IMMUNOCYTOCHEMICAL LOCALIZATION OF SECRETORY PROTEINS IN BOVINE PANCREATIC EXOCRINE CELLS

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ABSTRACT

The bovine exocrine pancreatic cell produces a variety of enzymes and proenzymes for export. Biochemical studies by Greene L. J., C. H. Hirs, and G. E. Palade (*J. Biol. Chem.* 1963. 238:2054) have shown that the mass proportions of several of these proteins in resting pancreatic juice and zymogen granule fractions are identical. In this study we have used immunocytochemical techniques at the electron microscope level to determine whether regional differences exist in the bovine gland with regard to production of individual secretory proteins and whether specialization of product handling occurs at the subcellular level. The technique used is a modification of one previously reported (McLean, J. D., and S. J. Singer. 1970. *Proc. Natl. Acad. Sci. U. S. A.* 65:122; Kraehenbuhl, J. P., and J. D. Jamieson. 1972. *Proc. Natl. Acad. Sci. U. S. A.* 69:1771) in which immunocytochemical reagents are applied to thin sections of bovine serum albumin-imbedded tissue and zymogen granule fractions. A double antibody technique was used in which the first step consisted of rabbit $F(ab')_2$ antibovine secretory protein and the detection step consisted of sheep $F(ab')_2$ antirabbit $F(ab')_2$ conjugated to ferritin. The results showed that all exocrine cells in the gland, and all zymogen granules and Golgi cisternae in each cell, were qualitatively alike with regard to their content of secretory proteins examined (trypsinogen, chymotrypsinogen A, carboxypeptidase A, RNase, and DNase). The data suggest that these secretory proteins are transported through the cisternae of the Golgi complex where they are intermixed before copackaging in zymogen granules; passage through the Golgi complex is apparently obligatory for these (and likely all) secretory proteins, and is independent of extent of glycosylation, e.g., trypsinogen, a nonglycoprotein vs. DNase, a glycoprotein.

Over the past three decades the enzymatic and biochemical properties of secretory proteins from the mammalian exocrine pancreas have been studied extensively (e.g., see references 5, 9, 15, 23, 31). As a consequence, we now have a reasonably detailed catalogue of the secretory products of this gland. Perhaps the most detailed accounting has been carried out on the guinea pig gland which produces at least 19 different polypeptide chains, 13 of which have been characterized with regard to enzymatic activity (31).

At the same time, numerous studies on the intracellular processing and mechanism of discharge of exportable proteins from the guinea pig exocrine pancreas have been carried out (summarized in references 14, 27). These investigations

have led to a general hypothesis which states that the transport and release of secretory products occurs via membrane-enclosed compartments of the exocrine cell. Recent detailed studies on individual exportable proteins in the same system have indicated kinetic parallelism for the transport and release of the majority of secretory proteins (33, 35, 37). Possible exceptions to this pathway have been suggested by others for several secretory proteins, leading to a reevaluation of the hypothesis mentioned (30).

Both biochemical and functional biologic studies have been carried out on total extracts of the gland, its collected secretion, and isolated subcellular fractions, or by autoradiography of intact cells whose exportable proteins have been radioactively labeled. These approaches necessarily represent the average of kinetic events or biochemical composition of the whole gland and tell us little of the function of individual cells in the population. Some of the physiological data leading to the notion of nonparallel transport of secretory proteins thus could be accounted for by regional differences in enzyme production within the gland, variations in the composition of secretory proteins among individual secretory granules in a given cell, or, as mentioned, alternate pathways for the transport of secretory proteins and individualized regulation of release by secretagogues (30).

With regard to these possibilities, two immunocytochemical studies at the electron microscope level have appeared which indicate at least qualitatively that each exocrine cell of the bovine pancreas produces trypsinogen (18) and ribonuclease (26), and that most, if not all, zymogen granules in a given cell contain these two proteins. In this report we extend our observations on the immunocytochemical localization at the electron microscope level of secretory proteins in the bovine pancreas to include four proteins in addition to our previously reported localization of trypsinogen (18). Additionally, the studies have been carried out on both tissue blocks and zymogen granule fractions isolated from the same gland, the latter obviating some of the sampling problems inherent in the use of tissue blocks. Finally, several technical improvements have been made in the localization procedure and are reported. The results lead to the conclusion that at any one time all cells in the gland and all zymogen granules and elements of the Golgi complex in a given cell are qualitatively identical with regard to their content of several secretory proteins.

