# DIAZOPHTHALOCYANINS AS REAGENTS FOR FINE STRUCTURAL CYTOCHEMISTRY

LOIS WITHROW TICE, M.D., and RUSSELL J. BARRNETT, M.D.

From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, and the Department of Anatomy, Yale University School of Medicine, New Haven, Connecticut

ABSTRACT

This paper reports the synthesis of 14 diazophthalocyanins containing Mg, Cu, or Pb as the chelated metal. To assess the usefulness of these compounds for fine structural cytochemistry, the relative coupling rates with naphthols were tested as well as the solubility of the resulting azo dyes. Three of the diazotates were reacted with tissue proteins in aldehyde-fixed material, and the density increases thus produced were compared in the electron microscope with those produced by staining similarly fixed material with the phthalocyanin dye, Alcian Blue. Finally, one of the diazotates was used as a capture reagent for the demonstration of the sites of acid phosphatase activity with the electron microscope.

Despite increasing application of histochemical methods to problems in electron microscopy, relatively few fine structural studies employing azo dye techniques have been reported. The most commonly used diazonium salts for histochemical tests at the light microscope level are unsuitable for such purposes, either because of their low inherent electron opacity or because the azo dyes produced by their coupling with naphthols are soluble in reagents ordinarily used in preparing tissues for electron microscopy.

Although one diazonium salt, diazotized basic fuchsin (hexazonium pararosaniline), has been applied to electron microscope studies by Davis, Lehrer, and Ornstein (1–3), most investigators have dealt with the technical problem of producing new reagents which might be better suited to fine structural cytochemistry. Seligman and coworkers have explored the increases in electron opacity produced by addition of iodine atoms to diazonium salts (4) and to other histochemical reagents (5–7). At the light microscope level, Burstone and Weisberger have tested a number of diazotizable amines from which azo dyes were produced and chelated with metal atoms to form insoluble and presumably electron-opaque complexes (8), but none of these has yet been tested with the electron microscope. Barrnett (9) tested a number of complex dye intermediates as diazonium salts in an effort to demonstrate alkaline phosphatase activity with an azo dye method at a fine structural level.

We are reporting the results of another approach to this problem, the use of diazonium salts whose density was increased by the inclusion of chelated metal atoms within the diazonium salt itself. For this purpose, a series of 14 diazophthalocyanins, containing Mg, Cu, or Pb as the chelated metal atom, were synthesized following the general methods of Haddock, Linstead, and coworkers for synthesis of this class of compounds (10–16). The relative coupling rates of these salts with unsubstituted naphthols and the solubility properties of the azo dyes so obtained were tested *in vitro*. Three diazophthalocyanins were then selected for studies of the electron opacity imparted by their direct reaction with tissue proteins, a fine structural analog of the coupled tetrazonium reaction (17). Finally, one of these salts was tested as a reagent for enzyme

TABLE I

*Properties of Diazophthalocyanins Synthesized and of Their Azo Dyes*

| Diazo-phthalocyanin synthesized* | Yield | Azo color dyes | | Solubility Azo dyes | | | | | | Coupling rates‡ | | Maximum pH at which coupling occurred |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | α-Naphthol | | | β-Naphthol | | | | | |
| | | α-Naphthol | β-Naphthol | ETOH | Acetone | PROP OX | ETOH | Acetone | PROP OX | α-Naphthol | β-Naphthol | |
| | *per cent* | | | | | | | | | | | |
| mono-(3-diazo-) Cu* | 90 | Red | Yellow | Sl | S | S | S | S | S | 4 | 2 | 4 |
| mono-(4-diazo-) Cu | 8 | Red | Yellow | Sl | S | S | Sl | S | S | 4 | 2 | 6 |
| di-(3-diazo-) Cu | Not recrystallized | Red | Yellow | I | S | S | S | S | S | 4 | 2 | 4 |
| tri-(3-diazo-) Cu | Not recrystallized | Red | Yellow | I | I | I | Sl | Sl | Sl | 1 | 1 | 4 |
| tetra-(3-diazo-) Cu | 36 | Red | Yellow | S | S | S | S | S | S | 4 | 2 | 4 |
| tetra-(4-diazo-) Cu | 40, 27 | Red | Yellow | I | S | S | S | S | S | 2 | 4 | 8 |
| mono-(3-diazo-) Mg | 16 | Yellow | Green-yellow | Sl | Sl | S | S | S | S | 5 | 3 | 4 |
| mono-(3-diazo-) Pb | 25, 50 | Yellow | Yellow | Sl | S | S | S | S | S | 3 | 3 | 6 |
| mono-(4-diazo-) Pb§ | 62, 64 | Orange | Red | I | I | I | I | I | I | 5 | 3 | 6 |
| di(4-diazo) Pb | 33 | Yellow | Yellow-green | I | S | S | S | Sl | Sl | 3 | 3 | 6 |
| tri-(4-diazo-) Pb‖ | 31, 25 | Orange-Red | Red | I | I | I | I | I | I | 5 | 3 | 8 |
| tri-(3-diazo-) Pb | Not recrystallized | Yellow | Yellow | S | S | S | S | S | S | 3 | 3 | 8 |

The following abbreviations were used: S, soluble; Sl, slightly soluble; I, insoluble; ETOH, ethyl alcohol; PROP OX, Propylene oxide.

* See legend for Fig. 1 for explanation of nomenclature used in naming these salts.

‡ Coupling rates were rated on an arbitrary scale from 1 (slowest) to 5 (fastest) using the coupling of Diazo Blue B with α naphthol at pH 8 as a standard, rated at 4. Reactions reported are those of the maximum pH at which coupling occurred.

§ Elemental analysis of the corresponding nitrophthalocyanin gave values of 65.74 per cent C, 3.36 per cent H, 16.08 per cent N; predicted values: 50.26 per cent C, 1.98 per cent H, 16.49 per cent N.

‖ Elemental analysis of the corresponding nitrophthalocyanin gave values of 45.80 per cent C, 1.74 per cent H, 17.77 per cent N; predicted values: 44.97 per cent C, 1.53 per cent H, 18.03 per cent N.

histochemistry at the electron microscope level in the demonstration of acid phosphatase activity.

## MATERIALS AND METHODS

### *Syntheses*

A typical synthesis (of tri-(4 diazo-) lead phthalocyanin) was conducted as follows: 2.8 gm phthalonitrile, 10.5 gm 4-nitrophthalonitrile (prepared from 4-nitrophthalimide) (10) were placed in a flask together with 8.0 gm lead metal powder and 20 ml methylnaphthalene. After thorough mixing, the flask was slowly heated to 180–200°C with occasional stirring. At temperatures of about 165°C a deep green color appeared, and a blue solid began to separate when the temperature reached 180°C. After 90 minutes, the mixture was cooled, the product was filtered, and solvent and unreacted phthalonitrile were removed by washing with acetone, water, dilute acid, and base. The product (tri-(4-nitro) lead phthalocyanin) was then recrystallized from boiling quinoline. The recrystallized product was again washed in acetone, acid, and base, and dried (yield: 25 per cent). Reduction to the aminophthalocyanin was carried out by stirring 5.0 gm nitrophthalocyanin with 15.0 gm sodium hydrosulfite in 160 ml 1 N NaOH 2 hours at 55–60°C. The product was filtered, stirred with boiling 20 per cent aqueous NaCl, washed with 10 per cent NaCl, water, and dried.

Although production of stabilized diazophthalocyanins has been reported (19–21), in these experiments diazotization of aminophthalocyanin was carried out immediately before use. 100 mg aminophthalocyanin was suspended in 3 ml 20 per cent HCl, which was then cooled to 4°C. 1 to 2 ml 1 per cent $NaNO_2$ was added dropwise to the suspension with continual stirring, and the mixture was stirred in the cold for 15 minutes. After filtration, the green diazo solution was used either for study of its coupling properties or for tissue experiments.

For the most part, the phthalocyanins investigated in this study contained either Cu or Pb as the chelated metal atom. To date, attempts to synthesize analogous series containing no metal, Na, Mg, or W have been unsuccessful. Although small yields of these compounds were sometimes obtained (Table I), they were usually too small to be satisfactorily purified and tested.

In studies of their coupling properties, solutions of the diazophthalocyanins were titrated to pH 4, 6 or 8 with cold NaOH and added to 0.1 M buffer (acetate, phosphate, or Tris) at the same pH in volumes sufficient to give final concentrations of approximately 1 mg diazonium salt/ml. After the pH was checked, $\alpha$- or $\beta$-naphthol, dissolved in acetone (4 mg/ml), was added rapidly to the solutions to yield final concentrations of 1 mg/ml. At each pH level, the relative coupling rate, as judged by the time required for the appearance of visible color change (compared with that required for coupling of Diazo Blue B at pH 8), and the color of the azo dye formed were recorded (Table I). The precipitated azo dyes were collected on filter paper, washed in acetate buffer at pH 5 to remove any uncoupled diazonium salt, and dried in air prior to studies of their solubility properties. Analysis for $Pb^{++}$ of the filtrate obtained after coupling of tri-(-3-diazo-) Pb phthalocyanin with $\alpha$-naphthol gave values of 0.004 mg/ml or $1 \times 10^{-5}$ M.

