CYTOCHEMICAL LOCALIZATION OF 5′-NUCLEOTIDASE IN SUBCELLULAR FRACTIONS ISOLATED FROM RAT LIVER

I. The Origin of 5′-Nucleotidase Activity in Microsomes

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ABSTRACT

A procedure has been developed for the cytochemical localization of 5′-nucleotidase in isolated, unfixed, rat liver microsomes. Membranes were incubated with adenosine 5′-phosphate (5′-AMP) and $Pb(NO_3)_2$ and then isolated on sucrose density gradients: all the phosphate released was recovered with the membranes by this procedure. Adenosine 2′-phosphate (2′-AMP) and adenosine 3′,5′-cyclic phosphate (3′,5′-AMP) were shown to be competitive inhibitors, but not substrates, for purified 5′-nucleotidase and were employed to determine the specificity of the cytochemical reaction. It was found that the incubation conditions for the cytochemical assay did not affect the specificity of 5′-nucleotidase. Microsomes incubated as controls with Pb^{2+}, or Pb^{2+} and 2′-AMP or 3′,5′-AMP were of the same density, although slightly denser than microsomes incubated without Pb^{2+}, and were unassociated with lead precipitate when examined by electron microscopy; microsomes incubated with Pb^{2+} and 5′-AMP were much denser and were stained heterogeneously with lead phosphate when examined by electron microscopy. Precipitates formed artificially from Pb^{2+} and inorganic phosphate did not resemble the reaction product. Microsomes were, therefore, separated on sucrose gradients and the subfractions were examined cytochemically. Lead precipitates were associated with the majority of rough-surfaced vesicles, and the reaction product was distributed heterogeneously in all fractions. Vesicles which stained like the membranes of the bile canaliculi in isolated plasma membranes were observed in the lightest subfraction. The reaction product was localized on the outside surface of the microsomal membranes, and was solubilized by low concentrations of ethylenediaminetetraacetic acid. It is concluded that 5′-nucleotidase is present in the endoplasmic reticulum and that the microsome fraction contains, in addition, vesicles derived from the plasma membrane.

INTRODUCTION

5′-Nucleotidase is highly concentrated in the plasma membrane of the hepatocyte, as determined by direct analysis of isolated membrane fractions (1) or by cytochemical procedures (2–4). Although 40–60% of the activity in homogenates is found in microsomal fractions (4), two recent reports (5, 6) have shown that a major part of this activity is localized in a specific fraction of vesicles of low density (probably derived from the bile canaliculi of the plasma membrane): this has been interpreted

to suggest (5, 6) that 5′-nucleotidase could be absent from the endoplasmic reticulum.

5′-Nucleotidase may be purified from both microsomal and plasma membrane fractions of rat liver (7), and some activity is recovered in all microsomal subfractions (5–10). In the course of studies designed to elucidate the site of synthesis and mechanism of intracellular transport of plasma membrane proteins, it became necessary to establish the precise subcellular localization of this enzyme. It was considered of particular importance to determine whether any of the activity of microsomes is present in membranes derived from the endoplasmic reticulum. The enzyme has, therefore, been studied by electron microscope cytochemistry, in a manner analogous to that described for the adenosine triphosphatase of erythrocyte ghosts (11) and for glucose-6-phosphatase of rat liver microsomes (12, 13).

A series of reports (14–18) have emphasized that there are numerous artifacts which may be observed when cytochemical reactions, which involve lead phosphate as a reaction product, are carried out using fixed tissue. Furthermore, since a variety of phosphatase activities are associated with hepatic membranes (19, 4), it is essential to establish the specificity of the reaction. In order to avoid the loss of enzymatic activity caused by fixation, and the problems caused by substrate permeability in intact cells, the results to be described here, therefore, have been obtained using unfixed, isolated cell fractions. The procedure was developed to ensure (*a*) that only inorganic phosphate (P_i)[1] liberated by the enzyme was associated with the membrane after the cytochemical reaction, and (*b*) that the staining observed could only be caused by specific 5′-nucleotidase activity. By carrying out biochemical experiments in parallel, it has been possible to provide strong supporting evidence for the cytochemical observations. Some of these results have been presented in a preliminary communication (20).

MATERIALS AND METHODS

Materials

Nucleotides were obtained from P-L Biochemicals Inc. (Milwaukee, Wis.). Isopropylidene adenosine was obtained from Aldrich (Cedar Knolls, N. J.), ^{32}P-phosphate (carrier-free) from New England Nuclear Corp. (Boston, Mass.), ^{210}Pb (SA 8.2 mCi/mg) from Amersham/Searle Corp. (Arlington Heights, Ill.), and special enzyme grade Tris from Schwarz/Mann (Orangeburg, N. Y.). All other chemicals were obtained from commercial sources and were of analytical grade when available. ^{32}P adenosine 5′-phosphate (5′-AMP) was prepared by the procedure of Symons (21) and purified by chromatography on Dowex-50 H^+ (Dow Chemical Co., Midland, Mich.), followed by chromatography on Dowex-1 Cl^-. Fresh preparations had a specific activity of 0.2 mCi/μmole and contained ~0.1% P_i.

Cell Fractionation

All operations were carried out in a cold room at 0–4°C, Microsomes and plasma membranes were isolated by procedures described elsewhere (22). Before incubation for cytochemical experiments, microsomes were washed once by resuspension and recentrifugation in 0.25 M sucrose, 10 mM Tris-acetate pH 7.5 (3 ml/g liver). Plasma membranes were washed in the same buffer, with the addition of 0.1 mM $Mg(NO_3)_2$ (5 ml/g liver). Microsomes were subfractionated using a linear density gradient from 1.1 to 1.6 M sucrose with a 1.75 M sucrose cushion at the bottom. All sucrose solutions contained 10 mM Tris-acetate pH 7.5. Microsomes corresponding to 750 mg liver were suspended in 3 ml 0.25 M sucrose, layered on the gradient, and centrifuged for 12 hr at 25,000 rpm in the Spinco SW25.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Samples were collected from the bottom of the tube, and the gradient was divided into four fractions of decreasing density: fractions I and II (the densest) consisted of rough microsomes, fraction IV (the least dense) of smooth microsomes, whereas fraction III contained both types of vesicle. After pooling fractions from three gradients, the membranes were obtained as a pellet by diluting the suspensions with water and centrifuging at 78,000 *g* for 2 hr in the Spinco 30 rotor. Each pellet was finally suspended in 1 ml 0.25 M sucrose, and appropriate samples were taken for biochemical and cytochemical assay.

Biochemical Assay of 5′-Nucleotidase

The assay for 5′-nucleotidase was routinely carried out in a total volume of 1 ml; incubation media are described in the legends to the figures and tables. Samples of 0.3 ml were withdrawn at appropriate time intervals and added to 0.3 ml 20% trichloroacetic acid (TCA). P_i was analyzed after removal of protein by centrifugation.

For enzyme kinetic studies, the assay measured the release of $^{32}P_i$ from ^{32}P 5′-AMP. The incubation contained 0.1 M Tris-Cl pH 8.5, 4 mM $MgCl_2$, various

[1] *Abbreviations:* 2′-AMP, adenosine 2′-phosphate; 3′,5′-AMP, adenosine 3′,5′-cyclic phosphate; 5′-AMP, adenosine 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate; TCA, trichloroacetic acid.

concentrations of substrate and inhibitor, and 4–10 mμg 5′-nucleotidase purified from microsomes (7). Samples of 0.3 ml were withdrawn at time intervals from 5 to 30 min and added to 0.7 ml 0.42% ammonium molybdate in 1 N H_2SO_4. Phosphomolybdate was extracted into isoamyl alcohol using successive portions of 0.5, 0.5, and 1.0 ml, and the radioactivity in the combined extracts was determined in a Beckman DPM 100 (Beckman Instruments, Inc., Fullerton, Calif.) scintillation counter after the addition of 2.5 ml Beckman D scintillation fluid. Under these conditions the reaction was linear with respect to both time and enzyme concentration up to 20 min, even at the lowest substrate concentrations.