MATERIALS AND METHODS

Materials were from the following sources: crystalline bovine serum albumin (BSA), rabbit γ-globulin, and 2 × crystallized horse spleen ferritin (Miles Laboratories, Inc., Kankakee, Ill.). Ferritin was further purified as described (19). Bovine pancreatic trypsinogen (1 × crystallized, EC 3.4.21.4), chymotrypsinogen A (5 × crystallized, EC 3.4.21.1), carboxypeptidase A (2 × crystallized, EC 3.4.12.2), RNase I (purest grade, EC 3.1.4.22), DNase I (chromatographically pure, EC 3.1.4.5), and pepsin (EC 3.4.23.1) (Worthington Biochemical Corp., Freehold, N.J.); Sephadex G25, G150, Sepharose 2B and 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.); Biogel A 5m (200-400 mesh) and Biogel A 50m (50-100 mesh) (Bio-Rad Laboratories, Richmond, Calif.); [*o*-(diethyl-aminoethyl)cellulose] (DEAE-cellulose) 52 (Whatman Chemicals, Div. W. & R. Balston, Maidstone, Kent, England); toluene-2,4-diisocyanate and 8% glutaraldehyde (E. M. Sciences, Fort Washington, Pa.); Aquacide II (Calbiochem., San Diego, Calif.); cyanogen bromide (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.). All other chemicals used were reagent grade. Lyophilized, desalted bovine pancreatic juice was the kind gift of Dr. L. J. Greene, Brookhaven National Laboratories, Upton, N.Y.

Immunization Schedules

RABBIT ANTISERA: 2.5 mg of bovine secretory proteins in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) were injected in multiple sites at weekly intervals for 4 wk. Blood was collected from the marginal ear vein at the end of the 5th wk and the serum stored at −20°C.

SHEEP ANTISERUM: sheep antirabbit $F(ab')_2$ antiserum was raised by six weekly injections of 2.5 mg rabbit $F(ab')_2$ in complete Freund's adjuvant. The serum was stored at −96°C. Rabbit $F(ab')_2$ was prepared as described below.

Preparation of $F(ab')_2$

FROM SERUM: 10 ml of specific rabbit or sheep antisera were adjusted to pH 4.5 with acetic acid and digested for 12 h at 37°C with 7 mg of pepsin. The digest was taken to pH 7.5 with 2.0 N NaOH. Specific $F(ab')_2$ from sheep antirabbit $F(ab')_2$ antiserum digests, and from rabbit antibovine secretory protein antiserum digests, were purified by affinity adsorption and gel filtration as described below.

FROM RABBIT γ-GLOBULIN: 100 mg of rabbit γ-globulin dissolved in 10 ml of acetate buffer pH 4.5 were digested for 12 h at 37°C with 1 mg of pepsin and

isolated as a 100,000 dalton fraction by gel filtration on Sephadex G150.

Affinity Adsorbents

Antigens were covalently coupled to cyanogen bromide-activated Sepharose 2B according to the method of Cuatrecasas (6). The composition of the adsorbents was 20 mg protein/g wet wt of packed gel for rabbit γ-globulin and for bovine secretory proteins. Adsorbents were stored at 4°C in 0.1 M Tris-HCl, pH 7.5 containing 0.05% sodium azide.

Preparation of First-Step Antibody Fragments

In contrast to our previous technique (18), first-step antibodies were rabbit $F(ab')_2$ rather than IgG, since the former do not bind to cellular Fc receptors and tend to aggregate less.

Reagents were prepared as follows: 10–20 ml of pepsin-digested antiserum were mixed with 4 g wet wt of appropriate insolubilized antigen for 1–2 h at 23°C with vigorous shaking. The charged adsorbent was exhaustively washed on a Buchner funnel with 0.1 M Tris-HCl, pH 7.5 until the A_{280nm} of the wash was <0.005. Antibody fragments and undigested antibodies were eluted with 30 ml of 3.0 M KSCN in 0.15 M KCl buffered to pH 6.0 with 10 mM sodium phosphate. The eluant was passed through a 0.45-μm Millipore filter (Millipore Corp., Bedford, Mass.) before use. The eluted antibodies, collected by suction of the adsorbent on a Buchner funnel, were rapidly concentrated to 10 mg/ml in an Amicon Ultrafiltration cell equipped with a PM-10 filter (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). $F(ab')_2$ was separated from undigested IgG aggregates, and KSCN by chromatography over a 1.5 × 100-cm column of Sephadex G150 (medium) eluted with 0.1 M Tris-HCl, pH 7.5 at 4.0 ml/h. The $F(ab')_2$ peak was collected, concentrated to 10 mg/ml protein, and stored in small samples at −96°C. For use, the sample was diluted to 0.1–1.0 mg/ml in 0.1 M Tris-HCl, pH 7.5 containing 1% BSA and centrifuged for 2 h at 50,000 rpm in a Beckman SW 50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.) to remove aggregates. Antibodies were not refrozen because this tended to induce aggregation.

Preparation of Second-Step Ferritin-$F(ab')_2$ Conjugates by Solid Phase Coupling

The technique used has been described before in detail (18) with the following modifications: first, rather than sheep antirabbit Fab, pepsin digests of sheep antiserum containing antirabbit $F(ab')_2$ prepared as described above were loaded onto rabbit γ-globulin Sepharose 2B and washed to remove nonadsorbed proteins. Coupling of the adsorbed $F(ab')_2$ to ferritin was performed with toluene-2,4-diisocyanate as before, except that ferritin was repurified before use (19).

