a volume of 0.2 ml, and incubation was carried out on a shaking incubator at 30 °C for 15 min. The reactions were stopped by putting the tubes in a boiling water bath for 5 min, followed by cooling in ice.

To prevent possible interference by excess ATP in the assay, cyclic AMP was isolated from the reaction mixture by ion exchange chromatography. Cyclic[^{3}H]AMP (approximately 15 000 dpm) was added to each sample to monitor recovery. The samples were then centrifuged if necessary and applied to 0.4 cm × 6.0 cm Dowex-50 columns equilibrated with 0.05 N HCl. Following a 1 ml wash with water, the cyclic AMP was eluted in 3 ml of water, Tris buffer was added to a final concentration of 0.01 M, and cyclic AMP was measured by the assay of Gilman[19]. The recovery of cyclic AMP was determined by counting the cyclic[^{3}H]AMP in 0.1 ml aliquots of the final eluates in 15 ml of Bray's scintillation fluid. The isolation and assay for cyclic AMP were validated by incubating aliquots of the final eluates with purified phosphodiesterase[20].

Fractions used as sources of adenyl cyclase activity were routinely diluted so that the concentration of protein was rate-limiting and cyclic AMP accumulation was proportional to protein concentration. Reagent and enzyme blanks were included with every experiment and contained negligible cyclic AMP activity. The standard unit of enzymatic activity referred to herein represents the accumulation of 1 μmole of cyclic AMP per min.

Phosphodiesterase assay. Resuspended cell fractions were diluted in a medium containing 1 mM $MgSO_4$, 1 mM glycylglycine buffer (pH 7.4) and 1 mM mercaptoethanol. The reaction mixture consisted of 12 μmoles of Tris–HCl buffer (pH 7.4), 0.6 μmole $MgSO_4$, 30 nmoles cyclic AMP, and 0.3 nmole cyclic [^{3}H]AMP (New England Nuclear, Boston, Mass., 4.4 Ci/mmole) in 200 μl. To this were added 100 μl of the diluted cell fraction. Incubation was carried out on a shaking incubator at 30 °C. After 20 min, 100 μg of lyophilized *C. atrox* venom (Ross Allen Reptile Institute, Silver Spring, Fla) in 100 μl was added, and the incubation continued for another 10 min. *C. atrox* venom contains a potent 5'-nucleotidase, but is without effect on cyclic AMP. Reactions were stopped by the addition of 0.5 ml of a solution chilled to 4 °C and containing 12 mM EDTA, 200 μM adenosine, and 0.2 μC [^{14}C]adenosine and immediate transfer to an ice-water bath. The samples were chromatographed on 0.4 cm diameter columns containing 1 ml of a 1:2 slurry of Dowex-1 equilibrated with 0.01 M Tris (pH 7.4). An additional 4.1 ml of 0.01 M Tris followed the samples, and 1.0 ml aliquots were counted in 15 ml of Bray's scintillation fluid for [^{3}H]adenosine and [^{14}C]adenosine.

Enzyme preparations were diluted such that hydrolysis of cyclic AMP did not exceed 15% of the total substrate. Under these conditions, the activity was directly proportional to protein concentration. The standard unit of enzyme activity is defined as 1 μmole of [^{3}H]adenosine produced per min.

RESULTS

The membrane systems and membrane-bounded organelles of the soil amoeba *A. palestinensis* were fairly typical of those normally found in eucaryotic cells and included endoplasmic reticulum, Golgi-like membranes, plasma membrane, food vacuoles (*i.e.*, phagocytic or digestive vacuoles), mitochondria, a contractile vacuole, a nucleus, and collapsed vesicles. Collapsed vesicles were usually small (approx. 150 nm in diameter), cup-like vesicles whose membranes, unlike other intracellular membranes, were identical in thickness and staining characteristics to plasma and food vacuole membranes, which they have been reported to be derived from and/or are precursors of[16].