Cytochemical Assay of 5′-Nucleotidase

Incubations were generally carried out in a volume of 3–5 ml in a medium containing 0.1 M Tris-acetate pH 7.5, 1 mM 5′-AMP, 1 mM $Mg(NO_3)_2$, and 1 mM $Pb(NO_3)_2$. All solutions for the cytochemical incubation medium were filtered through a 0.45 μ Millipore filter (Millipore Corporation, Bedford, Mass.) in order to remove dust and other particles which could form foci for lead precipitation, and the incubations were carried out in acid-washed tubes. Microsomes, microsome subfractions, or plasma membranes were added at a concentration such that not more than 40% of the 5′-AMP was hydrolyzed during 30 min incubation at room temperature. Reactions were stopped by cooling rapidly to 0°C in a salt-ice bath, and the entire incubation medium was layered over a density gradient formed from successive layers of 5 ml of 2.45, 2.2, 1.9, 1.6, and 1.35 M sucrose. All sucrose solutions contained 10 mM Tris-acetate pH 6.6, in order to permit the complete separation of the membranes from unreacted substrate. After centrifugation for 12 hr at 22,500 rpm in the Spinco SW25 rotor, the band or pellet containing the membranes was collected and diluted with water to lower the sucrose concentration to about 0.3 M. The membranes were centrifuged at 30,000 *g* for 20 min in a Sorvall RC2B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.), suspended in 1 ml 0.1 M sodium cacodylate buffer pH 7.2 containing 10 mM $MgCl_2$, and fixed in suspension by the addition of 1 ml 2% osmium tetroxide. This fixation procedure resulted in the best morphological preservation of both ribosomes and membranes in the preparations. After 4 hr at 0°–4°C, the membranes were obtained as a pellet by centrifugation at 40,000 rpm in the Spinco SW50 rotor, using cellulose nitrate tubes. The intact pellet was dehydrated in a series of graded ethanols, followed by propylene oxide, and embedded in Epon-Araldite. Sections were cut with a Porter-Blum microtome provided with diamond knives, so as to permit the examination of the entire depth of the pellet. Sections were examined in a Philips EM 300 electron microscope operated with a 30 μ objective and an anticontamination device. Unstained sections were examined at 60 kv, and sections stained with uranyl acetate and lead citrate at 80 kv. Staining the sections was found to have no effect on the localization of the reaction product, but markedly enhanced the contrast of the ribosomes. The author is very grateful for the skilled collaboration of Mr. James Apicella in the electron microscopy.

Analytical Procedures

P_i was estimated by the procedure of Chen et al. (23), and protein by that of Lowry et al. (24).

RESULTS

Development of the Cytochemical Incubation Medium

The routine assay for 5′-nucleotidase was carried out at 37°C in the presence of 10 mM $MgCl_2$, 10 mM 5′-AMP, and 0.1 M Tris-Cl pH 8.5. In preliminary experiments designed to determine conditions necessary to avoid the formation of nonspecific lead deposits, it was found that best results were obtained by reducing the 5′-AMP concentration to 1 mM and by substituting $Mg(NO_3)_2$ for $MgCl_2$ and Tris-acetate pH 7.5 for Tris-Cl pH 8.5. The preservation of the structure of the unfixed membranes was much improved by carrying out the incubation at room temperature (22°–25°C) instead of 37°C.

The effect of lead ion concentration on the activity of 5′-nucleotidase under the conditions of the cytochemical reaction is shown in Fig. 1. Lead ions were inhibitory at all concentrations, and at 1 mM Pb^{2+} the reaction was inhibited 55–60%. Similar results were obtained when the inhibition of purified 5′-nucleotidase by lead ions was studied.

Controls for the Cytochemical Procedure

The most satisfactory control for determining the specificity of the cytochemical reaction is to carry out the incubation in the presence of analogs of 5′-AMP which are not substrates for purified 5′-nucleotidase. Fig. 2 shows the Lineweaver-Burk plot from a kinetic analysis of the inhibition caused by adenosine 2′-phosphate adenosine (2′-AMP) and 3′,5′-cyclic phosphate (3′,5′-AMP). Both substrate analogs were clearly competitive inhibitors of 5′-nucleotidase. The results shown in Fig. 2 were obtained using enzyme purified from

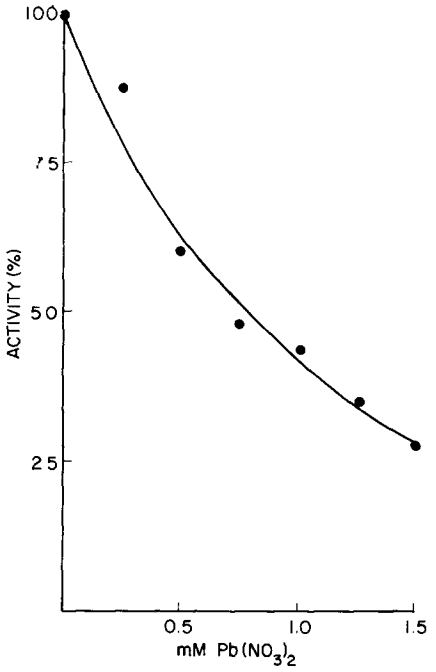

FIGURE 1 Effect of lead ion concentration on the activity of microsomal 5′-nucleotidase. The reaction was carried out at 22°C in 0.1 M Tris-acetate pH 7.5, 1 mM AMP, and 1 mM $Mg(NO_3)_2$. The specific activity of the control was 0.68 μmole phosphate released/30 min per mg protein.

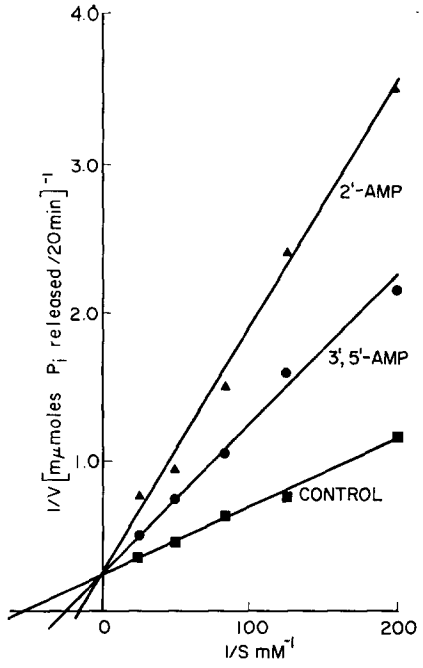

FIGURE 2 Kinetic analysis of the inhibition of 5′-nucleotidase purified from microsomes by 2′-AMP and 3′,5′-AMP. The reaction was carried out, as described in the text, with an enzyme concentration of 6.5 mμg/ml. ■——■, no inhibitor; ●——●, 0.5 mM 3′,5′-AMP; ▲——▲, 5 mM 2′-AMP. The Km for 5′-AMP was 0.02 mM, the Ki for 3′,5′-AMP was 0.455 mM, and the Ki for 2′-AMP was 1.66 mM.

microsomes and one inhibitor concentration. The same results were obtained at different inhibitor concentrations, and when the enzyme purified from plasma membranes was investigated.

Table I shows that, within the limits of the sensitivity of the assay, neither 2′-AMP nor 3′,5′-AMP was a substrate for 5′-nucleotidase purified from microsomes. In other experiments, it was shown by chromatographic analysis of the reaction products that both substrate analogs were unchanged after incubation with the enzyme. The same properties were also shown by the enzyme purified from plasma membranes.

Both 2′-AMP and 3′,5′-AMP were substrates for "nonspecific phosphatase"[2] present in the homogenate, in that more phosphate was released from the analogs than from β-glycerophosphate at the pH used in the cytochemical medium (Table I). In both the standard and cytochemical assay conditions, small amounts of phosphate were released by microsomes both in the absence of substrate and in the presence of 2′-AMP and 3′,5′-AMP. However, this activity was never more than a few per cent of that observed with 5′-AMP, showing that the altered pH and temperature and the presence of lead in the cytochemical incubation medium caused no major change in the properties of 5′-nucleotidase (Table I).