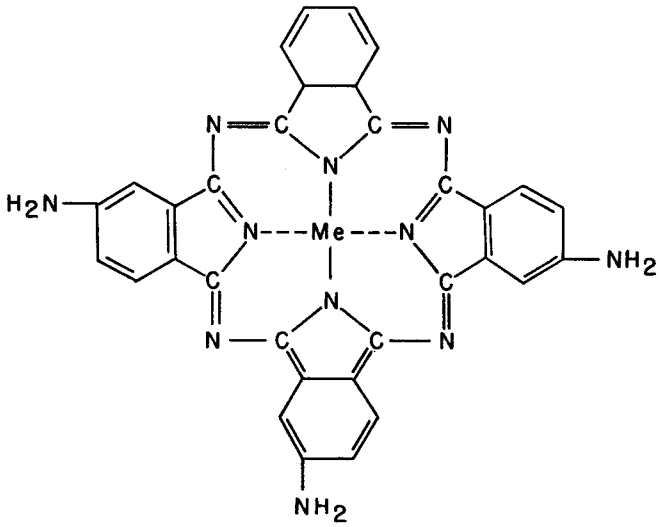



FIG. 1. Probable structural formula of a tri-(4-amino) metal phthalocyanin. Note that amino groups may be present on either the 3- or the 4- position of each aromatic nucleus in the phthalocyanin molecule; hence, either 3- or 4-substituted salts containing one to four diazonium radicals may be produced. In naming these compounds, mono-, di-, tri-, or tetra-, refers to the number of nitro, amino, or diazonium functions present, while 3- or 4- refers to the position of these functions on the benzene ring.

### *Tissue Experiments*

Thin slices of kidney, duodenum, and pancreas from normal male rats were fixed for 2 hours at 4°C in 4 to 6 per cent glutaraldehyde in cacodylate (kidney) or phosphate (duodenum and pancreas) buffers (0.067 M) at pH 7.2 (22). After fixation, the tissues were washed several hours in the corresponding buffer at pH 7.2 in the cold, and 10- or 60-$\mu$ sections, the former for light microscopy, the latter for electron microscopy, were cut on a freezing microtome. The sections were then incubated as described below, and washed for 15 to 30 minutes in several changes of acetate buffer (0.05 M) at pH 5. The thick sections were then processed for electron microscopy, with or without a second fixation in buffered 1 per cent osmium tetroxide. It was determined in preliminary experiments that the density created by direct reaction with tissue constituents was obscured by refixation of the reacted tissue sections in osmium tetroxide and, therefore, these tissues were not postfixed. After incubation for acid phosphatase, refixation in osmium tetroxide was used since the resulting azo dye could be clearly visualized, especially in overincubated tissues. After dehydration in ethanol and embedding in Epon, thin sections of this material, cut on an LKB ultramicrotome, were mounted on Formvar-coated grids, and studied with a RCA EMU 3-F or Siemens Elmiskop I.

The increased electron scattering of tissue constituents, produced by direct reaction with diazophthalocyanins, was studied using mono-(3-diazo-) Mg, Cu, or Pb phthalocyanin. Solutions of these salts, prepared as described, were titrated to pH 3–5 and added to acetate-HCl buffer (0.1 M) at the same pH to give final concentrations of approximately 2 mg/ml. Sections of duodenum and pancreas were treated in acetate-HCl buffer, at the pH to be used in the incubation, for 2 hours, immersed in the diazophthalocyanin solutions for 2 hours, thoroughly washed again in buffer at pH 5, and processed for electron microscopy without further staining. These preparations were compared with those in which the tissue was not stained but otherwise was similarly treated, with sections which had been stained in 0.5 per cent Alcian Blue for 2 hours at pH 2 or pH 3, and with sections which had been stained either with 1 mg/ml Garnet GBC at pH 5 or with Garnet GBC followed by mono-(3-diazo-) Pb phthalocyanin at pH 5. In early experiments using Alcian Blue for electron microscopy, it was found that penetration of the dye to the interior of a 50-$\mu$ frozen section was complete only after staining for 2 hours. Because of the likelihood that slow dye penetration was related to the large size of the phthalocyanin molecule, this incubation time was also used in experiments with diazophthalocyanins. In other experiments, staining with diazophthalocyanins was compared with stain-

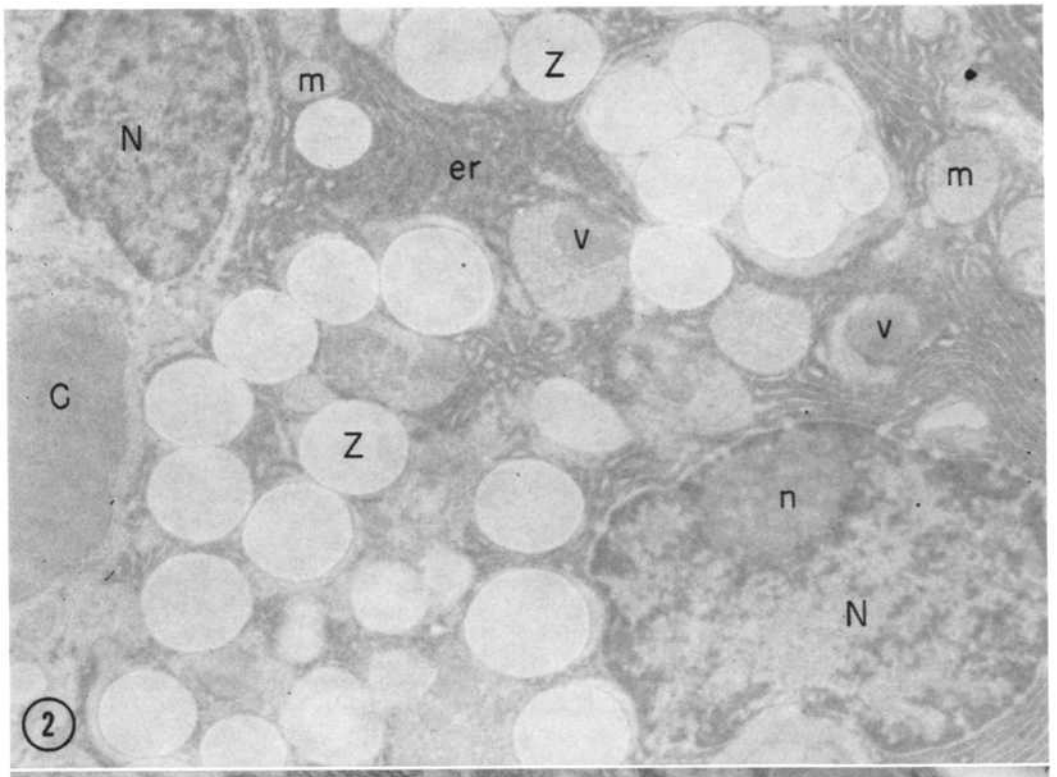
FIGURES 2 AND 3 Both figures are of sections of rat pancreas. In obtaining these figures, an attempt was made to obtain sections of equivalent thickness and to maintain identical photographic conditions. Both are of material fixed in glutaraldehyde, frozen sectioned, and embedded in Epon.

FIGURE 2 Unstained section. × 17,000.

FIGURE 3 Material treated like that in Fig. 2, except that the section was stained for 2 hours in mono-(3-diazo-) Pb phthalocyanin at pH 3. × 17,000.

In both sections pancreatic exocrine cells are shown, together with adjacent connective tissue. The unstained section (Fig. 2) presents the typical appearance of unosmicated material. Some mitochondrial change is evident, with lipid vacuoles (*v*) displacing the cristae to one side in some of the organelles. Other mitochondria appear fairly normal. In both cells, nuclei (*N*) with peripherally situated chromatin and obvious nucleoli (*n*) are conspicuous. In the cytoplasm of the exocrine cells, which occupies the greater part of the figures, there is abundant endoplasmic reticulum (*er*) together with mitochondria (*m*) and zymogen granules (*Z*).