Second, after elution of the conjugated and unconjugated $F(ab')_2$ with KSCN, the conjugate was purified in three steps: (*a*) the KSCN eluate was rapidly desalted on a Sephadex G-25 column (2 × 50 cm), run in 10 mM sodium phosphate buffer, pH 7.4 at 40 ml/h; (*b*) unconjugated $F(ab')_2$, ferritin conjugated $F(ab')_2$, and free ferritin were separated from each other by ion exchange chromatography on DEAE-cellulose (2). The elution profile of the DEAE column is shown in Fig. 1; (*c*) The conjugate was freed of polymerized ferritin by chromatography over a Sepharose 4B column as shown in Fig. 2. The portion of the eluted peak indicated by the horizontal bar was collected, concentrated to 1.0 mg/ml protein, and stored at 4°C in 0.1 M Tris-HCl, pH 7.5 containing 1% BSA and 0.05% sodium azide. Before use, the conjugate was centrifuged in a Beckman Microfuge, Model 52 (Beckman Instruments, Inc. Fullerton, Calif.) at 10,000 *g* for 2 min.

Tissue Preparation

Tissue pieces embedded in bovine serum albumin were prepared for thin sectioning according to the proce-

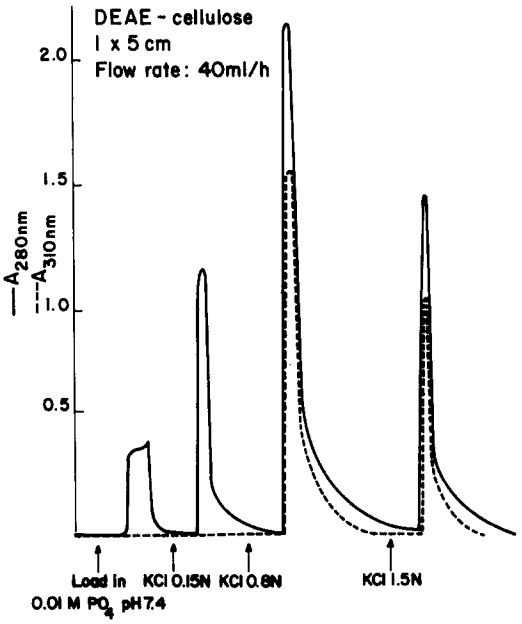

FIGURE 1 Elution profile of ferritin-$F(ab')_2$ conjugate from a DEAE-cellulose column (1 × 5 cm) equilibrated with 0.01 M sodium phosphate, pH 7.4. Solid line, A_{280nm}; dotted line, A_{310nm}. Unconjugated $F(ab')_2$ is eluted with the running buffer and with 0.15 N KCl. The conjugate is eluted with 0.8 N KCl, and free ferritin is eluted with 1.5 N KCl. $F(ab')_2$ detected by immunoelectrophoresis is associated with ferritin only under the envelope eluted with 0.8 N KCl.

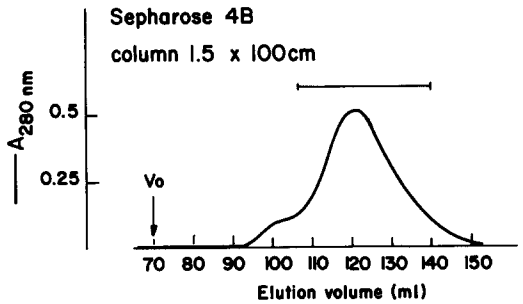

FIGURE 2 Elution profile of the conjugate obtained from DEAE-cellulose chromatography (Fig. 1, 0.8 N KCl fraction) subjected to gel chromatography on a Sepharose 4B column (1.5 × 100 cm) equilibrated with and eluted by 0.1 M Tris-HCl, pH 7.5 at 4°C with a flow rate of 6 ml/h. The void and included volumes of the column were 70 and 170 ml, respectively. The horizontal bar indicates the elution position of the conjugate. Polymers elute at approx. 100 ml.

dure developed by McLean and Singer (24) and modified slightly by us (18). Zymogen granule pellets were fixed in a cellulose nitrate centrifuge tube, cut into oriented strips, and embedded in BSA as for tissue. Sections collected on carbon-coated, Formvar-backed grids were stored dry until used for antigen localization.

Localization Sequences

The indirect localization sequence employed was identical to that used previously by us (18). Specific first step $F(ab')_2$ was used at 0.1 mg/ml, and control $F(ab')_2$ was applied at 10 mg/ml. The ferritin conjugate was used undiluted. Staining of the conjugate reacted grid was as described (19) with the addition of a final stain with bismuth subnitrate (1).