The other requirements, before meaningful cytochemical observations could be attempted, was to develop a technique by which only free phosphate liberated by the enzyme remained associated with the membranes to be examined by electron microscopy. These experiments were carried out by running cytochemical reactions with ^{32}P 5′-AMP. Membranes recovered from the incubation medium by centrifugation contained all the P_i liberated by the reaction, but about half the unreacted 5′-AMP as well. By using the sucrose density gradient procedure described in Ma-

[2] In this paper, nonspecific phosphatase is arbitrarily defined as release of phosphate not caused by specific 5′-nucleotidase activity.

TABLE I

Hydrolysis of Phosphate Esters by Rat Liver Homogenates, Microsomes, and 5′-Nucleotidase

	μmoles P_i released/30 min per mg protein (mean values from two experiments)			
	Homogenate	Microsomes		5′-Nucleotidase
Substrate	37°C, pH 7.5*	22°C, pH 7.5‡	37°C, pH 8.5§	37°C, pH 8.5‖
5′-AMP	0.705	0.610	3.2	2470
2′-AMP	0.102	0.011	0.017	<0.1
3′,5′-AMP	0.128	0.005	0.036	<0.1
β-Glycerophosphate	0.056	¶	¶	¶
None	0.098	0.009	0.028	0

* Substrate concentration 10 mM, 0.1 M Tris-acetate buffer, 10 mM $MgCl_2$.

‡ Substrate concentration 1 mM, 0.1 M Tris-acetate buffer, 1 mM $Mg(NO_3)_2$, 1 mM $Pb(NO_3)_2$.

§ Substrate concentration 10 mM, 0.1 M Tris-Cl, 10 mM $MgCl_2$.

‖ Substrate concentration 10 mM, 0.1 M Tris-Cl, 10 mM $MgCl_2$.

¶ Not assayed.

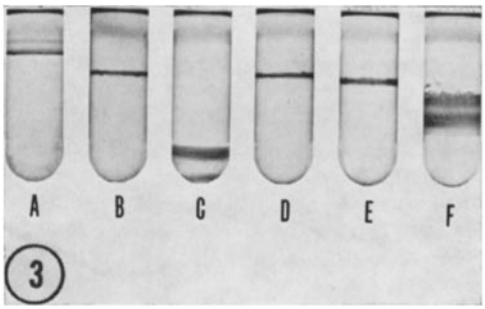

FIGURE 3 Isolation of microsomes on sucrose density gradients after cytochemical reaction. Approximately 2 mg microsomal protein were loaded on each gradient, as described in Materials and Methods for the cytochemical procedure. Tube A, unincubated diluted to 5.0 ml with 0.25 M sucrose; tube B, incubated at 25°C for 20 min in 5.0 ml cytochemical medium without substrate; tube C, as for tube B, except with 5′-AMP; tube D, the same, only with 3′,5′-AMP; tube E, the same, only with 2′-AMP; tube F, as tube C, except that a further 2 mg of unreacted microsomes were added at the end of the incubation.

terials and Methods, it was possible to obtain a complete separation of reaction product from 5′-AMP. Moreover, as shown in Fig. 3, this technique provided an immediate indication of the amount of reaction product associated with the membranes. Unreacted microsomes were not extensively fractionated on this gradient (tube A), and microsomes incubated in the absence of lead were of a similar density (not shown); microsomes incubated in the absence of substrate were denser (tube B) as a result of binding Pb^{2+} (see below), and appeared as a single band, probably due to aggregation. Incubation with 5′-AMP in the complete cytochemical medium caused a marked increase in the density of the microsomes (tube C); this increase progressed with the time of incubation, in that membranes incubated for 10 min were less dense, whereas membranes incubated for 30 min were recovered as a pellet. Microsomes incubated with 3′,5′-AMP (tube D) or 2′-AMP (tube E) showed little increase in density, as was expected from the biochemical assays. Evidence that considerable aggregation of the membranes occurred during this procedure was obtained by an experiment in which additional microsomes were added after incubation with 5′-AMP. The resulting mixture had an intermediate density, and was not fully resolved as a light and dense fraction (compare tubes B and C with tube F).

Table II shows that when microsomes were isolated from the incubation medium by this procedure, all the P_i was recovered bound to the membranes as an insoluble precipitate; the unreacted 5′-AMP, however, was completely solubilized and recovered at the top of the gradient. In this experiment the cytochemical incubation was carried out for 15 min to ensure that essentially no membrane was recovered in the pellet. This table also shows that 90% of the protein and 98% of the 5′-nucleotidase activity which could be recovered from the gradient were present in the membrane fraction. The light fraction, which is to be seen just below the meniscus in each gradient in Fig. 3,

TABLE II

Analysis of Sucrose Gradients after Cytochemical Reaction of Microsomes with ^{32}P-AMP

	Total radioactivity (^{32}P counts)	P_i radioactivity $\times 10^{-3}$	Protein (mg)	5′-Nucleotidase activity (units) (μmole P_i released/20 min)
Loaded on gradient	48.5	20.7	2.1	6.2
Recovered in light fraction	27.2	0.1		
Pellet from light fraction	0.4	0.1	0.15	0
Membrane fraction	19.6	19.4		
Pellet from membrane fraction	19.2	19.1	1.71	4.9
Gradient pellet	0.5	0.5	0.05	0.1

The gradient analyzed was similar to that shown in Fig. 3, tube C, except that the cytochemical incubation was carried out for 15 min in a volume of 2.5 ml. The light fraction and the membrane fraction (both ~5 ml) were removed from the gradient with a syringe, and the pellet was resuspended after decantation of the gradient. After adjusting the sucrose concentration in each sample to ~0.3 M and centrifuging to obtain a pellet, each sample was suspended in 1 ml 0.25 M sucrose. Total radioactivity (P_i + unreacted 5′-AMP) was determined after deproteinization with 10% TCA and extraction of TCA with ether. P_i radioactivity was measured in samples of the same extract after treatment with ammonium molybdate in 1 N H_2SO_4 and extraction of the phosphomolybdate into isoamyl alcohol as described in Materials and Methods. Enzyme activity was determined, after washing a 0.3 ml sample with 3 ml 1 mM EDTA, pH 7.5, (to remove Pb^{2+}), at 37°C in a medium containing 0.1 M Tris-Cl pH 8.5, 10 mM 5′-AMP, and 10 mM $MgCl_2$.

TABLE III

Binding of ^{210}Pb to Microsomes

Incubation conditions	μmole P_i/mg protein	μmole ^{210}Pb/mg protein	μmole ^{210}Pb/μmole P_i
−Substrate, 10 min	0.006	0.165	
−Substrate, 30 min	0.014	0.179	
−Substrate, 1 μmole P_i added at 30 min	0.314	0.695	1.64
−Substrate, 2 μmoles P_i added at 30 min	0.640	1.18*	1.56
5′-AMP, 10 min	0.260	0.578	1.59
5′-AMP, 30 min	0.660	1.13*	1.44

The experimental procedure was similar to that described for Table II, except that ~3 mg microsomal protein were incubated in a volume of 5 ml containing, in addition, 0.2 or 0.4 μCi ^{210}Pb. Membrane samples were recovered from sucrose gradients and deproteinized with 10% TCA. Appropriate samples of the TCA extracts were analyzed directly for P_i and for ^{210}Pb radioactivity. The ratio μmole ^{210}Pb/μmole P_i was calculated after subtracting μmole ^{210}Pb bound to membranes in the absence of substrate or added P_i. The results are the mean values from two experiments.

*Corrected for 0.52 μmole ^{210}Pb (total) recovered in the pellet from samples incubated for 30 min without substrate.

was not examined further since pellets of the material disintegrated during preparation for electron microscopy.

In order to investigate the binding of lead to the membranes, incubations were carried out with $^{210}Pb(NO_3)_2$. Table III confirms that lead bound to the membranes in the absence of substrate, and shows that the additional lead bound as lead phosphate, formed either after incubation with 5′-AMP or by the addition of P_i, was chemically

equivalent to the amount of phosphate, if all the lead phosphate was present as $Pb_3(PO_4)_2$.

Cytochemical Observations

In spite of the extensive manipulation, the structure of the microsomal membranes was reasonably well preserved. As was expected from the biochemical results, microsomes incubated in the cytochemical medium without substrate (Fig. 4), or with 2′-AMP (Fig. 5) or 3′,5′-AMP (Fig. 6), contained little or no lead precipitate associated with the membranes. These preparations were of a very uniform appearance when scanned from the top to the bottom of the pellet, and three separate preparations of microsomes subjected to the same procedure were indistinguishable.