The densities of the capillary lumina (*C*) in both figures should be compared for reference. In the stained section (Fig. 3) nuclear chromatin is increased in density, the nucleolus and nucleoplasm somewhat less so. Similarly, the profiles of endoplasmic reticulum are increased in density after staining, owing, in part, to increased density of the ribosomes and also, probably, of the intracisternal material. Mitochondrial cristae are more easily visible. However, the most striking changes are produced in the zymogen granules which, after staining, approach the mitochondria in relative electron opacity, while in the unstained section, they are much lighter than these organelles.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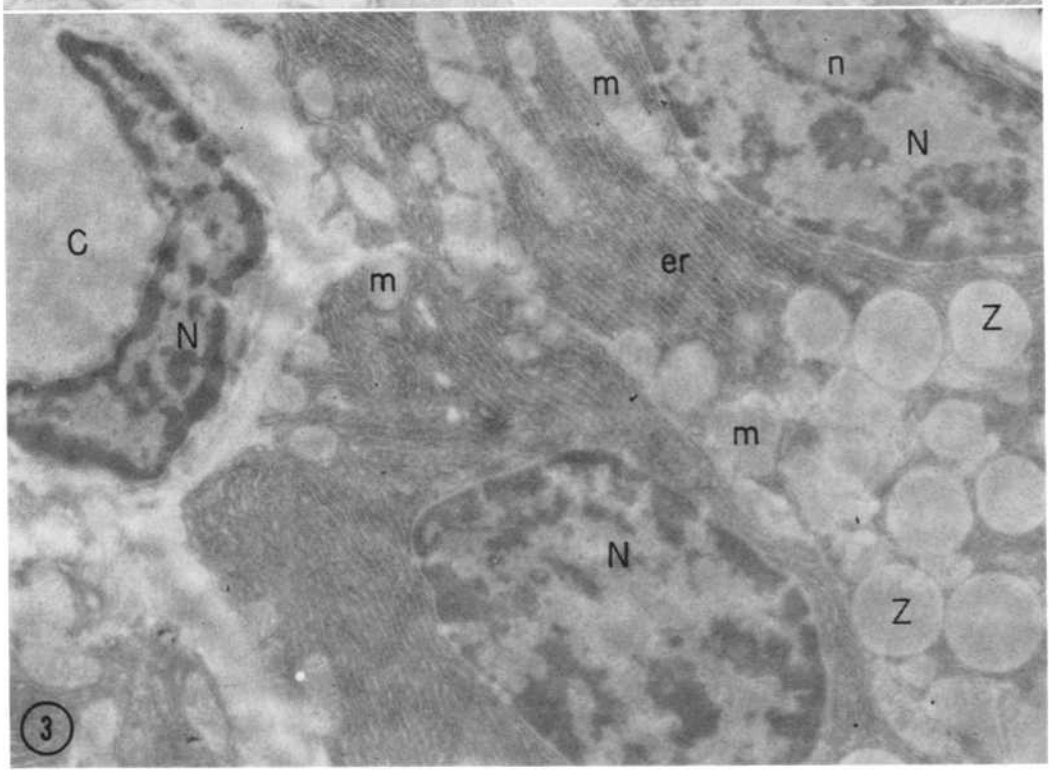
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ing by $MgCl_2$(0.1 mg/ml), $CuCl_2$ (0.1 mg/ml), and Pb acetate (0.2 mg/ml) for 2 hours at pH 5.

Acid phosphatase activity was demonstrated in sections of kidney using α-naphthyl phosphate as substrate and tri-(4-diazo-) Pb phthalocyanin as capture reagent. This substrate was selected to permit more direct comparison of results in tissue with the *in vitro* data. The incubating media were prepared by diluting the filtered diazotate, at pH 5, to the desired volume with 0.05 M acetate buffer at pH 5 or 5.5. The substrate was then added to this solution, the pH adjusted if necessary, and the clear or slightly turbid medium was used without further filtration. The final concentration of α-naphthyl phosphate was 0.5 mg/ml, that of diazophthalocyanin, 1 mg/ml. Control experiments included incubations from which the substrate was omitted, incubations in which an acid phosphatase inhibitor, NaF ($10^{-3}$ M), had been added to the medium, and incubations in which the usual substrate, α-naphthyl phosphate, had been replaced by β-glycerophosphate at the same or higher concentrations. Incubation times in all these experiments ranged from 20 to 40 minutes. Because of the possibility that contaminant lead ion might be responsible for production of density, thick sections of the Epon-embedded material, cut parallel with the thin sections, were routinely checked for the appearance of black precipitate after treatment with ammonium sulfide.

## RESULTS

The products obtained in the syntheses described here closely corresponded in their properties to those of the nitrophthalocyanins described by Haddock, Linstead, *et al.* (10–15). They were dark blue solids, insoluble in water, dilute acids or bases, or in many common organic solvents, but somewhat soluble in dioxane, dimethyl formamide, and quinoline. They dissolved in concentrated $H_2SO_4$ to give an intensely blue-green solution, and decomposed on addition of concentrated $HNO_3$. The corresponding aminophthalocyanins (Fig. 1) were of a more greenish hue and, with the exception of the fact that they were much less readily soluble in quinoline, possessed similar solubility characteristics. Solutions of the amines in sulfuric acid were yellow-green, changing to a rather bright blue when diluted with water.

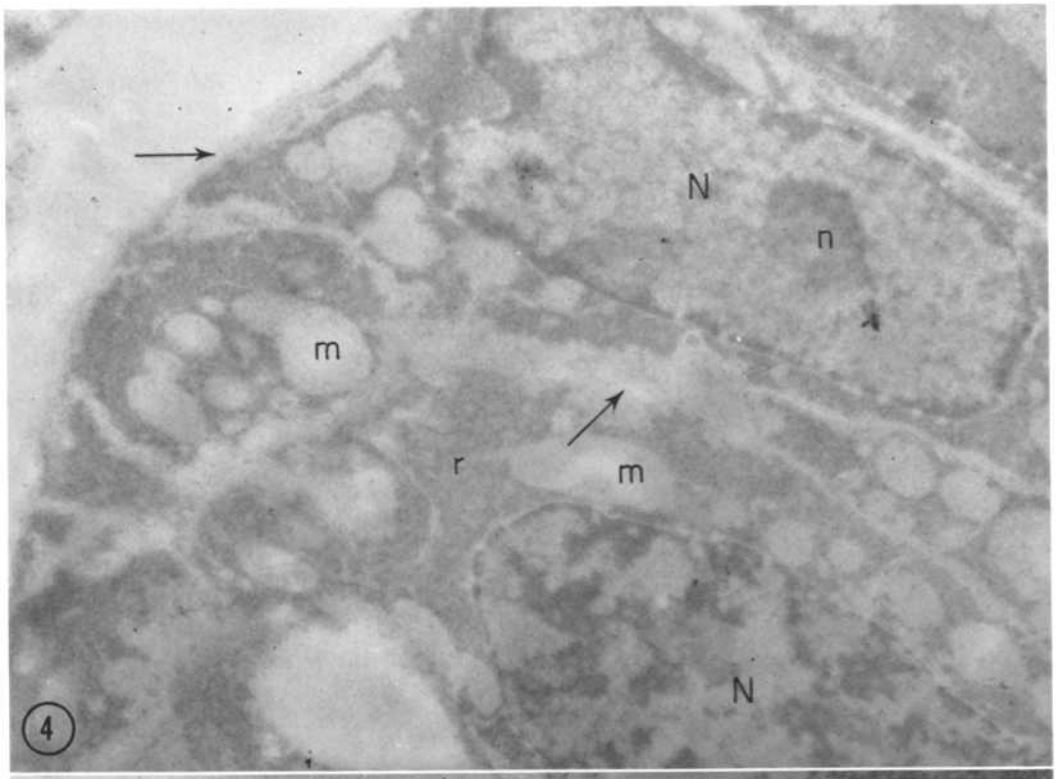
Specific information on each of the diazophthalocyanins synthesized is given in Table I. These compounds coupled with naphthols relatively rapidly at acid pH, but increases in coupling rate with increasing pH were sharply limited for some of these compounds by isodiazotate formation at pH levels approaching neutrality. Coupling rates were not markedly influenced by the contained metal atom, nor did they vary consistently with the number of diazonium groups present on the molecule. In comparative tests, coupling was generally faster with 4-diazophthalocyanins than with 3-diazophthalocyanins, and faster, regardless of pH, with α- than with β-naphthol. The majority of the azo dyes obtained were slightly to completely soluble in alcohol, acetone, and propylene oxide; a few, particularly those derived from coupling of mono-(4-diazo-) Pb, tri-(4-diazo-) Pb, or tri-(3-diazo-) Cu phthalocyanin with α-naphthol, were

FIGURES 4 AND 5 Sections of intestinal epithelium, fixed in glutaraldehyde and embedded in Epon. Both figures are taken from the extreme periphery of the original frozen sections, sites at which maximal staining might have been expected to occur.

FIGURE 4 Unstained section. × 13,000.

FIGURE 5 Section from material stained for 2 hours at pH 5 with mono-(3-diazo-) Pb phthalocyanin. × 13,000.

In the unstained section (Fig. 4) the base of the intestinal epithelium is shown. Underlying connective tissue, artificially separated from the epithelium, is barely visible at the upper left. Nuclei (*N*) containing inconspicuous nucleoli (*n*) are visible together with mitochondria (*m*) and numerous small particles, presumably ribosomes (*r*). At the base and lateral surfaces of the cells, linear densities of plasma membranes (arrows) are barely visible or apparently not detectable. After reaction with phthalocyanin (Fig. 5), the nuclei, nucleoli, and ribosomes appear increased in density. In addition, the ground cytoplasm, particularly apparent in processes of underlying connective tissue cells (*ct*), and in the lateral aspects of the intestinal epithelial cells, appears to have gained in contrast with respect to the adjacent embedding medium. In these sites, the cells appear outlined by clearly visible linear densities (arrows) which may represent stained plasma membranes.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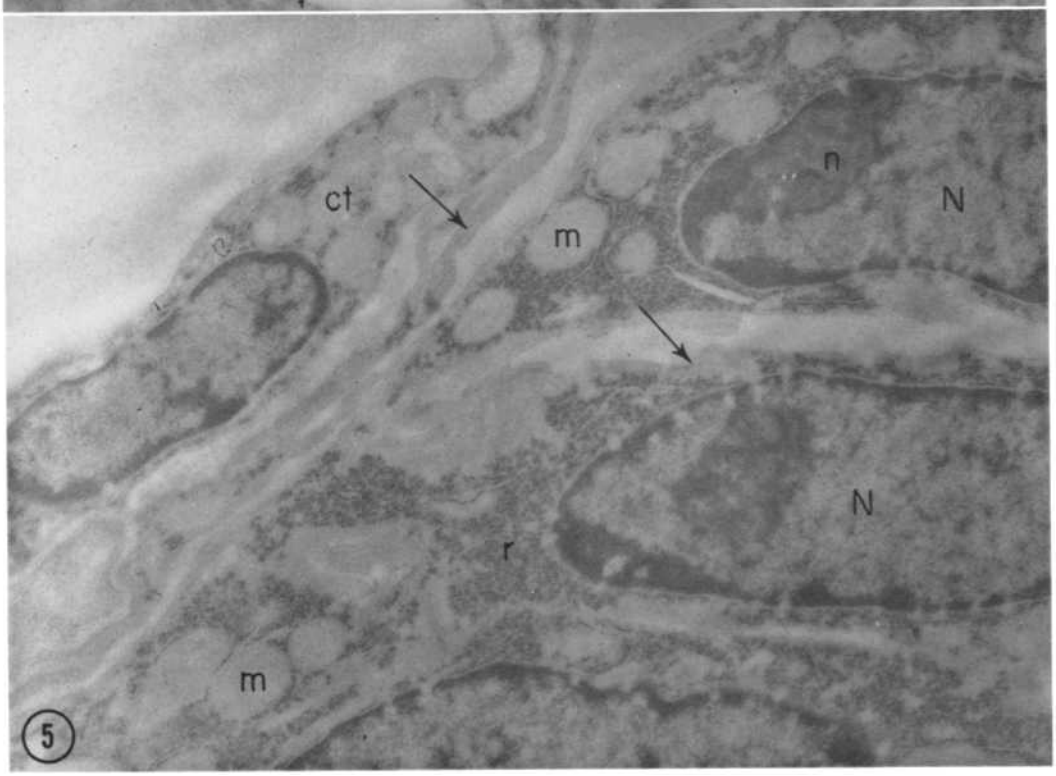
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either completely insoluble in these solvents or appeared to contain insoluble components.