Most of the phosphodiesterase activity was in the soluble fraction, 75% of the activity having been recovered in the postmicrosomal supernatant (Table I). This supernatant was also the only fraction to show increased specific activity of phosphodiesterase over that of the homogenate.

The data in Table I indicate that most of the adenyl cyclase was associated with particulate fractions since only 1% of the activity was recovered in the postmicrosomal supernatant. Only 14% of the adenyl cyclase activity was recovered in the 2000 × *g* pellet, and the specific activity of this fraction was lower than that of the homogenate. This was surprising since this pellet has been shown to contain most of the plasma membranes[16]. In fact, the bulk of the adenyl cyclase activity was recovered in the microsomal pellet (37%), the 12 500 × *g* pellet (21%), and the discards (20%). However, only in the microsomes and in the 12 500 × *g* pellet was an enrichment of adenyl cyclase specific activity apparent.

Further fractionation of the 2000 × *g* pellet into plasma membranes, nuclei

TABLE I

ADENYL CYCLASE AND PHOSPHODIESTERASE IN MAJOR FRACTIONS OF *A. palestinensis*

The homogenization, fractionation and enzymatic assays were performed exactly as described in Methods. Each fraction was assayed a minimum of four times. The data presented here are from one cell preparation and are very representative of the total of eleven experiments

Fraction	*Protein*		*Adenyl cyclase*			*Phosphodiesterase*		
	mg	*% recovered*	*munits**	*% recovered*	*Spec. act.*	*munits***	*% recovered*	*Spec. act.*
Homogenate	1335.0	—	88.0	—	0.07	1466.7	—	1.10
2000 × *g* pellet	361.8	27.1	12.6	14.4	0.04	19.0	1.3	0.05
12 500 × *g* pellet	117.0	8.8	18.6	21.1	0.16	31.6	2.2	0.27
Microsomes (100 000 × *g* pellet)	239.8	18.0	32.7	37.1	0.14	65.7	4.5	0.27
Postmicrosomal supernatant	455.6	34.1	0.9	1.0	0.002	1100.5	75.0	2.42
Glycogen	19.2	1.4	0.9	1.0	0.05	17.3	1.2	0.9
Discards I and II	215.0	16.1	20.0	22.7	0.09	126.7	8.6	0.59
Total		105.5		97.3			92.8	

* 1 munit of adenyl cyclase activity is defined as the accumulation of 1 nmole of cyclic AMP per min.
** 1 munit of phosphodiesterase activity is defined as 1 nmole of [^{3}H]adenosine produced per min.

TABLE II

ADENYL CYCLASE IN FRACTIONS OF 2000 × *g* PELLET

The gradient centrifugation of the fresh 2000 × *g* pellet was carried out as described in Methods. Each fraction was assayed a minimum of four times, and these data are the values found in one experiment which was representative of five.

Fraction	*Protein*		*Adenyl cyclase*		
	mg	*% recovered*	*munits*	*% recovered*	*Spec. act.*
2000 × *g* pellet	361.8	—	12.6	—	0.04
Plasma membranes	65.9	18.2	2.6	20.6	0.04
Nuclei	32.1	8.9	0.4	3.4	0.01
Discard III	144.7	40.0	10.4	82.5	0.07
Total		67.1		106.5	

and Discard III (the latter containing mostly mitochondria and microsomes) showed that most of the adenyl cyclase activity of this fraction was associated with the discard (Table II). The plasma membrane fraction used in these studies was contaminated with mitochondria and rough endoplasmic reticulum (Fig. 2), since no washing steps were carried out[16]. In fact, when the plasma membrane fraction was washed once by low-speed centrifugation in 0.25 M sucrose-TM, no adenyl cyclase activity remained. This may have been due to the loss of the mitochondrial and microsomal contaminations, or to inactivation of the adenyl cyclase system.