In contrast, microsomes incubated in the cytochemical medium with 5′-AMP showed extensive lead precipitates associated with the membranes (Fig. 7). The distribution of the reaction product was quite heterogeneous, in that some membranes were very densely stained, whereas others were hardly stained at all. The lead precipitates appeared to be localized on the outside (cytoplasmic surface) of the membranes, in contrast to the reaction product obtained from glucose-6-phosphatase (12, 13). The micrograph shown in Fig. 7 was selected from the upper half of the pellet, where the lead precipitates could be distinguished most clearly. Although the membranes at the bottom of the pellet showed essentially the same distribution of reaction product, they were aggregated into much larger clusters, and were therefore more difficult to examine.

In order to compare the appearance of the enzyme reaction product with artificially produced lead phosphate precipitates, microsomes were incubated (*a*) with Pb^{2+} but without substrate, and sodium phosphate added at the end of the incubation, and (*b*) with 5′-AMP but without Pb^{2+}, and $Pb(NO_3)_2$ added after the reaction. In both cases, the membranes coprecipitated with the lead phosphate, and were recovered as a pellet from the sucrose gradients. The pellets prepared for microscopy were not of uniform appearance: in both preparations lead phosphate precipitates were concentrated at the bottom of the pellet and observed only occasionally at the top. Representative fields from the middle of the pellet are shown in Fig. 8 (precipitate formed from Pb^{2+} and phosphate) and Fig. 9 (precipitate formed from phosphate released from 5′-AMP and Pb^{2+}). Both types of dense precipitate were randomly associated with the membranes and showed little resemblance to the nucleotidase reaction product. In addition, a few vesicles in Fig. 9 were associated with a precipitate probably caused by a low level of cytochemical reaction which occurred at 0°C. Low levels of reaction product were always observed in preparations in which microsomes were incubated in the complete medium with 5′-AMP at 0°C.

As an additional control, microsomes were incubated with Pb^{2+} in the absence of substrate, isolated on sucrose gradients, and then suspended in the cytochemical medium, without lead or substrate. After the addition of P_i, the membranes were reisolated on sucrose gradients and then examined in the electron microscope. Their appearance was identical to that of membranes incubated with Pb^{2+}, but without substrate. It is, therefore, unlikely that lead ions bind to specific sites on the membranes which then become preferential sites for precipitating the phosphate released from 5′-AMP.

These results indicated that this approach was appropriate for the cytochemical localization of 5′-nucleotidase, and that the enzyme was heterogeneously distributed within the microsomal fraction. Microsomes were, therefore, subfractionated on a linear sucrose gradient, as described in Materials and Methods, and divided into four fractions of increasing density. The results presented are from one of two experiments which yielded identical results. As was expected from other results (5, 6), the specific activity of 5′-nucleotidase increased as the density of the microsomal subfraction decreased (Table IV).

Fraction I, the densest, consisted of a relatively homogeneous rough microsomal fraction (Fig. 10): after incubation with 2′-AMP, only occasional lead precipitates could be seen, some of which appeared to be localized inside the membranes (Fig. 11). Again, no precipitates were seen after incubation in the absence of substrate (Fig. 12), but after incubation with 5′-AMP a considerable amount of reaction product was associated with the membranes. In a typical field (Fig. 13), vesicles with ribosomes still attached and with lead precipitate present on at least one part of the circumference were frequently observed; very heavily stained areas were also observed occasionally (Fig. 14). Although the reaction product showed a distinctly heterogeneous distribution, no clusters of vesicles entirely free of lead precipitate were ob-

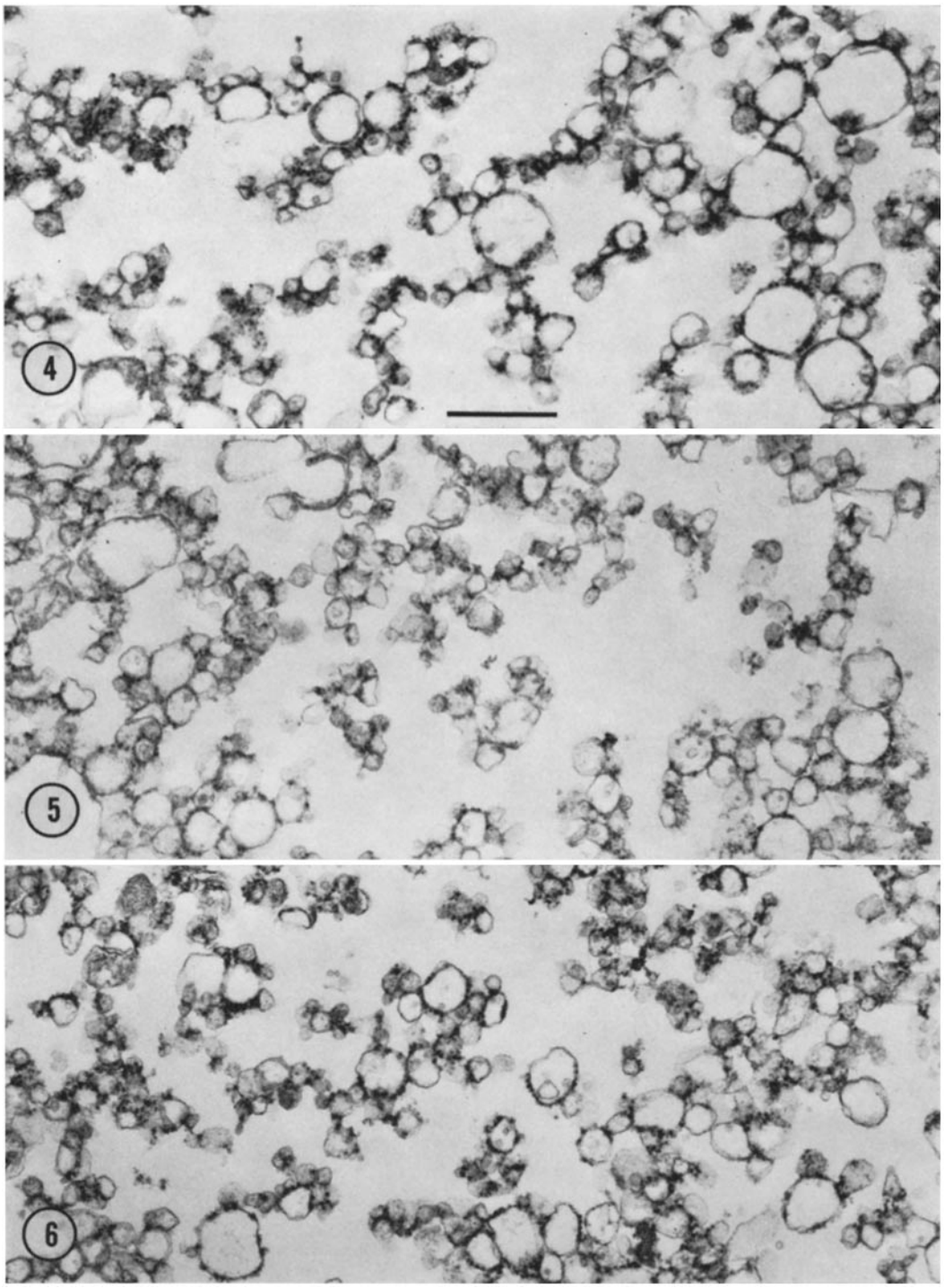

FIGURE 4 Representative field in a preparation of microsomes incubated for 30 min at 25°C in the cytochemical medium in the absence of substrate. The bar represents 0.5 μ. × 31,000.

FIGURE 5 Same as Fig. 4, except that the incubation medium contained 2′-AMP. × 31,000.

FIGURE 6 Same as Fig. 4, except that the incubation medium contained 3′,5′-AMP. × 31,000.