### *Tissue Experiments: Direct Coupling with Tissue*

The distribution of density in tissues fixed in glutaraldehyde has already been examined in some detail (22), and, with one exception, the results obtained with unstained material confirmed the published findings. Most inherent density was contributed by particulate components, presumably nucleoprotein, both in the nucleus and associated with the endoplasmic reticulum (Fig. 2). Mitochondrial cristae were barely visible, but other linear membranous densities of the sort seen after osmium fixation were absent. Although the membranous component of the endoplasmic reticulum was not visible, a single, moderately dense line often appeared between the rows of linearly arranged ribosomes in the endoplasmic reticulum, as well as within the nuclear envelope. This "intermediate line" was most often seen in pancreatic exocrine cells, but at times was also visible in the endoplasmic reticulum of other cell types. It resembled the mitochondrial component described by Pease (23) in formalin-fixed material.

Sections which had been treated with diazophthalocyanins at pH 5 superficially resembled unstained sections in patterns of density distribution (Fig. 3). They differed from unstained material in that many densities had been increased, with a resulting increase in contrast in some cellular components. Although the difference between stained and unstained material was immediately apparent in the fluorescent screen of the microscope, it was particularly striking when sections of comparable thickness were photographed under similar conditions (Figs. 2 to 5). The increased electron opacity was most noticeable in components of the nucleus and in the endoplasmic reticulum, in which both the RNP particles and the intermediate line were increased in opacity (Fig. 10). Mitochondrial membranes appeared more prominent after staining (Fig. 3), and membranes of the Golgi apparatus were often visible. Pancreatic zymogen granules, which in unstained sections appeared as bodies of rather low inherent density (Fig. 2), were increased in opacity (Fig. 3).

At the periphery of the sections, where reaction to saturation had presumably occurred, the hyaloplasm was increased in opacity (Fig. 5). At times, in these locations, cells were outlined by a linear density, which was particularly apparent after staining with Pb diazophthalocyanin, and less marked, although still present, after staining with Cu or Mg diazophthalocyanin. Further interior in the sections, this density was less striking but often suggested. Such linear densities, which might have represented stained plasma membrane, were not seen in unstained material.

The changes produced by staining with diazophthalocyanins were greater when tissues were incubated at pH 5 than at lower pH levels. Increases in contrast were somewhat greater when Pb

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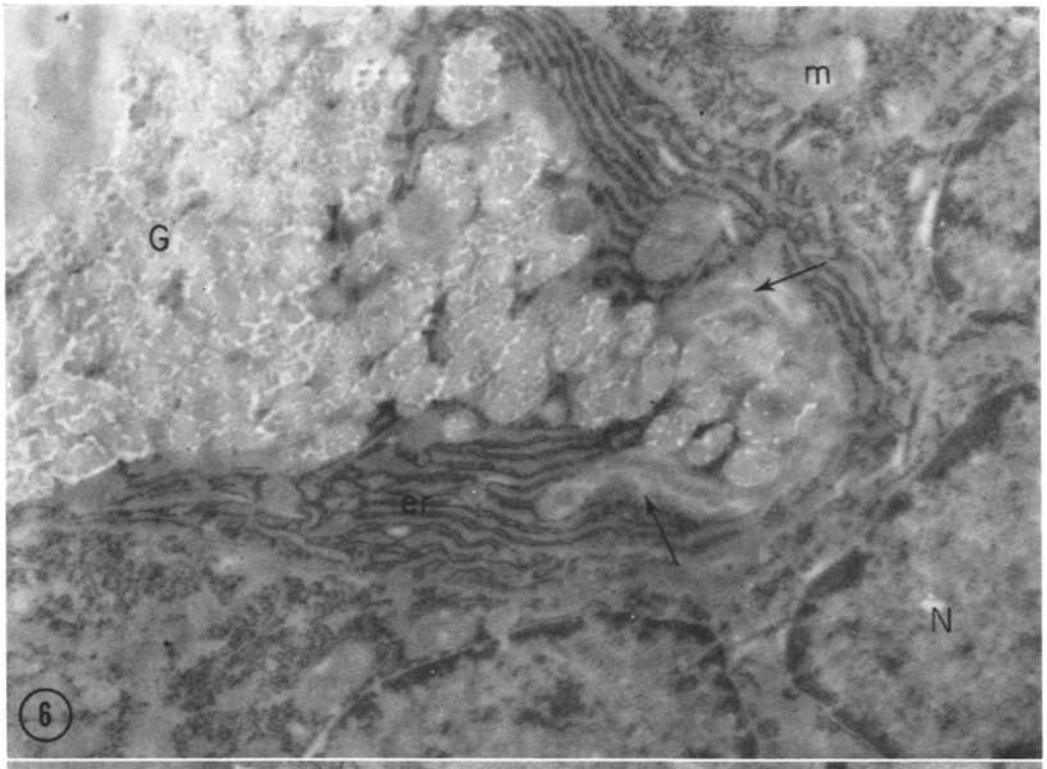
FIGURES 6 AND 7 are of goblet cells from intestinal epithelium. In Fig. 6, the section was stained for 2 hours in mono-(3-diazo-)me Mg phthalocyanin, pH 5; in Fig. 7, the section was stained with 0.5 per cent Alcian Blue for 2 hours at pH 3.

After both staining procedures, staining of chromatin and of elements of the endoplasmic reticulum (*er*) is a conspicuous feature. In Fig. 6, showing tissue treated with diazophthalocyanin, the density of the secretory material within the goblet cell (*G*) is only slightly increased over that of unstained material. In Fig. 7, showing tissue treated with Alcian Blue, the secretory material within both the goblet (*G*) and the Golgi apparatus (*g*) is markedly more electron opaque than in Fig. 6. After staining with diazophthalocyanin (Fig. 6), membranes of the Golgi complex are faintly visible (arrows) and moderately dense cytoplasm surrounds the most basal secretory droplets. In Fig. 7, showing a section stained with Alcian Blue, the Golgi lamellae are not seen.

Note also the appearance of mitochondria (*m*) in the two sections. In Fig. 6, mitochondria appear as bodies having roughly the same density as the surrounding cytoplasm. Cristae are very faintly visible, as is usual in unosmicated sections of intestinal epithelium. After staining with Alcian Blue, as in Fig. 7, mitochondria appear as amorphous bodies, without visible cristae, and are distinctly greater in density than the surrounding cytoplasm. Fig. 6, × 17,000. Fig. 7, × 19,000.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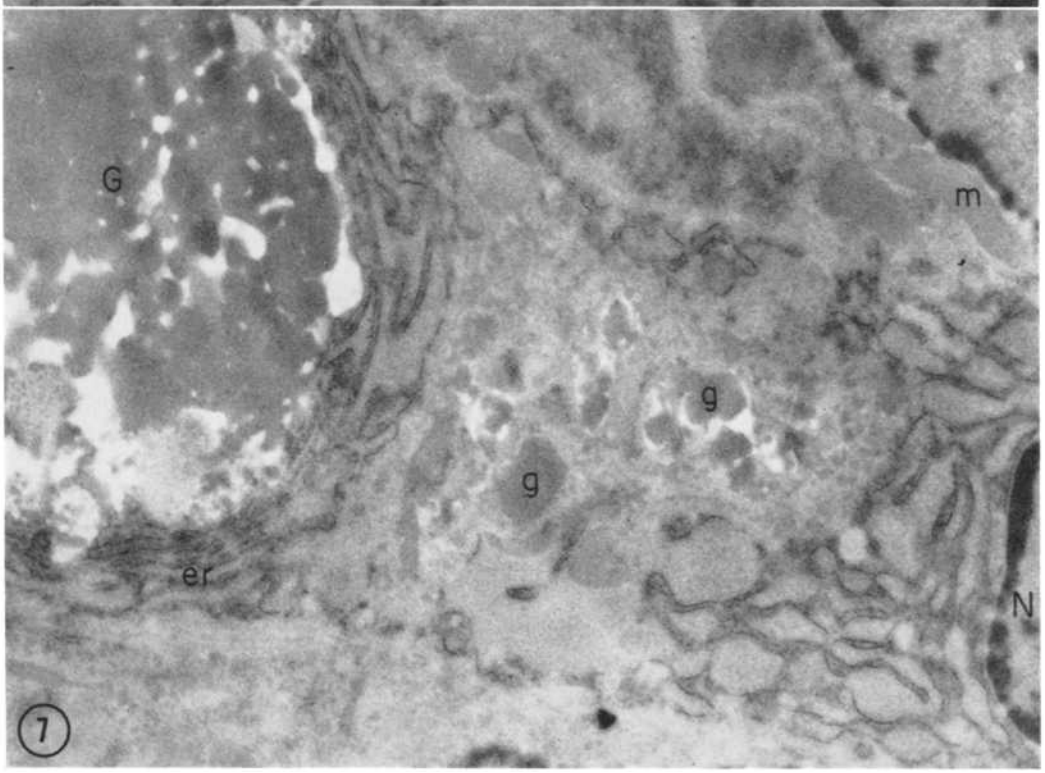
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diazophthalocyanins were employed (Figs. 3 and 5) than when Cu (Fig. 10) or Mg (Fig. 6) phthalocyanins were used as reagents, although no qualitative differences between the density increases induced by the three analogs were observed.