Animals and Cell Fractionation

Four male Holstein calves, 150–200 pounds, were used in this study. After an overnight fast, the animals were anesthesized with sodium pentobarbital intravenously, and the pancreas was removed. Small tissue pieces from the tail, body, and head of the pancreas were removed immediately, diced in fixative, and randomly mixed. 10 g each of tissue from the head, body, and tail of the same gland were removed, trimmed free of fat, placed in iced 0.3 M sucrose and homogenized. The homogenates from these regions were pooled and zymogen granule fractions isolated as previously described (36).

RESULTS

Properties of Immunologic Reagents

FIRST-STEP ANTIBODY: In our previous study of antigen localization on thin sections of BSA embedded pancreas, we found that affinity purified specific IgG gave the best detection sensitivity against a reasonably low cytoplasmic background except for high levels of nonspecific staining over the nucleus, erythrocytes, and collagen. In this study $F(ab')_2$ has been used which possesses the advantage that nonspecific background over the above mentioned structures is absent, due presumably to removal of the cytophilic Fc fragment of IgG.

All affinity purified preparations of rabbit $F(ab')_2$ antibovine secretory proteins were checked for specificity and absence of cross reactivity by immunoelectrophoresis (34) and double diffusion in agar (25). Fig. 3*a* illustrates immunoelectrophoretic patterns for $F(ab')_2$ preparations directed against chymotrypsinogen A, trypsinogen, and DNase I. The wells contained 10 mg/ml of bovine pancreatic juice. Single precipitin lines were obtained, indicating monospecificity. Identical results were obtained with $F(ab')_2$ anti-RNase and anticarboxypeptidase A (not shown); alkaline extracts of zymogen granules (9) gave patterns similar to juice. Double immunodiffusion tests of each $F(ab')_2$ antibody against its antigen and all others used in this study indicated absence of cross reactivity. Examples of cross reactivity tests between $F(ab')_2$ directed against trypsinogen, chymotrypsinogen A, and DNase I are shown in Fig. 3*b*; all permutations of antibody and antigens were tested (not all shown) with similar results.

The $F(ab')_2$ preparations were free of IgG and Fc fragments as determined by immunoelectrophoresis against guinea pig antirabbit Fc antiserum.

PROPERTIES OF THE CONJUGATE: Our previous studies employed a ferritin conjugate prepared by solid phase coupling of ferritin to specific sheep Fab antirabbit IgG. In this study we have used sheep $F(ab')_2$ antirabbit $F(ab')_2$ in the conjugate for the following reasons: (*a*) the second step antibody is directed specifically against the first step antibody, i.e., rabbit $F(ab')_2$; (*b*) bivalent antibodies in the conjugate should possess higher affinity for the first step reagent (13); and (*c*) multivalent antibody conjugates facilitate direct immunologic testing of the conjugate. Based on the spectral properties of the conjugate (OD contributed by ferritin iron at A_{310nm} and the extinction coefficients at A_{280nm} for ferritin and $F(ab')_2$), we have calculated that 11% of the $F(ab')_2$ and 3.3% of the ferritin initially present in the solid phase reaction mixture were coupled.