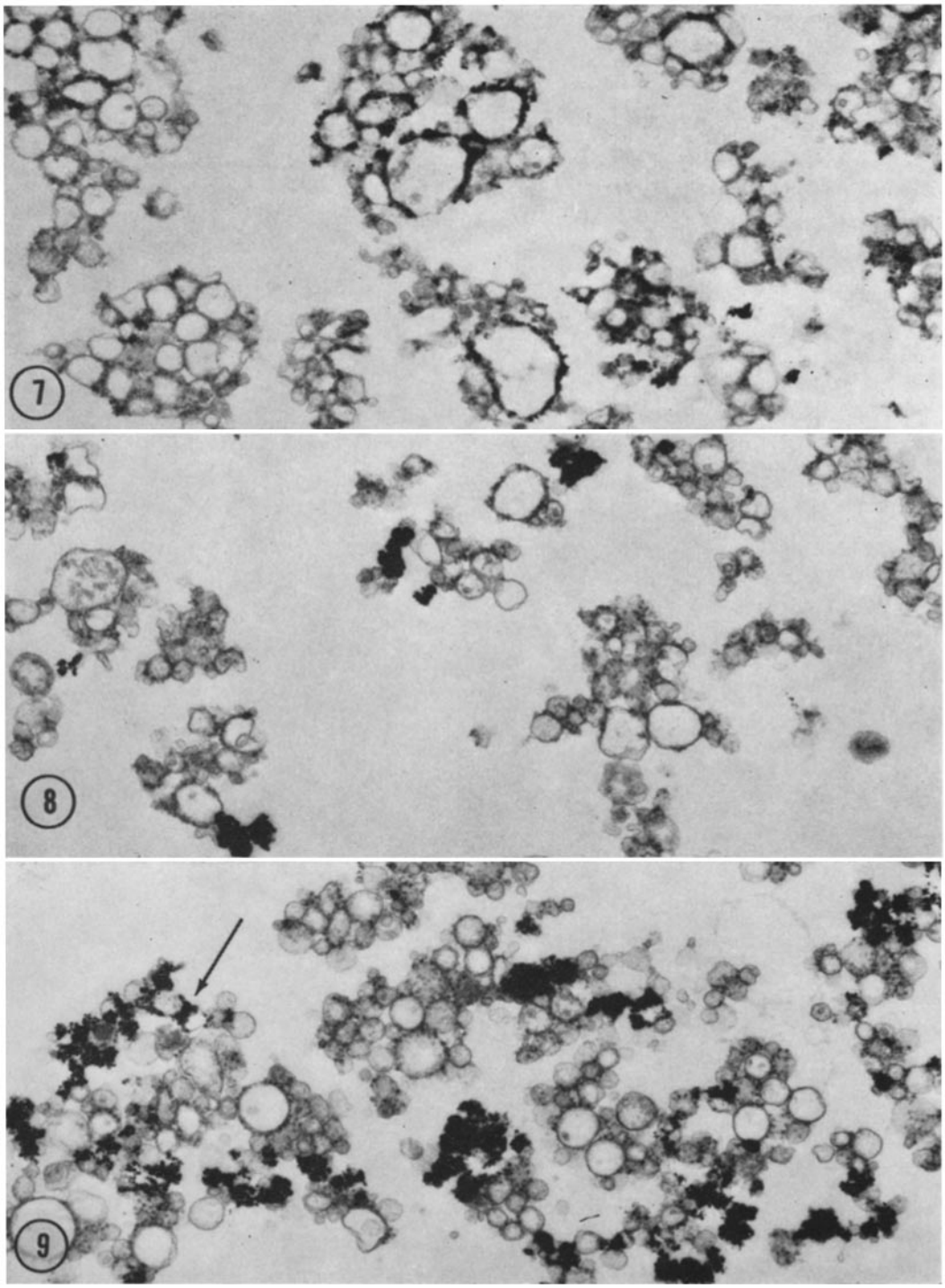

FIGURE 7 Representative field from the upper half of the pellet in a preparation of microsomes incubated for 30 min at 25°C in the cytochemical medium containing 5′-AMP. × 31,000.

FIGURE 8 Representative field from the middle of the pellet in a preparation of microsomes incubated as described for Fig. 4. 0.3 μmole P_i/μmole Pb^{2+} was added slowly over a period of 2 min at the end of the incubation, but before cooling, to produce an artificial lead precipitate. × 31,000.

FIGURE 9 Representative field from the middle of the pellet in a preparation of microsomes incubated in the cytochemical medium with 5′-AMP but without Pb^{2+} for 20 min at 25°C. Pb^{2+} was added after cooling the reaction to produce an artificial lead precipitate. Approximately 20 min elapsed between the addition of Pb^{2+} and the start of the centrifugation. The arrow points to a lead deposit which is similar to the cytochemical reaction product. × 31,000.

TABLE IV

5'-Nucleotidase Activity in Microsomal Subfractions

Fraction	Protein recovered	Activity recovered	Specific activity	Relative specific activity
	%	%		
I (densest)	14	7.5	0.22	0.54
II	18	12.5	0.29	0.71
III	24	28	0.48	1.19
IV (lightest)	21	34	0.68	1.66

Microsomes were separated on a sucrose gradient as described in Materials and Methods. The specific activity was measured under the conditions for the cytochemical reaction, and represents μmole P_i released/20 min per mg protein. The relative specific activity was calculated as the ratio:specific activity of fraction per specific activity of original microsomes.

served at any level of the pellet. In a series of fields selected at random through the depth of the pellet, approximately 5% of the vesicles were heavily stained, 22% were unstained, and the remainder were associated with at least one precipitate of lead phosphate.

Fraction II also contained mainly rough microsomes (Fig. 15) and, after incubation with AMP, appeared similar to Fraction I (Fig. 16). Fraction III contained both rough- and smooth-surfaced vesicles (Fig. 17); the reaction product in this fraction was also distributed heterogeneously (Fig. 18).

Fraction IV, the least dense, consisted almost entirely of smooth-surfaced vesicles (Fig. 19). The reaction product was again distributed heterogeneously, and larger vesicles, which stained very heavily, were also observed (Fig. 20). One commone fature of many of these large vesicles was that they were incompletely surrounded by lead precipitate. A similar appearance was observed in membranes of the bile canaliculi when isolated plasma membranes were incubated in the same manner (Fig. 21), suggesting that the heavily stained vesicles in the microsome fraction originated from the bile canaliculi. The control incubations for plasma membranes showed an absence of lead phosphate deposits, as in the case of the microsomes; a detailed description of the cytochemical localization of 5'-nucleotidase in this fraction will be presented elsewhere.[3]

[3] Widnell, C. C. Manuscript in preparation.

Localization of the Reaction Product

In electron micrographs the reaction product appeared on the outside of the vesicles. This was most clearly seen in areas where the precipitate was not too extensive (e.g., Fig. 13). Since the lead deposits obtained from glucose-6-phosphatase are found inside the membranes and are not solubilized by low concentrations of ethylenediaminetetraacetic acid (EDTA) (12, 13), the solubilization of 5'-nucleotidase reaction product by low concentrations of EDTA was investigated. After incubation for glucose-6-phosphatase and treatment with EDTA at a concentration of 3 μmoles/μmole phosphate, only 8% of the phosphate was recovered in the supernatant. In contrast, 95% of the phosphate associated with the membranes after incubation for 5'-nucleotidase was solubilized at an EDTA concentration of 2 μmoles/μmole phosphate (Fig. 22). 1 μmole EDTA released 0.58 μmole phosphate, compared with a theoretical maximum of 0.67 μmole.

Effect of Fixation on 5'-Nucleotidase Activity

In order to investigate the effect of fixation on 5'-nucleotidase, plasma membranes and rough microsomes were treated with 2% glutaraldehyde in the presence of 5'-AMP for 15 min (the time required to obtain the membranes as a pellet) and then assayed with 5'-AMP and 2'-AMP both under optimal conditions and under the conditions of the cytochemical incubation. Table V shows that 5'-nucleotidase was extensively inactivated (~95%) by this procedure, whereas the nonspecific phosphatase was inactivated to a much lower extent (~60%).