When tissue sections were stained with Garnet GBC, nuclei at the periphery of the section appeared somewhat increased in density. If sections were stained with Garnet GBC followed by lead diazophthalocyanin (Fig. 1), no alterations were seen in the density distribution from that produced by Garnet GBC alone. After treatment with inorganic metal salts at pH 6, only nuclear material and ribosomes were increased in opacity, and these increases were relatively slight compared with those produced by diazophthalocyanins.

After staining with Alcian Blue, the pattern of tissue electron opacity was qualitatively different from that of unstained material or from that seen after reaction with diazophthalocyanins. Both the nuclei and the particulate components of the endoplasmic reticulum were strikingly increased in opacity at higher pH. Mitochondria appeared as amorphous dense profiles (Fig. 7) which, apart from their shape, size, and position, lacked the usual identifying characteristics of the organelle. In material treated with Alcian Blue at pH 2, nucleoprotein and mitochondrial staining was markedly reduced.

The different results produced by staining with Alcian Blue and by reaction with diazophthalocyanins were perhaps most dramatically illustrated at the striated border of the intestinal epithelium. In tissues which had been stained with Alcian Blue, the striated border appeared as a negative image (Fig. 8) with no apparent staining of either microvilli or the subcuticular zone, but with staining apparently confined to material lying between the microvilli. This was in sharp contrast to the image seen after reaction with diazophthalocyanins, in which the striated border appeared as a positive image, due to increases in density of the microvilli (Fig. 9). After staining with Alcian Blue, goblet cell mucins were increased in opacity (Fig. 7). At the base of the epithelium, the basement membrane proper appeared unstained, but beneath it, and in the spaces between connective tissue cells, small aggregates of amorphous intercellular material were increased in density. After reaction with diazophthalocyanins, goblet cell mucins (Fig. 6) and intercellular materials were only slightly increased in opacity to the electron beam. After either of these staining procedures, collagen fibrils appeared as sharply outlined negative images.

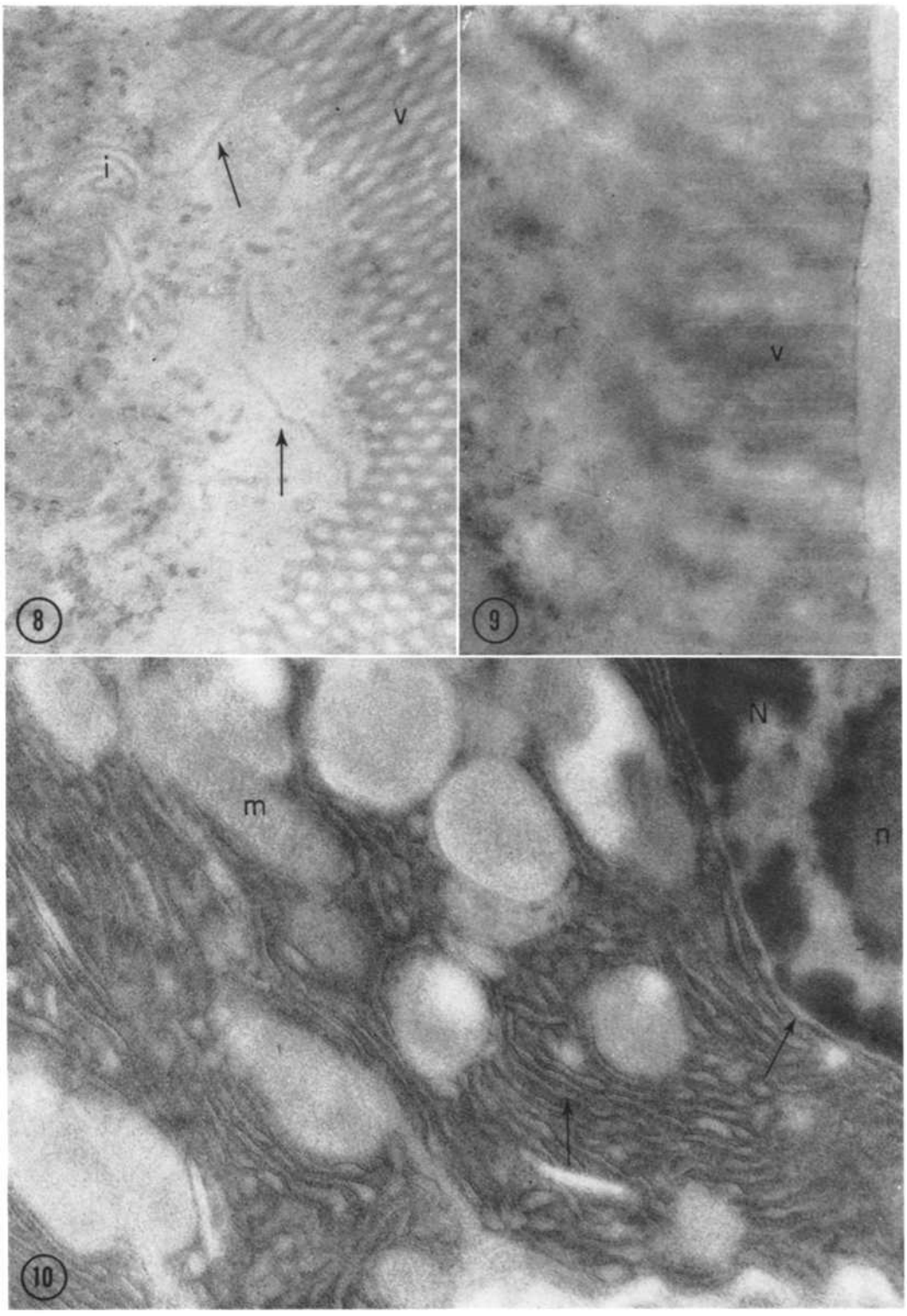
### *Acid Phosphatase*

Only a few of the diazophthalocyanins tested had azo dyes which were insoluble in the organic solvents usually used in electron microscopy (Table I). One of these, tri-(4-diazo-) lead phthalocyanin, was tested in experiments with enzymes. After incubations of frozen sections with $\alpha$-naphthyl phos-

FIGURE 8 Oblique section of glutaraldehyde-fixed duodenum stained for 2 hours with 0.5 per cent Alcian Blue, pH 3. At the right of the section, microvilli (*v*) appear as negative images apparently due to preferential uptake of stain by material between them. Material in the intercellular spaces at the lateral aspects of the epithelial cells is increased in density, and the outline of the lateral interdigitions (*i*) is clearly visible. This outline is fainter just above the lateral interdigitations, at the site of the terminal bar (arrows). The subcuticular zone is relatively unaffected by the staining procedure. Beneath it, small clusters of ribosomes and short segments of rough-surfaced endoplasmic reticulum are intensely stained. × 14,500.

FIGURE 9 Section of glutaraldehyde-fixed intestinal epithelium stained with mono-(3-diazo-) Pb phthalocyanin for 2 hours at pH 5. After this procedure, microvilli appear as positive images, while material in intervillous spaces remains unstained. × 150,000.