In electron micrographs, the four microsomal fractions (Fig. 3) appeared quite similar to those described in a previous report[16], despite the fact that in this study microsomes were not washed prior to gradient centrifugation. Most of the adenyl cyclase activity (60.6%) was recovered in Band 4, and the specific activity of the

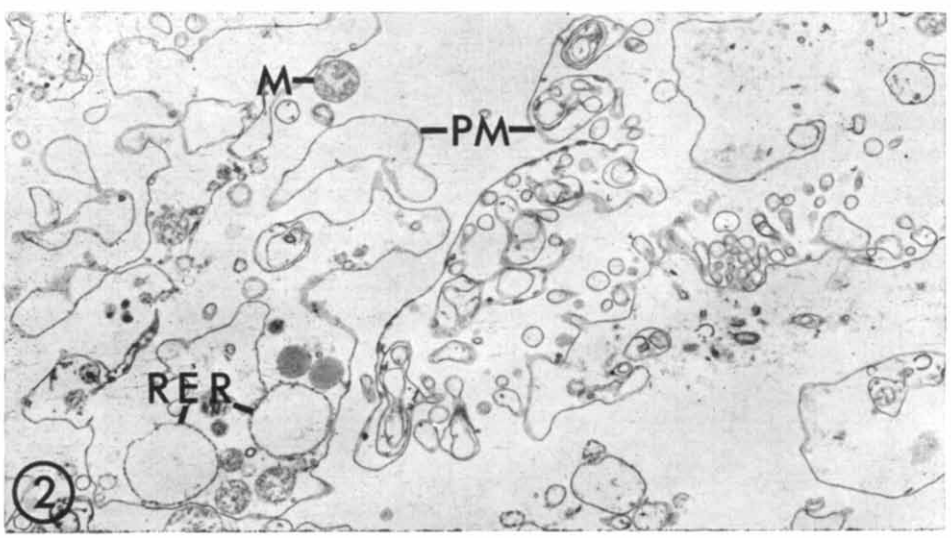

Fig. 2. Electron micrograph of a section through a pellet of the plasma membrane fraction. Vesicles and large fragments of plasma membrane (PM) are numerous. In addition, lipid droplets, rough endoplasmic reticulum (RER), and mitochondria (M) in various stages of swelling contaminate the fraction (0.04 units adenyl cyclase/g protein). Magnification × 13 600.