DISCUSSION

These experiments were designed to determine whether 5'-nucleotidase is present in both the plasma membrane and the endoplasmic reticulum. Since a major fraction of the activity is recovered in the plasma membrane, and since fixation, even with low concentrations of glutaraldehyde in the presence of 5'-AMP, causes extensive inactivation, the enzyme has been investigated in isolated, unfixed cell fractions. It was reasoned that if the enzyme could be detected on rough microsomes, its presence in the endoplasmic reticulum would be confirmed. The cytochemical procedure was, therefore, developed as an attempt to permit the unequivocal localization of 5'-nucleotidase

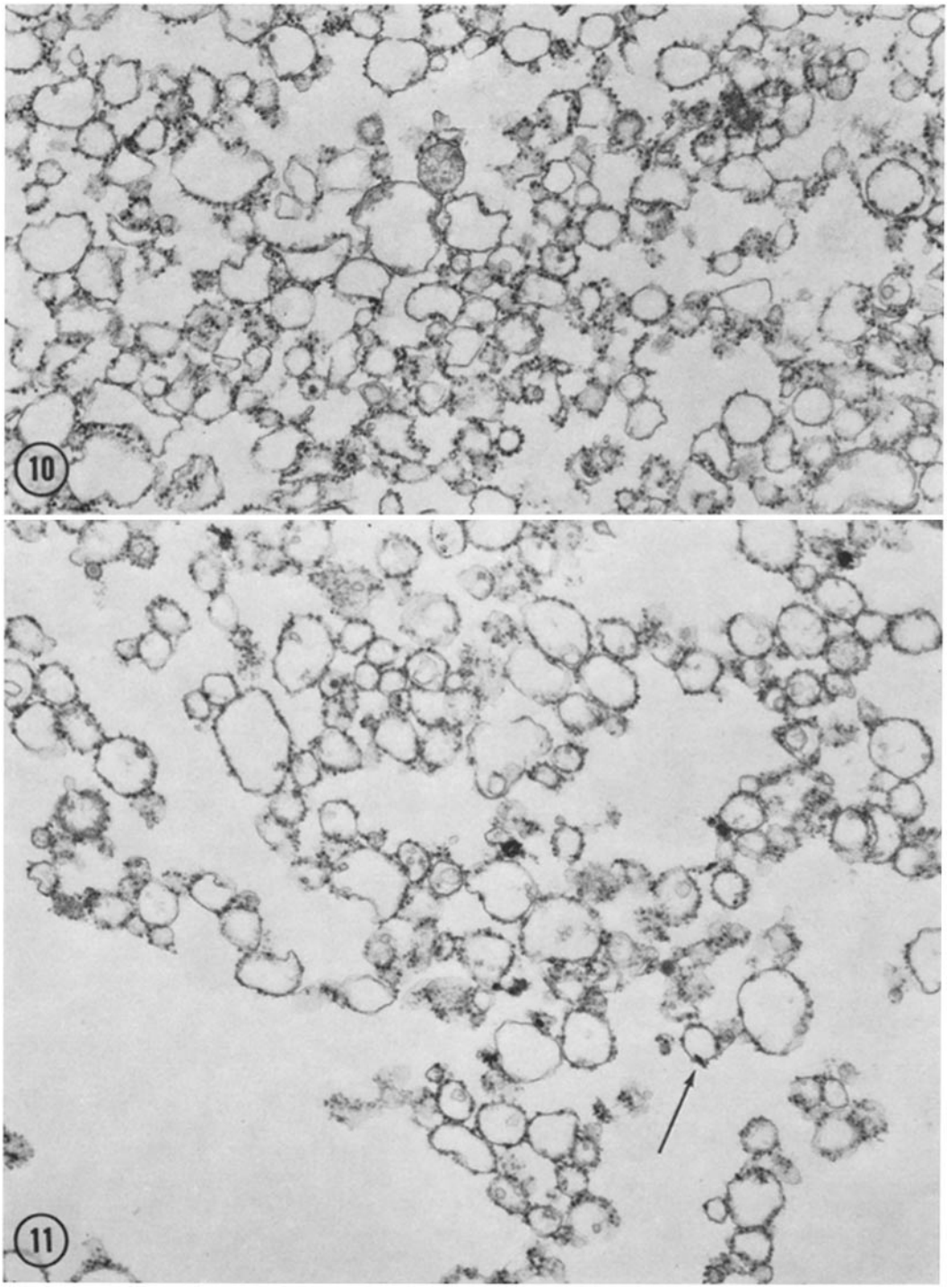

FIGURE 10 Microsomal subfraction I was fixed without incubation in the cytochemical medium. × 31,000.

FIGURE 11 Microsomal subfraction I was incubated for 30 min at 22°C in the cytochemical medium with 2′-AMP. The arrow points to a lead precipitate inside a vesicle. × 31,000.

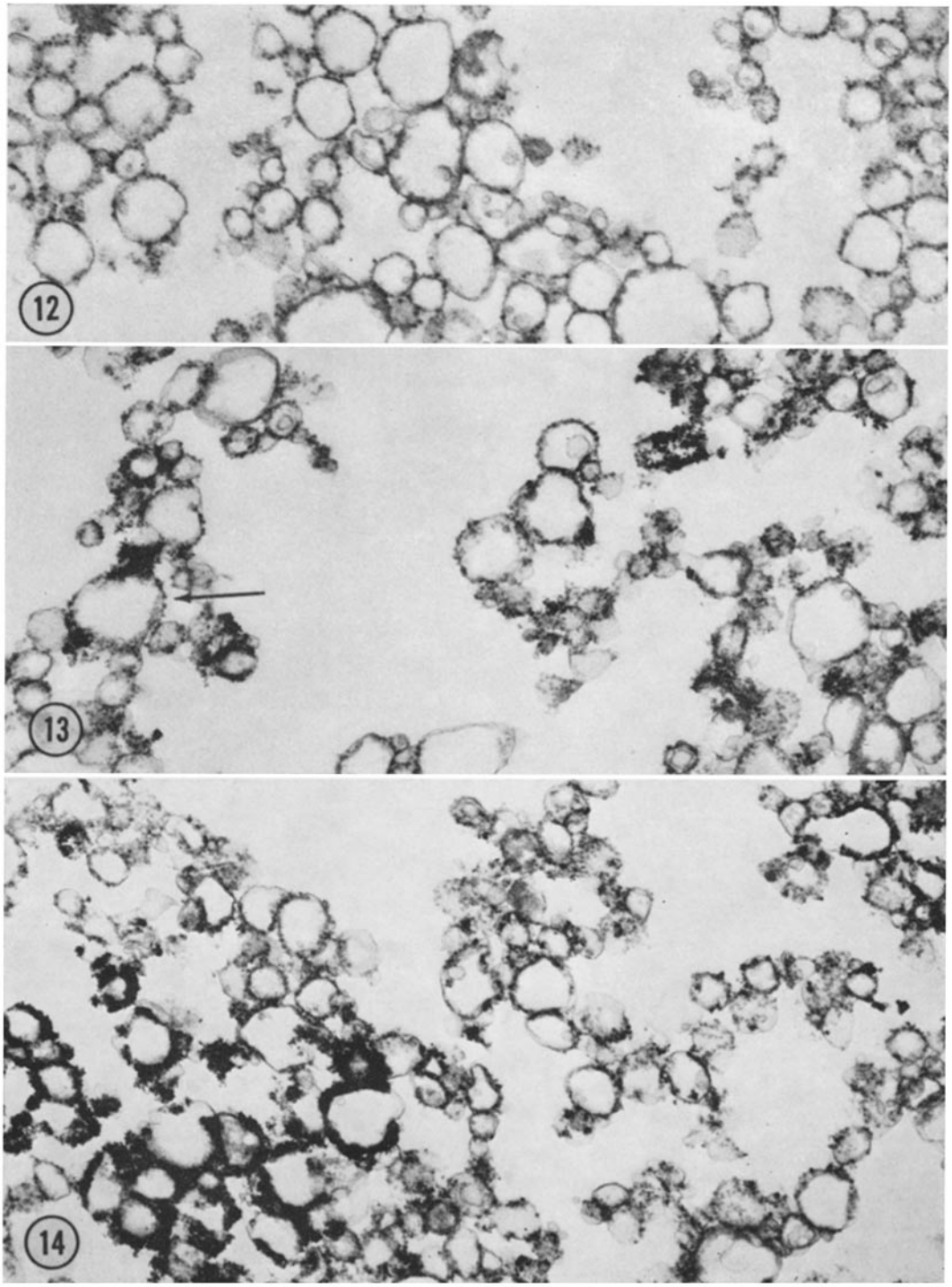

FIGURE 12 Same as Fig. 11, except the medium contained no substrate. × 31,000.