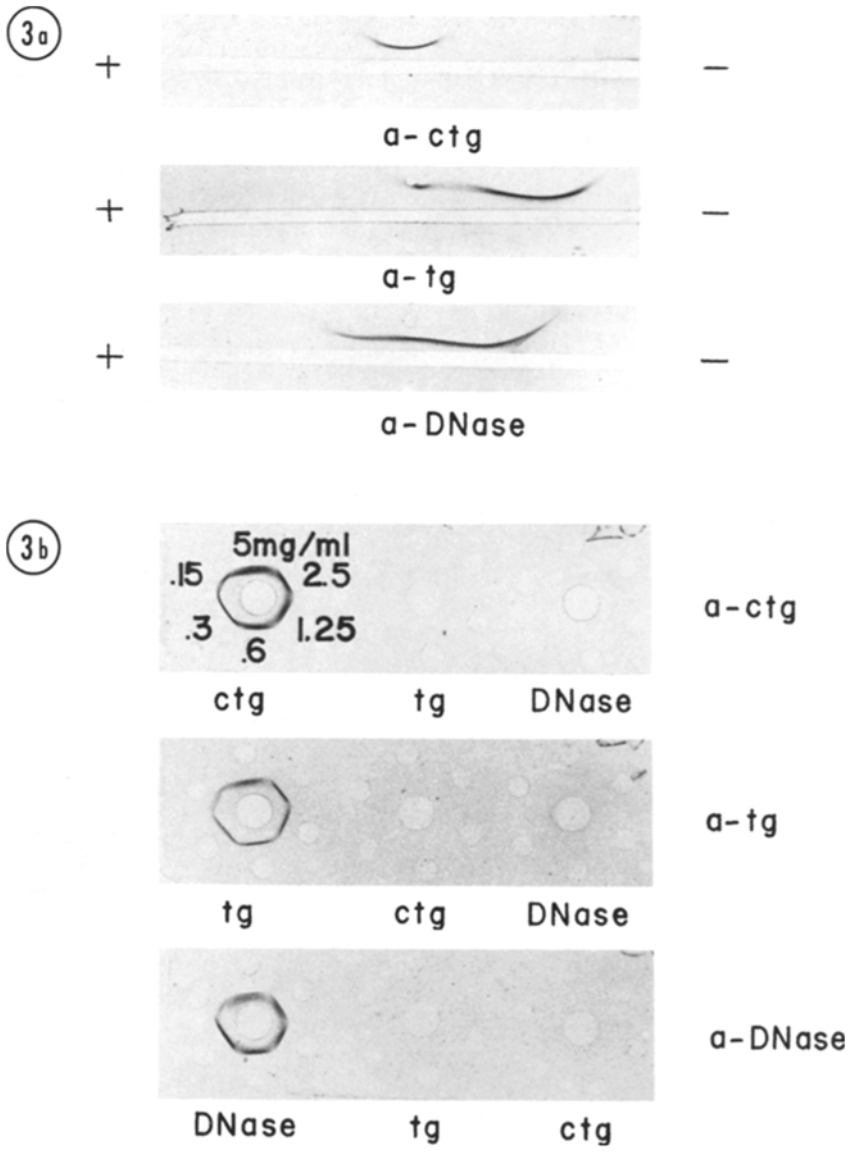

FIGURE 3 (*a*) Immunoelectrophoretic patterns of rabbit F(ab')$_2$ fragments (1 mg/ml) directed against bovine chymotrypsinogen (a-ctg), trypsinogen (a-tg), and deoxyribonuclease (a-DNase) present in the troughs and tested against bovine pancreatic juice (10 mg/ml) in the wells. A single line is obtained with each antibody fragment preparation indicative of its monospecificity. (*b*) Double immunodiffusion of rabbit F(ab')$_2$ (1 mg/ml) directed against bovine chymotrypsinogen (upper panel), trypsinogen (middle panel) and DNase (lower panel) tested against each antigen. The antibody fragment preparations are in the central wells; the antigens serially diluted from 5 mg/ml to 0.15 mg/ml are in the outer wells. The antibody fragment preparation reacts only with the related antigen; no cross reactivity is observed.

These calculations indicate that the molar composition of the conjugate is approx. 6 F(ab')$_2$ molecules per ferritin molecule. The calculated mass of the conjugate is thus approx. 1.2×10^6 daltons, which agrees with that derived from chromatography on Sepharose 4B (Fig. 2) using the formula for molecular weight determination of Laurent and Killander (21).

Immunoelectrophoresis of the conjugate followed by development of precipitin lines with

guinea pig antiserum directed against sheep $F(ab')_2$ indicated that its electrophoretic mobility was altered and intermediate between that of free ferritin and unconjugated sheep $F(ab')_2$ (Fig. 4*a*). The absence of amido black stained precipitin lines in the region of unconjugated $F(ab')_2$ suggests, within the limits of detection, that the conjugate does not contain free $F(ab')_2$. Similar electrophoretic runs (not shown) in which the trough is filled with guinea pig antiserum against horse spleen ferritin indicate absence of precipitin lines in the region of free ferritin. The amido black stainable precipitin line also stains with Prussian blue, indicating the presence of ferritin iron. The broad stained zone on either side of the well is residual ferritin conjugate trapped in the agar of the plate which was not extracted with the isotonic saline wash before staining.

If normal rabbit serum is subjected to immunoelectrophoresis followed by development of precipitin lines using the conjugate in the trough a strong amido black stained precipitin line in the position of rabbit IgG is seen with a smaller arc toward the anode representing immunoglobulins moving in the β-region (Fig. 4*b*). Again, the amido black stainable line is Prussian blue positive, indicating the presence of ferritin iron. This result demonstrates that the conjugate is able to precipitate the antigenic determinants against which it is directed. Whether precipitin line formation by the conjugate is due to retention of bivalency by the coupled $F(ab')_2$ is unknown, though it is likely that some is converted to monovalent antibody during conjugation (4) to ferritin, which, nevertheless, acts as a multivalent ligand in the precipitin test. However, in previous studies (unpublished) we were unable to obtain immunoprecipitates with Fab-ferritin conjugates of similar composition (18).