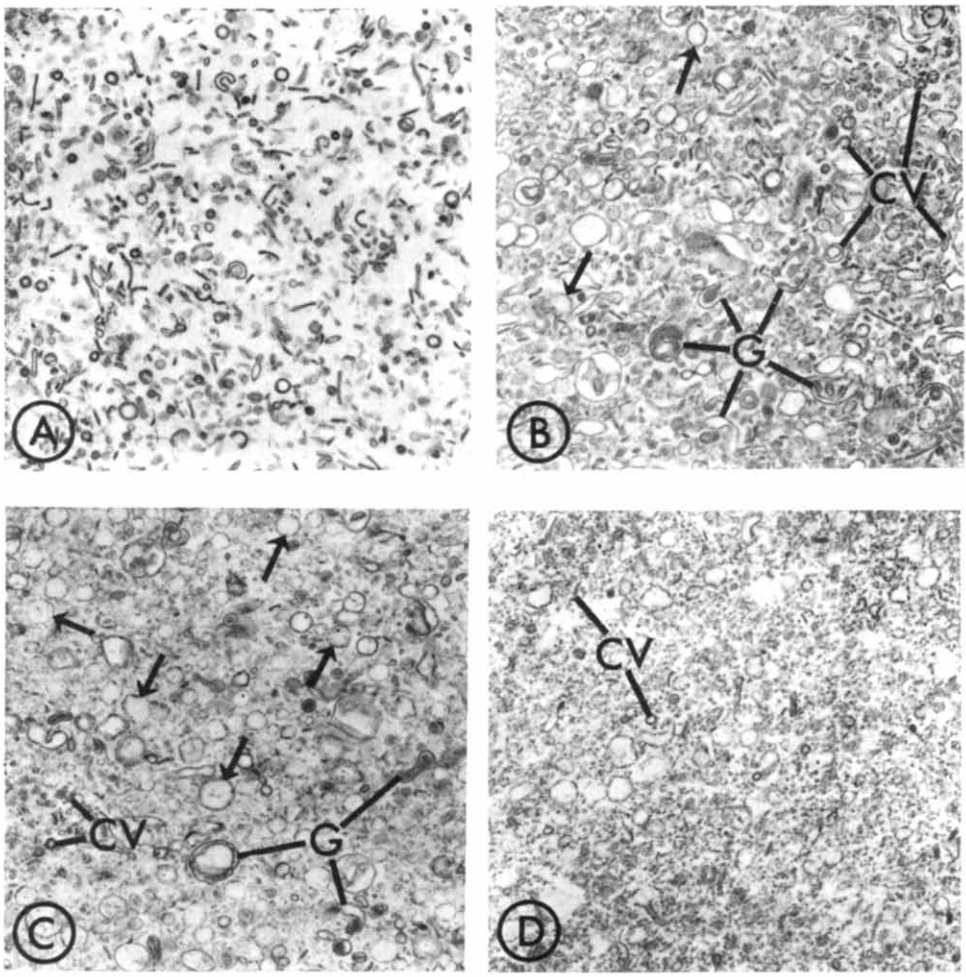

Fig. 3. Electron micrographs of sections through pellets of Bands 1 (A), 2 (B), 3 (C), and 4 (D) of the microsomal subfractions. A. The entire pellet of Band 1 is composed of collapsed vesicles (0.06 units adenyl cyclase/g protein). B. Band 2 is enriched with Golgi-like membranes (G). Collapsed vesicles (CV) are found throughout the band and a small amount of rough endoplasmic reticulum (arrows) is also present (0.14 units adenyl cyclase/g protein). C. Band 3 is a mixture of collapsed vesicles, Golgi-like membranes and rough endoplasmic reticulum (0.26 units adenyl cyclase/g protein). D. Band 4 is composed almost entirely of rough endoplasmic reticulum. A few collapsed vesicles can be seen (0.43 units adenyl cyclase/g protein). Magnification × 20 000.

enzyme increased in this band (Table III). It was composed almost exclusively of rough endoplasmic reticulum, except for a few collapsed vesicles (Fig. 3). It seemed unlikely that these vesicles were responsible for the enzyme activity in Band 4, since Band 1, which was composed almost entirely of collapsed vesicles, had a low adenyl cyclase specific activity and less than 2% of the total activity. Although a large amout (47.6%) of enzyme activity was recovered in Discard V, the specific activity of this fraction was lower than that of the starting material.

Since 21% of the adenyl cyclase activity of the homogenate was recovered in

TABLE III

ADENYL CYCLASE IN FRACTIONS OF MICROSOMAL (100 000 × *g*) PELLET

The experimental details are in Methods. These data are from one of six experiments in which the recoveries and changes in specific activity were very similar.

Fraction	*Protein*		*Adenyl cyclase*		
	mg	*% recovered*	*munits*	*% recovered*	*Spec. act.*
Microsomes (100 000 × *g* pellet)	44.4	—	6.06	—	0.23
Band 1 (collapsed vesicles)	1.9	4.3	0.11	1.8	0.06
Band 2 (Golgi-enriched)	2.0	4.5	0.29	4.7	0.14
Band 3 (membrane mixture)	0.5	1.1	0.12	2.0	0.26
Band 4 (rough endoplasmic reticulum)	8.7	19.5	3.67	60.6	0.43
Discard V	18.8	42.3	2.88	47.6	0.15
Total		71.7		116.6	

the 12 500 × *g* pellet, the pellet was further fractionated on a sucrose gradient as described in Methods. The entire gradient contained diffuse material, a pellet, and three fairly distinct bands in the middle third of the tube. Most of the adenyl cyclase activity was present in Band F3 (Table IV), which was highly enriched in rough endoplasmic reticulum (Fig. 4). Its specific activity (0.50 units/g protein) was the highest of all the fractions of the 12 500 × *g* pellet, and was enriched 7-fold over the original homogenate. Band F1, which was enriched with intact mitochondria, had a lower specific activity than the starting material and accounted for only 12.5% of the total activity. Little activity was recovered in Band F2 and none in the pellet. Although Discard IV did not show the enrichment in specific activity that Band F3 did, its content of adenyl cyclase was very high. However, these experiments were complicated by an apparent activation of adenyl cyclase, for greater than 100% of the starting activity was present in this fraction when assayed immediately following the centrifugation.