FIGURE 13 Representative field from the middle of the pellet in a preparation of microsomal subfraction I incubated in the cytochemical medium with 5′-AMP for 30 min at 22°C. The arrow points to a rough-surfaced vesicle with the reaction product clearly visible. × 31,000.

FIGURE 14 Same as Fig. 13, except that the field was selected to show a group of heavily stained vesicles. × 31,000.

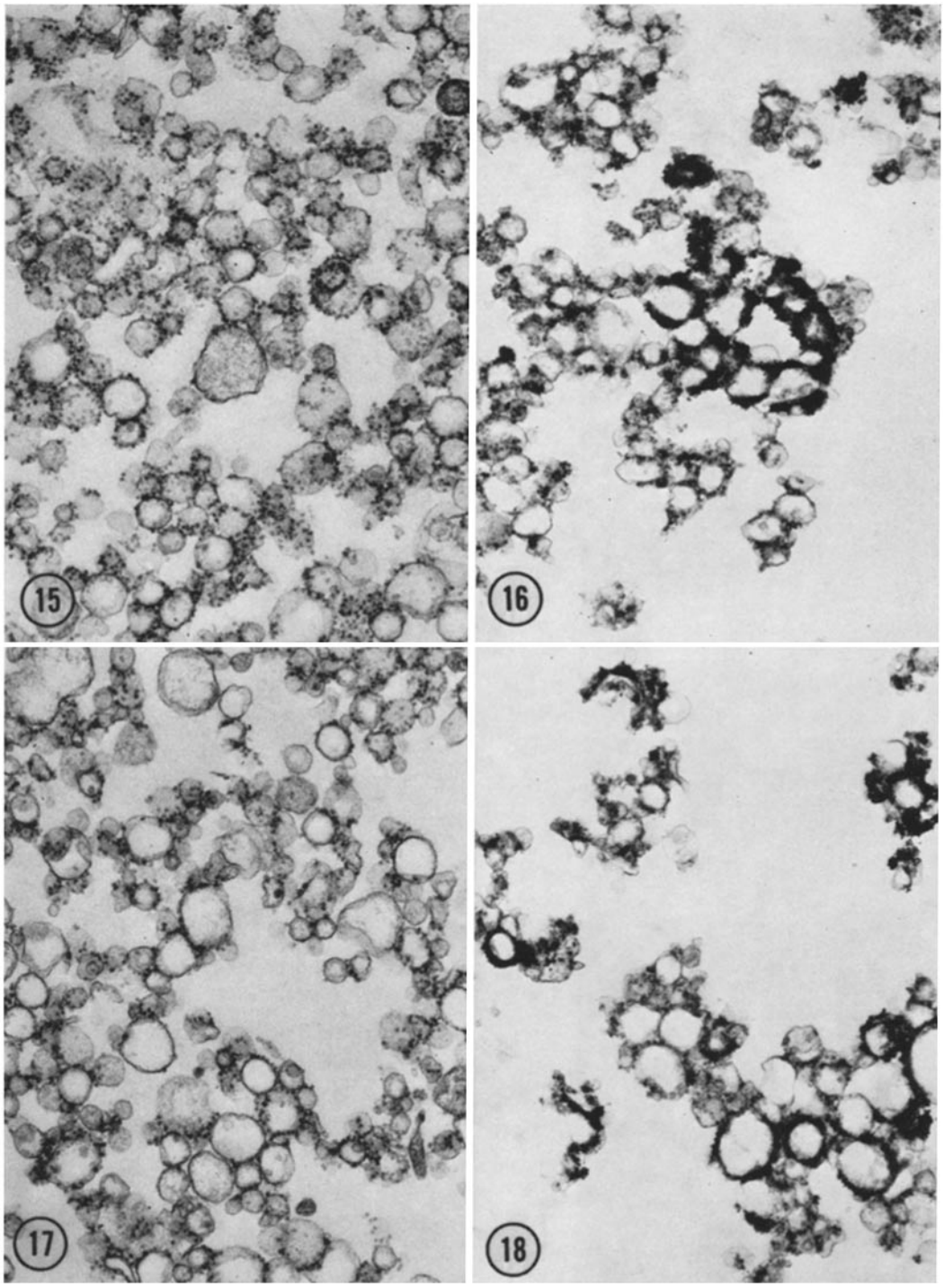

FIGURE 15 Microsomal subfraction II was fixed without incubation in the cytochemical medium. × 31,000.

FIGURE 16 Microsomal subfraction II was incubated in the cytochemical medium with 5 -AMP for 30 min at 22°C. Field selected to show a group of densely stained vesicles. × 31,000.

FIGURE 17 Microsomal subfraction III was fixed without incubation in the cytochemical medium. × 31,000.

FIGURE 18 Microsomal subfraction III was incubated in the cytochemical medium with 5′-AMP for 20 min at 22°C. Representative field from the upper half of the pellet. × 31,000.

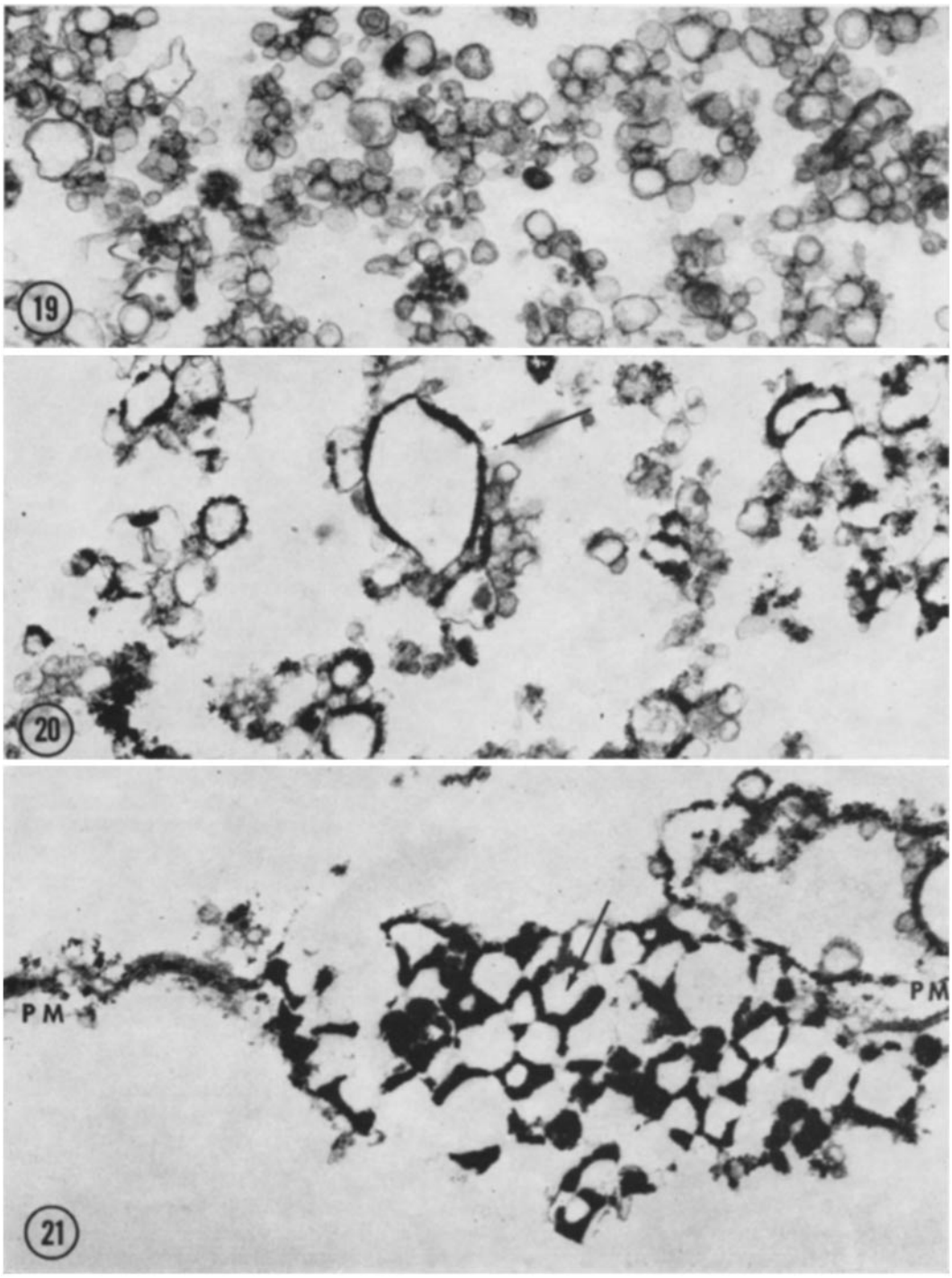

FIGURE 19 Microsomal subfraction IV was fixed without incubation in the cytochemical medium. × 31,000.