FIGURE 10 Section of pancreas stained with mono-(3-diazo-) Cu phthalocyanin for 2 hours at pH 3. The nucleus (*N*) and part of the nucleolus (*n*) of an exocrine cell appear at the upper right of the figure. Mitochondria (*m*) and zymogen granules are surrounded by profiles of intensely stained rough-surfaced endoplasmic reticulum. Within many of these and within the nuclear envelope, a single "intermediate line" is seen (arrows).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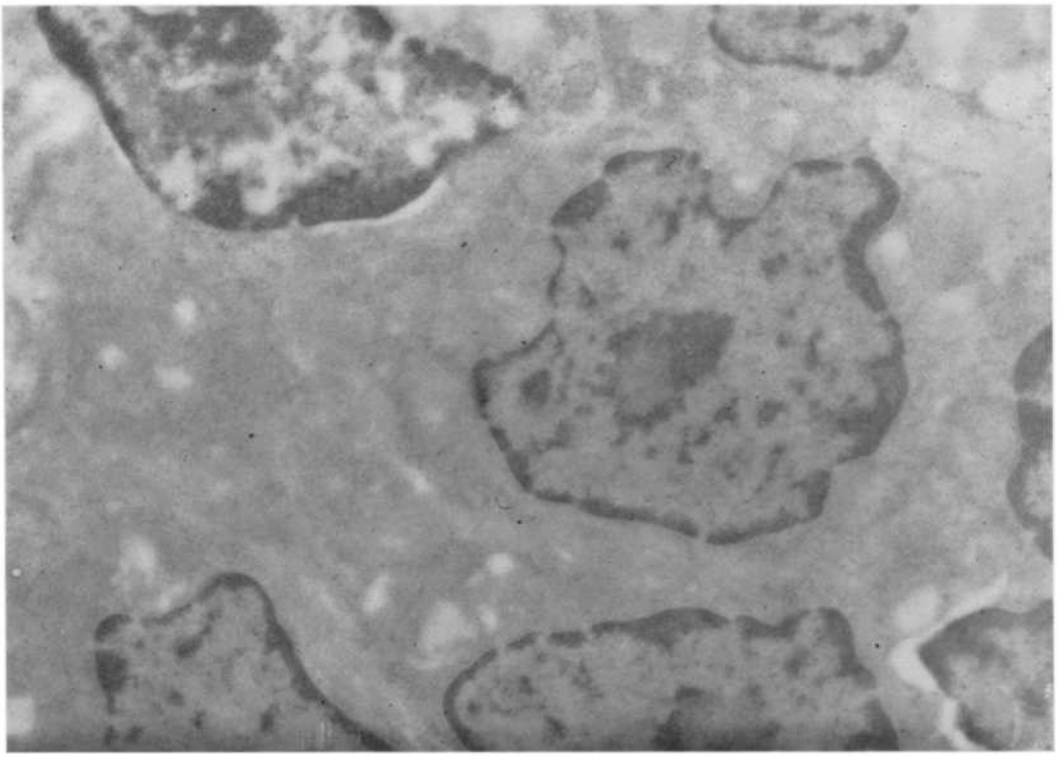


FIGURE 11 Section of intestinal epithelium fixed in glutaraldehyde and stained with Garnet GBC prior to staining with mono-(3-diazo-) Pb phthalocyanin for 2 hours at pH 5. Although nuclear chromatin shows a slight increase in density due to the first reaction, the expected increase in density due to the second was blocked. × 13,000.

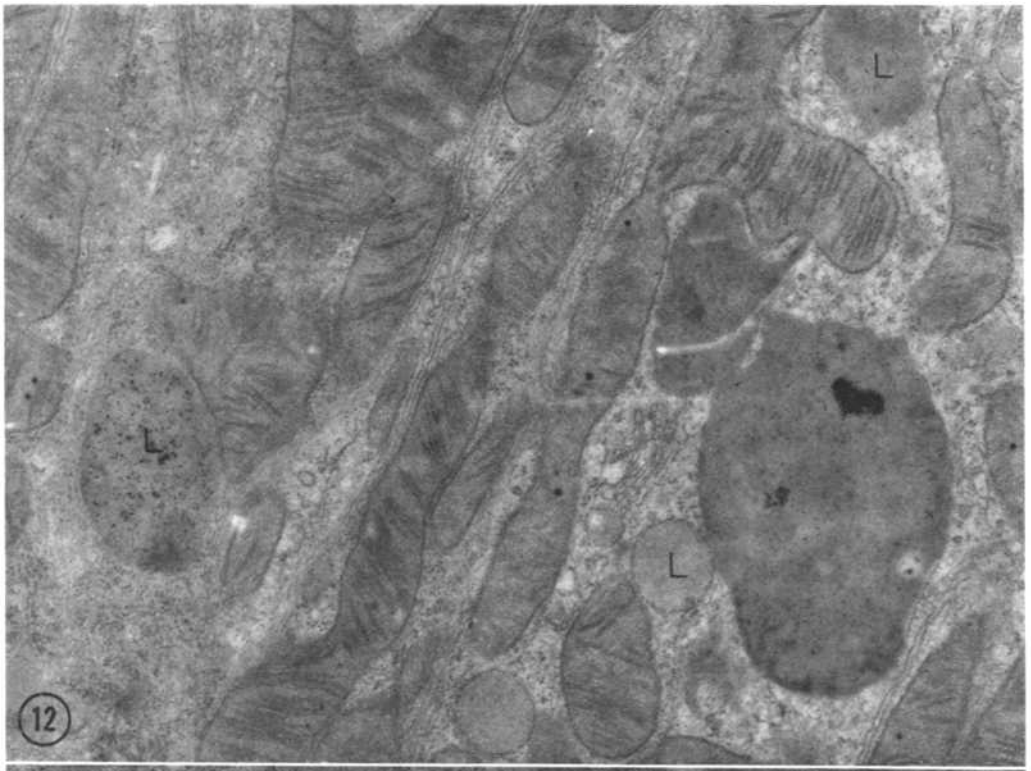
phate and this diazotate, small discrete deposits of pale orange-red final product were seen in the cytoplasm of the kidney tubule epithelial cells. The color was barely visible in thick sections of Epon-embedded material. No color changes were noted after treatment of sections with ammonium sulfide.

Examination of thin sections in the electron microscope revealed precipitated material within

FIGURES 12 AND 13 Sections of rat kidney fixed in glutaraldehyde, treated with tri-(4-diazo-) Pb phthalocynin at pH 5 for 30 minutes, and subsequently postfixed for 1 hour in 2 per cent buffered $OsO_4$.

FIGURE 12 In this section of distal convoluted tubule, mitochondria are seen oriented parallel to the deep infoldings of plasma membrane of the cell. In addition, several types of dense bodies (*L*) are included in the section. Some of the larger dense bodies contain small flecks of extremely dense material scattered throughout a matrix of moderate and somewhat variable density. Smaller bodies, also bounded by a single membrane, retain a homogeneous matrix of low electron opacity. × 28,000.

FIGURE 13 Control section of rat kidney incubated in medium containing $\alpha$-naphthyl phosphate and tri-(4-diazo-) Pb phthalocyanin, at pH 5, and NaF ($10^{-3}$ M). No final product is present, and dense bodies show their natural density. Compare with Figs. 14 to 16. × 31,000.


L
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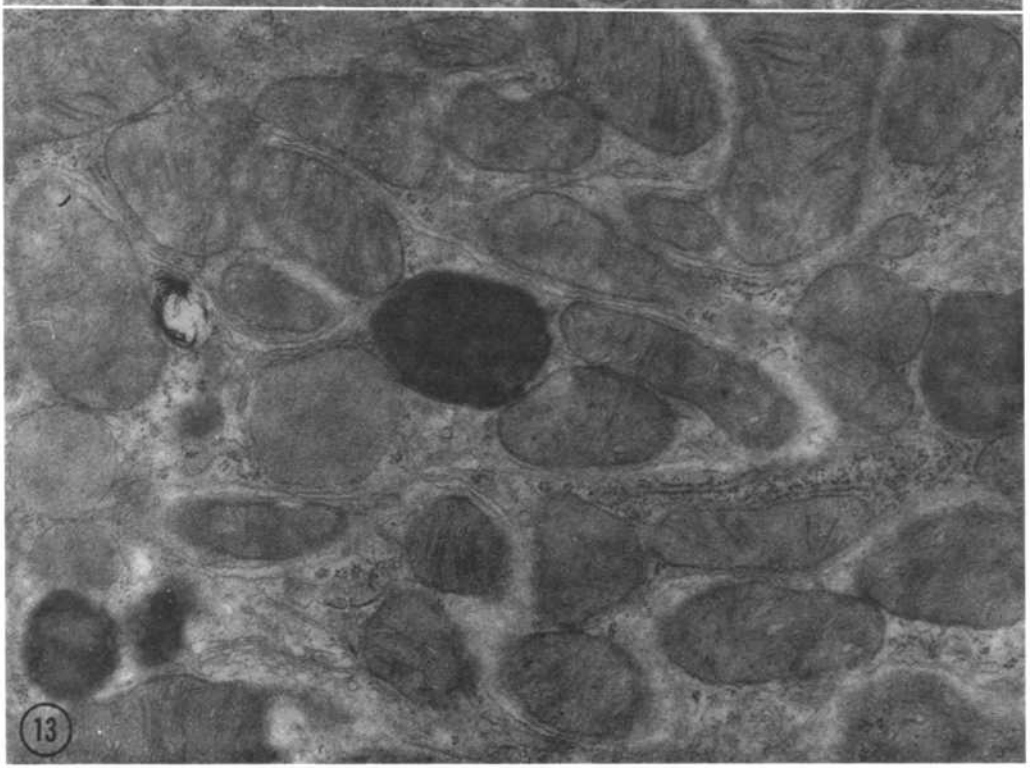
12