TABLE IV

ADENYL CYCLASE IN FRACTIONS OF 12 500 × *g* PELLET

The details of the experiments are described in Methods. The data presented here are from one of four experiments in which the distributions and specific activity changes of adenyl cyclase were very similar. Each fraction was assayed a minimum of four times. N.D., not detectable.

	Protein		*Adenyl cyclase*		
	mg	*% recovered*	*munits*	*% recovered*	*Spec. act.*
12 500 × *g* pellet	35.6	—	4.0	—	0.11
Band F1	6.2	17.4	0.5	12.5	0.08
Band F2	2.0	5.6	0.17	4.3	0.08
Band F3	4.0	11.2	2.01	50.3	0.50
Pellet	0.4	1.1	N.D.	0	—
Discard IV	17.9	50.2	5.15	128.8	0.29
Total		85.5		195.9	

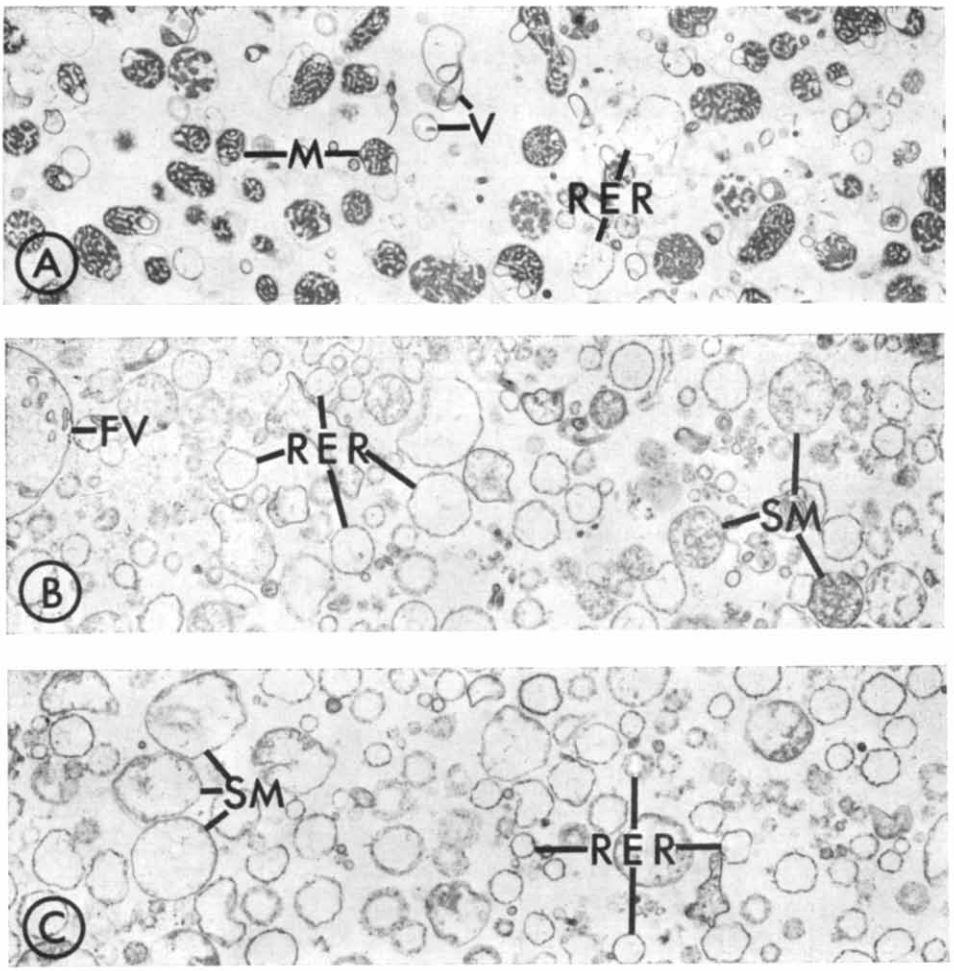

Fig. 4. Electron micrographs of sections through pellets of Bands F1 (A), F2 (B), and F3 (C) of the 12 500 × *g* pellet subfractions. A. Intact mitochondria (M) are enriched in band F1, although small vesicles (V) and some rough endoplasmic reticulum (RER) are present (0.08 units adenyl cyclase/g protein). B. Swollen mitochondria (SM), food vacuoles (FV) and a large amount of rough endoplasmic reticulum are present in band F2 (0.08 units adenyl cyclase/g protein). C. Band F3 is highly enriched with rough endoplasmic reticulum. Some swollen mitochondria can also be seen (0.50 units adenyl cyclase/g protein). Magnification × 20 000.

DISCUSSION

The enzymatic activity, ultracentrifugal and electron microscopic data presented here strongly suggest that the primary association of adenyl cyclase in *A. palestinensis* is with the rough endoplasmic reticulum. Although some activity was associated with other fractions, most notably the 12 500 × *g* pellet, electron micrographs revealed sufficient contamination of these fractions with rough endoplasmic reticulum to account for the observed enzymatic activity. This also appeared to be true for the plasma membrane fraction. However, it is possible that the incubation

conditions used for determining adenyl cyclase activity, while apparently optimal for the enzyme in the rough endoplasmic reticulum, were not suitable for the expression of its activity in the plasma membranes.