Localization

CHOICE OF FIXATION CONDITIONS: Since cross-linking reagents such as glutaraldehyde and formaldehyde have been reported by others to reduce the antibody binding capacity of several proteins (10), before initiating our localization studies on aldehyde fixed pancreas, we set about (*a*) to determine the lowest concentration of glutaraldehyde which was compatible with reasonable tissue preservation and (*b*) to quantitate the effect of glutaraldehyde on retention of antibody binding capacity of several of the bovine secretory proteins to be localized on tissue sections. For the latter purpose, a model consisting of secretory proteins covalently coupled to agarose beads was used. The beads were exposed to glutaraldehyde at increasing concentrations and for times and at temperatures corresponding to those used for albumin embedment of tissues and cell fractions. After removal of excess fixative by washing and quenching of residual aldehyde groups, the ability of the insolubilized antigens to extract specific

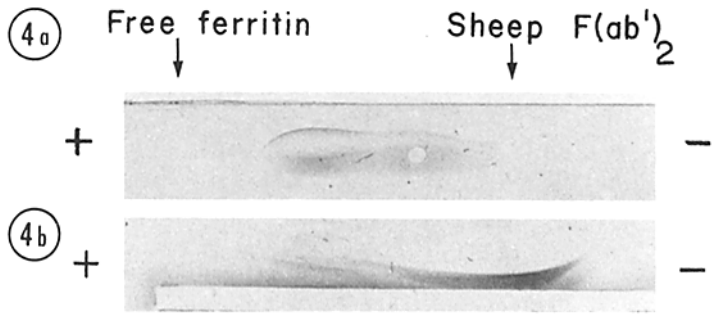

FIGURE 4 (*a*) Immunoelectrophoretic pattern of purified ferritin-sheep $F(ab')_2$ conjugate. The well contained 1 mg/ml conjugate. After electrophoresis, the trough was filled with guinea pig anti-sheep $F(ab')_2$ antiserum. A single precipitin line is observed after amido black staining. The arrow on the left indicates the position where free ferritin would run, while that on the right shows the position of free sheep $F(ab')_2$. When reacted with Prussian blue on a parallel plate, the Amido black stainable band is stained blue indicating the presence of ferritin iron. (*b*) Immunoelectrophoresis of rabbit serum tested against the purified conjugate. The well contained normal rabbit serum; after electrophoresis, the trough was filled with sheep $F(ab')_2$ antirabbit-ferritin conjugate. A precipitin line in the position of rabbit IgG is observed, indicating that the antigen-precipitating capacity of the conjugate is preserved. Plate stained with Amido black. The same line stains with Prussian blue in parallel plates, indicating the presence of ferritin iron.

antibodies from rabbit antiserum was tested and compared to that of unfixed antigen. The results are shown in Table I. For trypsinogen and DNase I, increasing the glutaraldehyde concentration leads to a rapid depression of antibody binding capacity which plateaus at between 40–50% of control above approx. 0.5% glutaraldehyde. Chymotrypsinogen A and carboxypeptidase A are less sensitive to fixation, retaining approx. 70% of antibody binding capacity even at 2% glutaraldehyde, whereas RNase antibody binding was unaffected by fixation. As we have shown elsewhere (19), the effects of glutaraldehyde (at 0.5%) on antibody binding capacity are maximal within 15 min.

TABLE I

Effect of Glutaraldehyde on Antibody Binding Capacity of Insolubilized Antigens

Antigen	Concn glutaraldehyde	Control
	Percent	*Percent*
Trypsinogen	0.25	75
	0.5	58
	1.0	54
	2.0	52
Chymotrypsinogen A	0.25	85
	0.5	80
	1.0	75
	2.0	72
Carboxypeptidase A	0.25	82
	0.5	82
	1.0	80
	2.0	70
DNase	0.25	60
	0.5	44
	1.0	42
	2.0	40
RNase	0.25	106
	0.5	100
	1.0	110
	2.0	120

The antigens were attached to cyanogen bromide-activated Sepharose 4B as described in Materials and Methods and exposed for 16 h at room temperature to glutaraldehyde solutions in 0.1 M Na phosphate, pH 7.4, (0.5 g wet wt adsorbent per experimental point). Excess glutaraldehyde was removed from the absorbents by repeated washes with 0.1 M Tris-HCl pH 7.5, and unreacted aldehyde groups were quenched with 10 mM $NaBH_4$ in 0.1 M Tris-HCl pH 7.5. After additional washes with Tris, each adsorbent was exposed to 0.5 ml of appropriate antiserum in 1.0 ml of 0.1 M Tris-HCl pH 7.5 for 1.5 h at room temperature. After repeated washing with Tris to remove unbound antiserum, each adsorbent was eluted with KSCN as described under Materials and Methods and the amount of specific antibody recovered determined by A_{280nm}. Data are expressed relative to control adsorbents not exposed to glutaraldehyde and are averages of two separate exp.