13

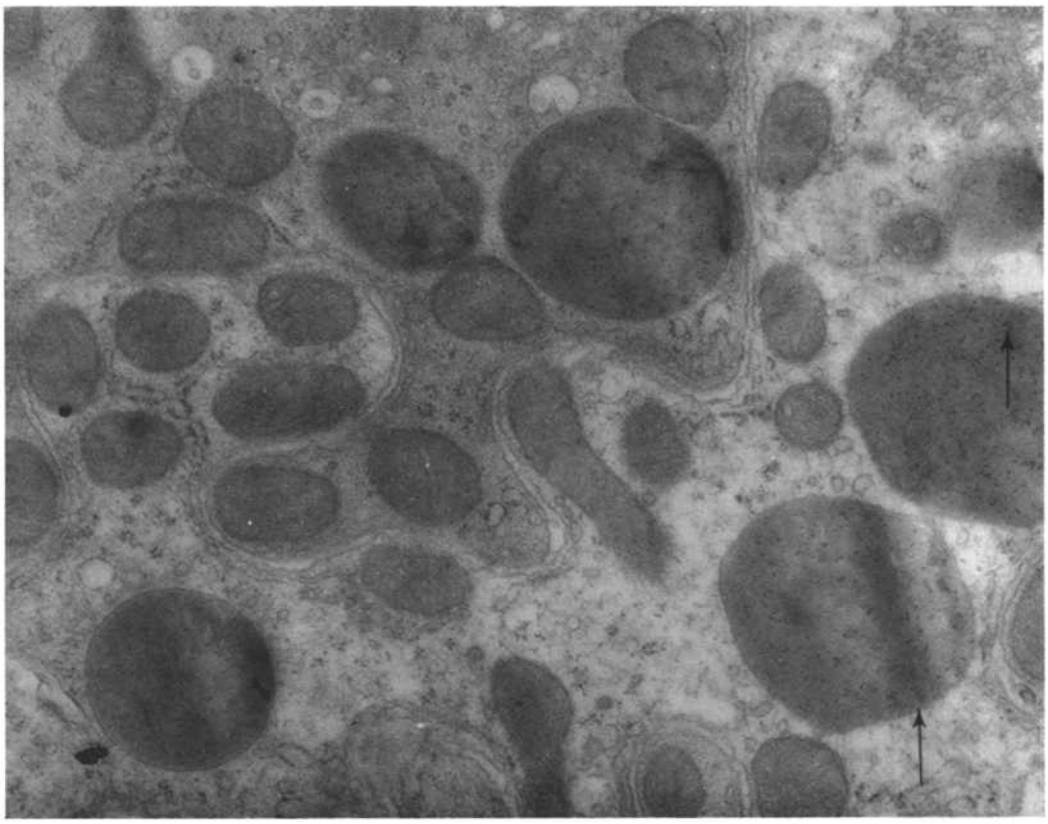
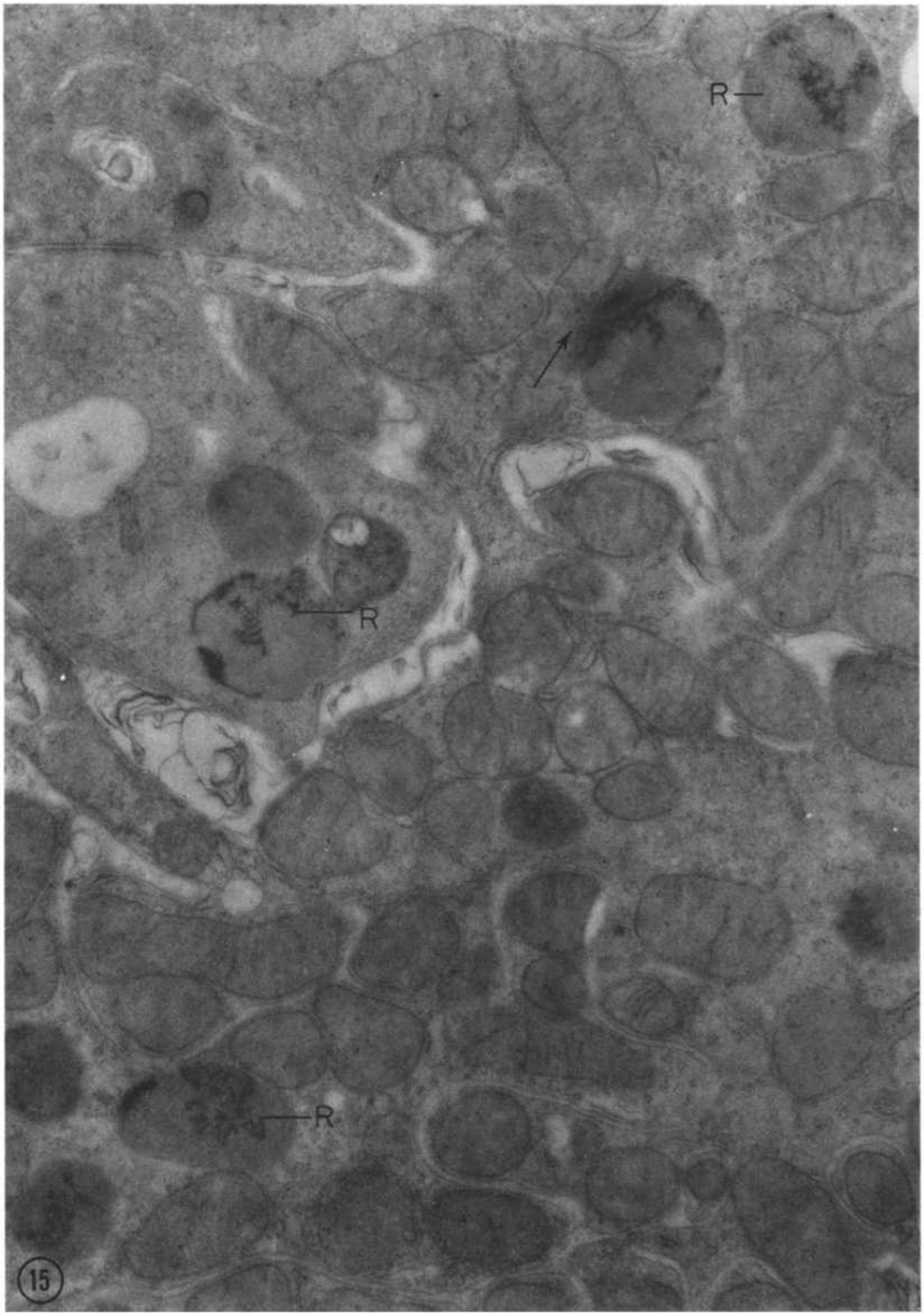


FIGURE 14 Section incubated for 30 minutes in medium, at pH 5, containing 2 mg/ml tri-(4-diazo-) Pb phthalocyanin and 1 mg/ml $\alpha$-naphthyl phosphate. This field, at the periphery of the original frozen section, contains portions of several proximal tubule cells. Mitochondria and small vesicular elements are visible. Some of the latter are scattered throughout the cytoplasm, while others lie in chain-like fashion parallel with the plasma membrane. In addition, small clusters of ribosomes and short segments of rough-surfaced endoplasmic reticulum are seen, as well as Golgi complex and several multivesicular bodies. Several dense bodies contain small, angular flecks of moderately electron-opaque material scattered throughout the matrix which have an appearance quite distinct from that of the endogenous densities ordinarily found within the organelle. These deposits, found only in sections incubated with substrate and diazophthalocyanin, are presumed to represent final product, precipitated azo dye. In a few sites, coalescence of precipitate (arrows) has resulted in an appearance similar to that seen in Fig. 15. × 22,000.

FIGURE 15 Oblique section of distal convoluted tubule incubated for 30 minutes, at pH 5, with tri-(4-diazo-) Pb phthalocyanin and $\alpha$-naphthyl phosphate. This field is further interior in the frozen section than that in Fig. 14. In addition to the other organelles usually found within the renal tubule cells, several dense bodies appear in the field. The majority of these contain aggregates of angular, moderately electron-opaque material, presumably precipitated azo dye (*R*———). With one exception (arrow), these precipitates are confined to the dense bodies. No localization of final product to other cytoplasmic structures is seen. × 20,000.