The specific activities of adenyl cyclase in the microsomal fractions increased from Band 1 through Band 4 (Table III), just as the frequency of appearance of rough endoplasmic reticulum increased in electron micrographs (Fig. 3). In this respect it was interesting that the ratio of RNA to protein, used as an indicator of the presence of rough endoplasmic reticulum, has been reported to increase from Band 1 to Band 4 in a manner similar to the adenyl cyclase specific activity[16]. This, in addition to the morphological data, strongly suggests a relationship between the presence of adenyl cyclase and the presence of rough endoplasmic reticulum.

With these observations in mind, it was surprising that little activity was found in the nuclear fraction; since the nuclear envelope is often considered to be similar to, if not continuous with, cytoplasmic rough endoplasmic reticulum. Three of the several possible explanations for the low activity in the nuclear fraction include: (a) the amount of nuclear envelope relative to rough endoplasmic reticulum is very low in these cells; (b) the nuclear envelope has no adenyl cyclase; or (c) adenyl cyclase in the nuclear envelope is denatured or inhibited by unknown factors.

Our initial experiments demonstrating adenyl cyclase activity in the microsoma fraction (Table I) suggested that the enzyme might be associated with collapsed vesicles, which may be derived from or give rise to plasma membrane[16]. However, this possibility is excluded by the microsomal fractionation experiments which demonstrated that the highest concentration of collapsed vesicles was found in Band 1, which had less than 2% of the total microsomal adenyl cyclase and a very low specific activity.

The significance of the localization of adenyl cyclase of *A. palestinensis* in the rough endoplasmic reticulum is unclear at this time, just as it is unclear what role the system might play in the organism. However, based on our present understanding of mammalian and bacterial systems, it seems likely that adenyl cyclase in this soil amoeba represents part of a mechanism for adjusting to environmental changes. Indeed, Raizada and Krishna Murti[21] have presented data suggesting that in *Hartmanella culbertsoni*, a similar soil amoeba, cyclic AMP is involved in the control of encystment. Further, based on models provided by higher organisms, it is likely that in *A. palestinensis* cyclic AMP will also be found to be involved in the control of a number of metabolic activities, for example, glycogenolysis, glycogen synthesis, and lipolysis.

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