We next examined bovine pancreas fixed at concentrations of glutaraldehyde ranging from 0.25% to 2.0% by conventional thin sections of Epon-embedded material. For glutaraldehyde concentrations less than 2.0%, the tonicity of the fixative was maintained constant by addition of appropriate sucrose concentrations. Tissue fixed with 0.25% glutaraldehyde showed obvious extraction of zymogen granule content resulting in either a mottled appearance of the content or a halo of extraction surrounding a dense core. The morphologic appearance of tissues fixed with 0.5% to 2.0% glutaraldehyde was identical, and no obvious extraction of granule content was noted. All concentrations of formaldehyde up to 4% showed extraction of granule content and rupture or absence of their limiting membrane, ruling out this aldehyde as sole fixative for our purposes since unknown and possibly selective extraction and/or translocation of secretory proteins to the cytosol were of concern and may have led to artifactual localizations. Similar effects of fixation conditions have been observed for the guinea pig pancreas (19).

Based on these two sets of observations, a glutaraldehyde concentration of 0.5% was chosen since it offered satisfactory tissue preservation with an acceptable preservation of antibody binding capacity.

LOCALIZATION OF SECRETORY PROTEINS ON THIN SECTIONS OF PROTEIN-EMBEDDED BOVINE PANCREAS: Figures 5–9 illustrate typical localization of bovine secretory proteins on thin sections of BSA-embedded pancreas. Fig. 5 is a low magnification field of a section through an acinus reacted in the first step with rabbit $F(ab')_2$ antichymotrypsinogen. While uniform staining by the second-step ferritin conjugate of all zymogen granules may not be evident in the reproduction, it is intended to show the reasonably good tissue preservation afforded by BSA embedment with the exception of some obvious "ghosts" where zymogen granules have been mechanically dislodged during thin sectioning. The indicated area of this micrograph is shown at higher magnifica-