R
R
R
15

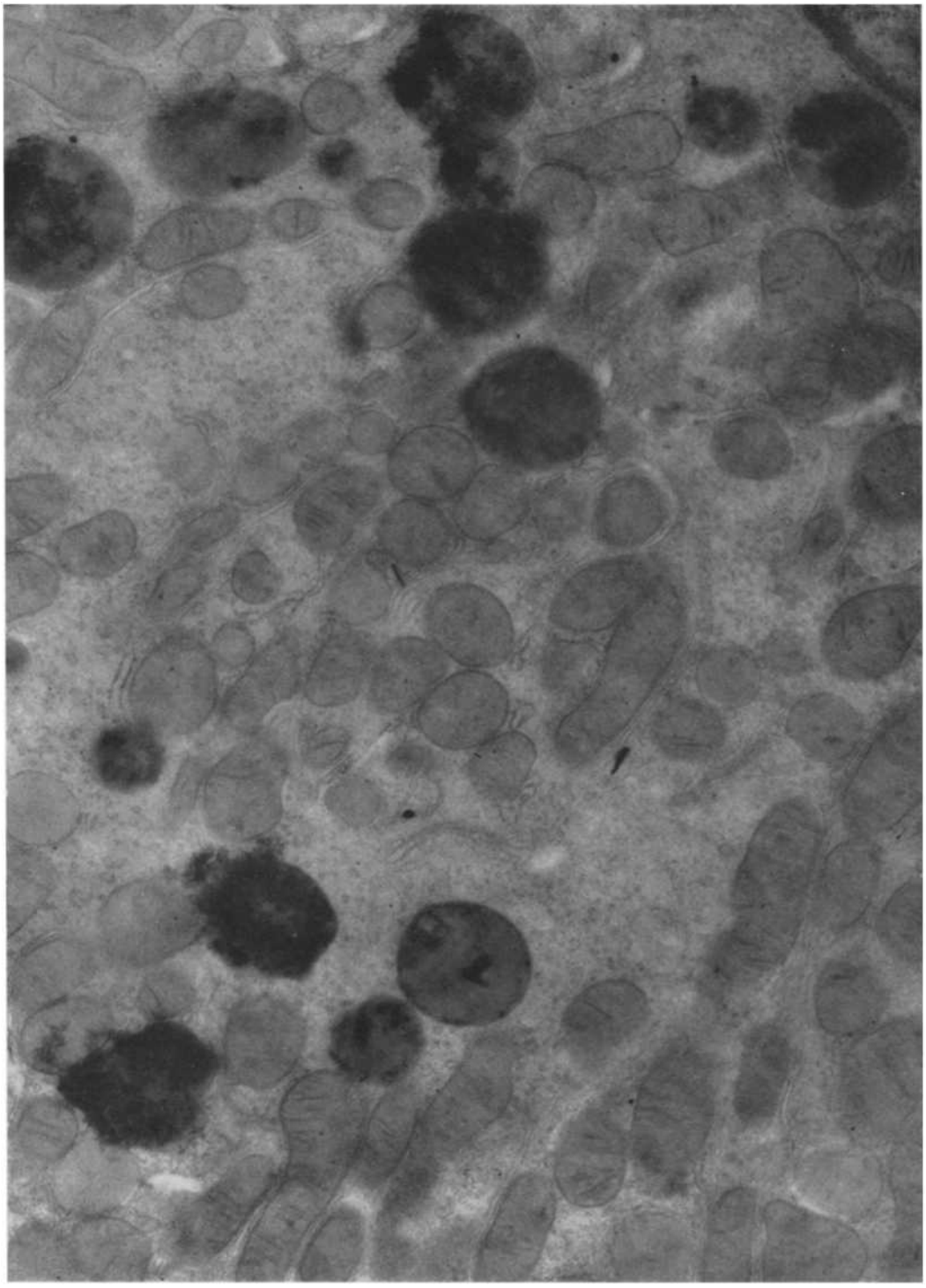


FIGURE 16 Section of proximal tubule from material incubated for 1 hour with diazophthalocyanin with double the usual amount of substrate in an attempt to obtain overreaction and diffusion of final product. The majority of the dense bodies in the field are overlaid by deposits of azo dye, which retains the configuration seen in less intensely reacted material. Although these precipitates extend beyond the confines of the dense bodies, no evidence of migration of final product with artificial localization to the nucleus or to other cytoplasmic structures is seen. × 21,500.

or closely applied to the dense bodies of the renal tubules. The appearance of these deposits, which were presumed to represent precipitated azo dye, varied somewhat from the outer to the inner regions of the frozen sections. At the outermost parts of the sections, the amorphous matrix of many dense bodies was stippled with small, angular flecks of material of moderate electron opacity (Fig. 14). Because of irregularities in their size and shape, such deposits could easily be distinguished from the endogenous, finely granular, dense deposits found in some dense bodies of the distal convoluted tubule cells (Fig. 12). They did not occur after treatment of sections with diazophthalocyanins alone (Fig. 12), and were few or absent if sections were incubated in a medium containing both substrate and the inhibitor, NaF (Fig. 13). These deposits were not seen if $\beta$-glycerophosphate were used as substrate instead of $\alpha$-naphthylphosphate.

Further interior in the sections, deposits of final product, presumed to be azo dye, appeared as somewhat angular, branching aggregates of moderately electron-opaque material. Under normal conditions of incubation (0.5 mg substrate/ml; 1 mg diazophthalocyanin/ml), precipitated azo dye was found almost exclusively within the dense bodies (Fig. 15). Only if deliberate attempts were made to obtain overreaction of sections by prolonging incubation and by doubling the usual substrate concentration was spread of final product beyond the dense bodies seen (Fig. 16).

## DISCUSSION

The primary question asked in this study was whether diazophthalocyanins were sufficiently electron-opaque to be useful histochemical reagents for electron microscopy. The results indicated that easily detectable density increases were produced by reactions with these salts. If staining with the diazonium solutions were due to reaction of diazonium salt with tissue proteins, it could be assumed (as a first approximation) that the reaction was occurring with available groups of proteins (residues of tyrosine, tryptophan, and histidine (18)). With a few exceptions, these amino acids are present in tissue proteins in relatively low concentrations. Hence, the degree of staining observed could have resulted from reaction of diazonium salt with relatively few sites in the tissue sections. Therefore, if detectable density increases could be produced in this reaction, useful density increases could probably be obtained by coupling these diazotates to other, more specific, naphtholic reagents for the demonstration of groups in protein (24–28).

It was possible that the observed increases in density were not produced by reactions of the diazonium salts with groups in protein, but were due to some other staining reaction. The possibilities included staining by contaminant metal ions, staining by unknown impurities in the reaction mixture, and staining by the diazophthalocyanins *via* some molecular function other than the diazonium group. Although washed and recrystallized product was used, very small quantities of metal ions were still present. However, the density increases produced by staining with diazophthalocyanins were qualitatively different from and greater than those produced by staining, with approximately 40 times the concentration of metal ions found in the diazo solutions. They were by no means limited to particulate components of the nucleus and endoplasmic reticulum—sites which were most affected by metal ions. In fact, patterns of density were most altered at sites (cell surfaces, contents of cisternae of the ER, pancreatic zymogen granules) at which little, if any, staining by metal ions occurred in unosmicated material.

Staining by unknown, undiazotized impurities in the final product again could not be excluded altogether, but was rendered unlikely by the fact that pretreatment of sections with one diazonium salt (Garnet GBC) effectively blocked subsequent reaction with the diazophthalocyanin mixtures. Since different results were obtained with diazophthalocyanins and with Alcian Blue, it also appeared that water-soluble phthalocyanins were dependent for their staining properties on their substituted groups, and that the phthalocyanin moiety itself did not enter into the reaction. In addition, the qualitative similarities in staining after Mg, Cu, and Pb diazophthalocyanins suggested that the reaction did not vary qualitatively with the chelated metal atom. For these reasons, it was concluded that most of the observed density increases were due to combination of tissue constituents with diazophthalocyanins (or closely related compounds) reacting as diazonium salts, and were not due to other staining reactions.

An incidental result, which warrants further investigation, concerns the use of Alcian Blue for electron microscopy as a stain possessing some degree of specificity for acid mucopolysaccharides. Initially, the experiments with Alcian Blue were used as a means of determining, in a preliminary

way (29), whether increases in electron opacity could be produced by reaction with phthalocyanins. Later, these results were used to assess the mechanism of tissue reaction with phthalocyanins. Although the specificity of Alcian Blue staining was not of primary concern in these studies, the electron microscopic results were fairly consistent with those obtained with light microscopy (30, 31). They can be presumed, therefore, to parallel these results in having a moderate, though limited specificity for acid mucopolysaccharides. Hence, this dye may be tentatively added to the list of stains for electron microscopy which have some degree of specificity (reviewed in 32, also 33–35).

If metal atoms are to be used to increase the electron opacity of azo dyes, the use of diazonium salts already containing firmly chelated metal atoms would seem to have certain advantages over methods in which chelation is carried out after coupling. With such methods, non-specific staining by metal salts would, of necessity, always be a problem. With this approach, in which firm chelation is accomplished before the histochemical reaction, non-specific staining due to reaction with metal ions can be minimized. With both approaches, however, increases in density due to coupling of diazonium salts with tissue constituents must be differentiated from density increases due to precipitation of final product.

Although sublimation of phthalocyanins is known to occur at high temperatures *in vacuo* (36), in our experience the "staining" produced by Alcian Blue, by the reaction of diazophthalocyanins with groups in protein, and by the azo dyes produced by coupling with naphthol in the acid phosphatase reaction was stable to prolonged observation at 50 or 80 kv. Repeated photography of all material at these accelerating voltages showed identical distributions of density increases or of final product.

Somewhat different requirements are imposed on diazonium salts to be used for localizing non-enzymatic constituents at a fine structural level and those to be used for enzyme histochemistry. The tissue experiments with diazophthalocyanins were, correspondingly, divided into two groups. The results of the first group of experiments have already been discussed. In them, density production, stability, and the mechanism of reaction of diazophthalocyanins were primary considerations. In the second group of experiments, considerations of coupling rate and insolubility of the azo dye produced assumed crucial importance, and the diazonium salt used in those studies was selected because of the insolubility of its derivative azo dyes. Although the azo dyes derived from one other lead phthalocyanin (mono-(4-diazo-) Pb phthalocyanin) possessed similar favorable solubility characteristics, the salt used (tri-(4-diazo-) Pb phthalocyanin) was selected because it had more than one diazonium group which theoretically might favor this type of investigation. Although the relatively faint colors of the azo dyes made light microscopic study somewhat difficult, the electron microscopic results gave ample confirmation of their suitability as reagents for electron microscopy, since relatively good localization of enzyme activity could be obtained under less than optimal conditions. On this basis, the present investigation indicates that electron-opaque diazotates have some promise in studies requiring a combined histochemical and electron microscopic approach. Further reagents will be synthesized and tested.

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