FIGURE 20 Microsomal subfraction IV was incubated in the cytochemical medium with 5′-AMP for 20 min at 22°C. Selected field from the lower half of the pellet, showing a large, densely stained vesicle (arrow). × 31,000.

FIGURE 21 Isolated plasma membranes were incubated in the cytochemical medium with 5′-AMP for 30 min at 22°C. Selected field showing a bile canaliculus with the plasma membrane (*PM*) extending from each side. The arrow points to a vesicle similar in appearance to the one arrowed in Fig. 20. × 31,000.

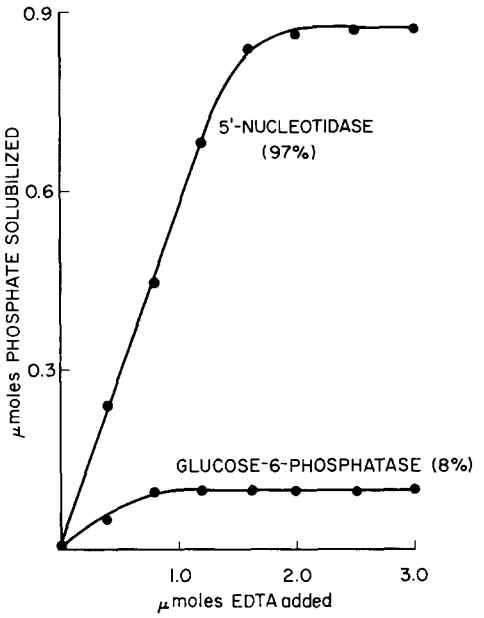

FIGURE 22 Solubilization of cytochemical reaction products by EDTA. Microsomes were incubated for 30 min at 25°C either in 0.1 M Tris-acetate pH 7.5, 1 mM 5'-AMP, 1 mM $Mg(NO_3)_2$, and 1 mM $Pb(NO_3)_2$ or in 0.1 M Tris-maleate pH 6.6, 1 mM glucose-6-phosphate and 1 mM Pb $(NO_3)_2$. After incubation the membranes were centrifuged and suspended in 0.25 M sucrose. Samples containing ~ 1 μmole P_i were treated with various concentrations of EDTA for 2 hr at 2°C. The membranes were again centrifuged, and P_i was determined in both the pellet and supernatant after deproteinization with TCA.

The procedure was considered satisfactory for the following reasons: (*a*) no lead precipitates were associated with membranes incubated with Pb^{2+}, but without substrate. (*b*) Only occasional deposits of lead phosphate were observed when membranes were incubated with analogs of 5'-AMP which were not substrates for 5'-nucleotidase. (*c*) Only P_i was associated with the membranes as lead phosphate after their isolation on sucrose density gradients. (*d*) The additional lead bound to membranes after incubation with 5'-AMP was chemically equivalent to the P_i. (*e*) Artificially produced lead precipitates did not resemble the reaction product either in their appearance or in their distribution in membrane pellets. The localization reported here, therefore, depends on the assumption that lead phosphate is precipitated at the site on the membrane occupied by the enzyme. Migration of lead phosphate would invalidate the conclusion that the enzyme is present in the endoplasmic reticulum. This possibility is considered very unlikely since vesicles with little or no lead precipitate were observed adjacent to very heavily stained membranes (Fig. 20) and since no evidence was obtained for binding sites on the membranes, specific for Pb^{2+}, which might subsequently attract P_i.

The failure to demonstrate 5'-nucleotidase activity in the endoplasmic reticulum by cytochemical procedures using intact tissue (2–4) is probably caused by inactivation of the enzyme during fixation. The fraction of the activity lost

TABLE V

Effect of Fixation by Glutaraldehyde on the Activity of 5'-Nucleotidase in Plasma Membranes and Rough Microsomes

Sample	μmoles P_i released/20 min per mg protein (mean values from two experiments)			
	37°C, pH 8.5		25°C, pH 7.5 + Pb^{2+}	
	5'-AMP	2'-AMP	5'-AMP	2'-AMP
Plasma membranes	23.2	0.059	2.84	0.022
Fixed plasma membranes	0.81	0.023	0.14	0.008
Rough microsomes	1.3	0.026	0.27	0.014
Fixed rough microsomes	0.038	0.011	0.022	0.009

Membranes were fixed in suspension in a medium containing 0.1 M cacodylate buffer pH 7.2, 2% glutaraldehyde, 1 mM 5'-AMP and 1 mM $MgCl_2$, and immediately obtained as a pellet by centrifugation at 100,000 *g* for 15 min. The pellet was washed by resuspension and resedimentation in 0.25 M sucrose and 10 mM Tris-acetate pH 7.5 and finally suspended in the same medium. Appropriate samples were taken for assay as described in Table I and Materials and Methods. The rough microsome fraction was obtained by pooling subfractions I and II.

after treatment with 2% glutaraldehyde (~95%) was similar in isolated plasma membranes and rough microsomes. However, the activity of 5'-nucleotidase in fixed plasma membranes was half that found in unfixed rough microsomes and, therefore, at a level which could be detected cytochemically. Since the activity with 5'-AMP was 15 times that with 2'-AMP, a cytochemical reaction carried out with fixed plasma membranes would represent specific 5'-nucleotidase activity. On the other hand, the activity in fixed rough microsomes was such that an incubation of several hours would be required before an observable cytochemical reaction product could be obtained. Furthermore, in this case the activity with 5'-AMP was little more than twice that with 2'-AMP, so that it would be very questionable to assign specific 5'-nucleotidase activity to any precipitates obtained using fixed rough microsomes.

Lead ions at a concentration of 1 mM have been shown to cause hydrolysis of RNA (25), and this has been suggested as a possible explanation for the loss of ribosomes after incubation for glucose-6-phosphatase in the presence of 2 mM Pb^{2+} (12, 13). Ribosomes could still be seen attached to membranes after incubation in the cytochemical medium described here. This could be caused by any combination of the presence of Mg^{2+}, the lower Pb^{2+} concentration, and the shorter incubation times.

In addition to demonstrating the presence of 5'-nucleotidase in rough microsomes, these results provide further support for the suggestions that the enzyme is distributed heterogeneously in the microsome fraction (5), and that vesicles derived from the plasma membrane are present in microsomes (5, 6). However, they also indicate that only a direct approach can be used to establish the precise subcellular localization of membrane-bound enzymes.

The distribution of 5'-nucleotidase in microsomes is markedly different from that of glucose-6-phosphatase (12, 13), which is present to a similar extent in all vesicles derived from the endoplasmic reticulum. This apparent heterogeneous distribution could well be exaggerated by the localization of the reaction product on the outer surface of the membrane. Other sections through vesicles which showed little or no lead precipitate could have revealed more extensive deposits of lead phosphate, so that the fraction of unstained vesicles in a section (22% in the case of rough microsomes) almost certainly represents a maximum value for the total population. An analysis of the distribution of reaction product is further complicated by the fact that the enzyme could not be assayed cytochemically under optimum conditions. However, unless the enzyme is inactivated preferentially at specific sites on the microsomal membrane, it is clear that activity is not distributed uniformly on the surface of the vesicles. Further studies using intact cells will be necessary to determine whether the distribution observed here reflects that of the enzyme on the endoplasmic reticulum *in situ*.

The physiological significance of the enzyme's presence in the endoplasmic reticulum remains to be determined.

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