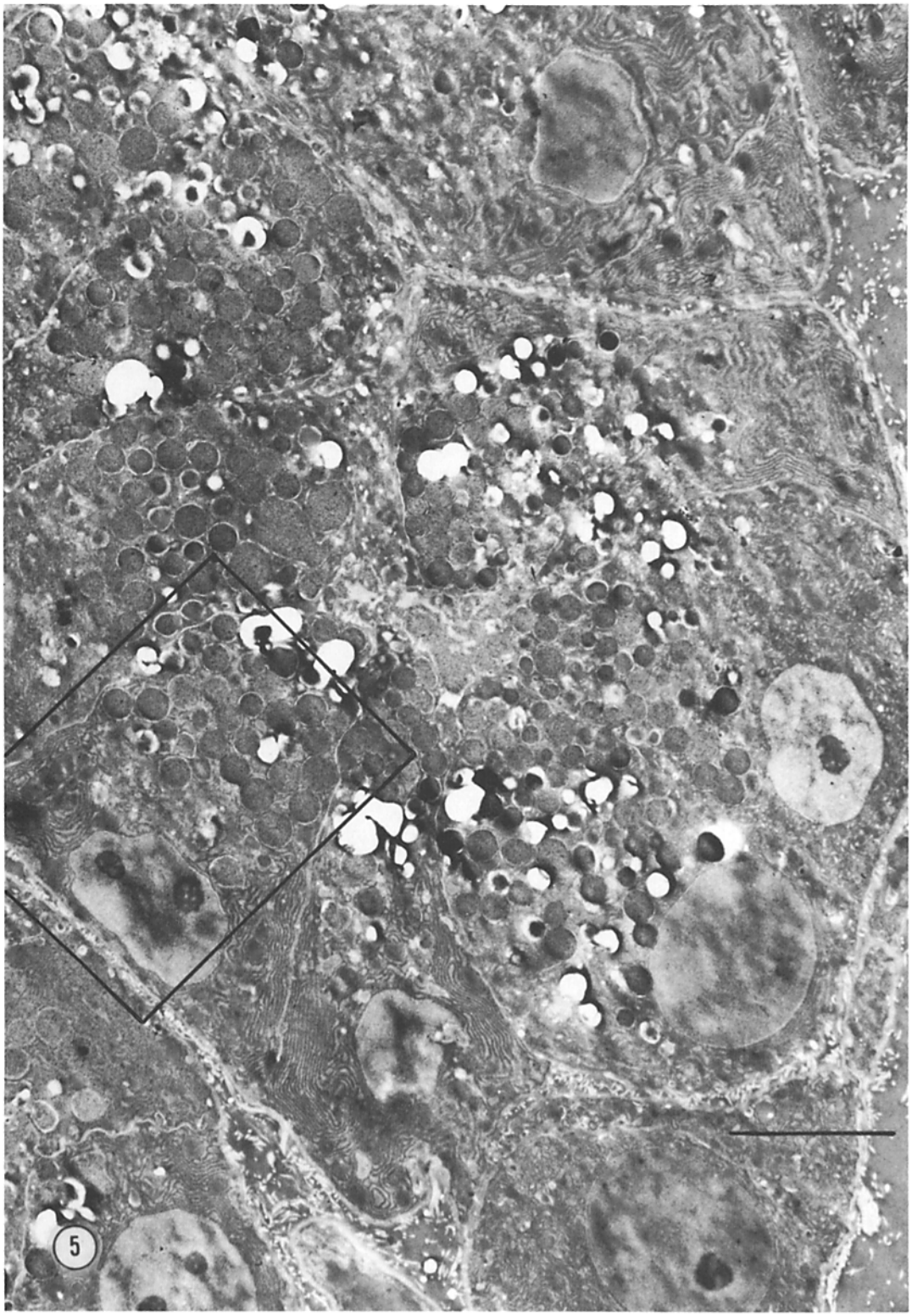

FIGURE 5 Thin section of BSA-embedded bovine pancreas. First-step reaction with $F(ab')_2$ antichymotrypsinogen 0.1 mg/ml. × 5300. Bar, 5 μm.

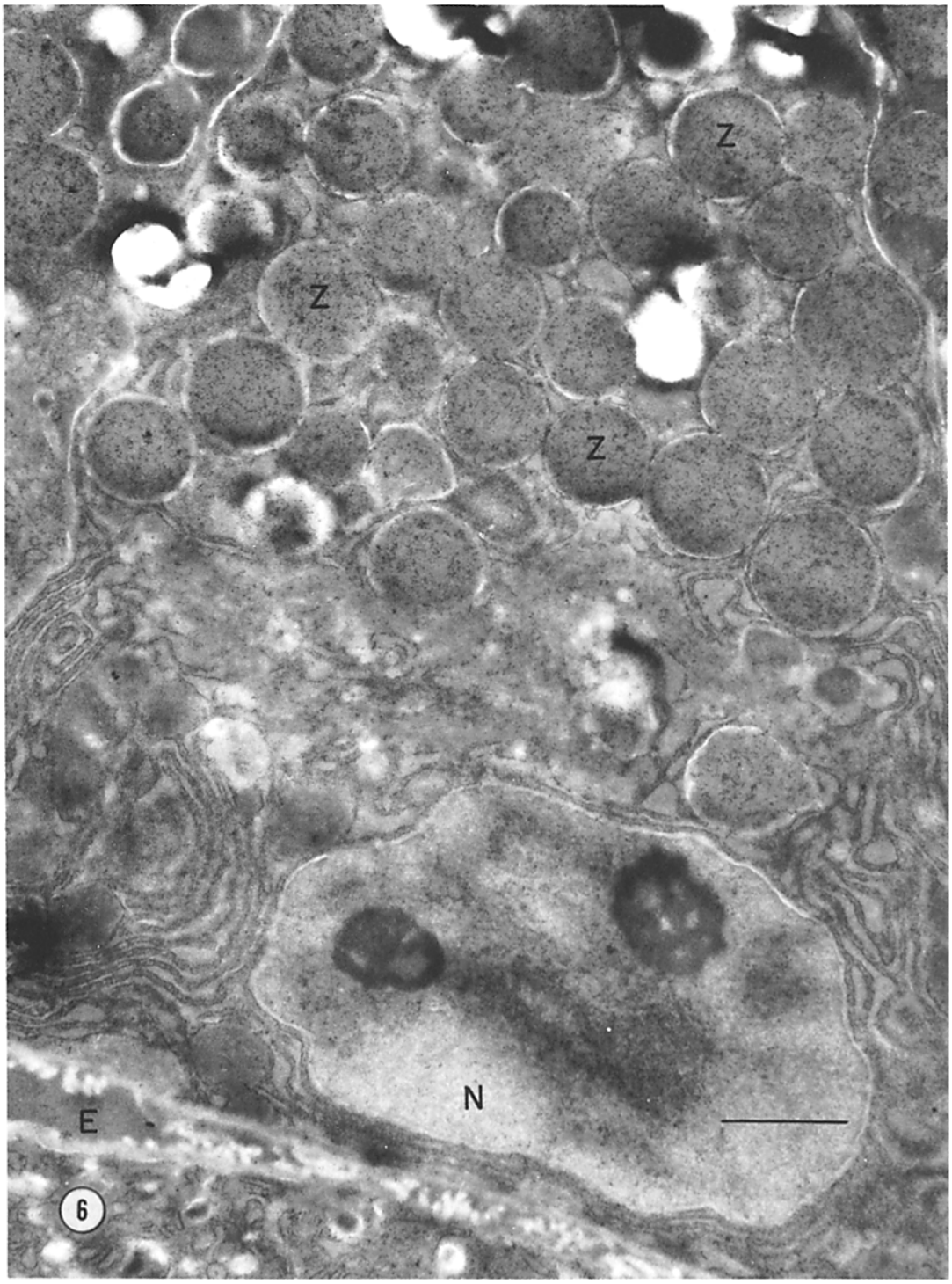

FIGURE 6 Area indicated in Fig. 5 shown at × 19,000. Bar, 1 μm. zymogen granules (*Z*); nucleus (*N*); extracellular space (*E*).

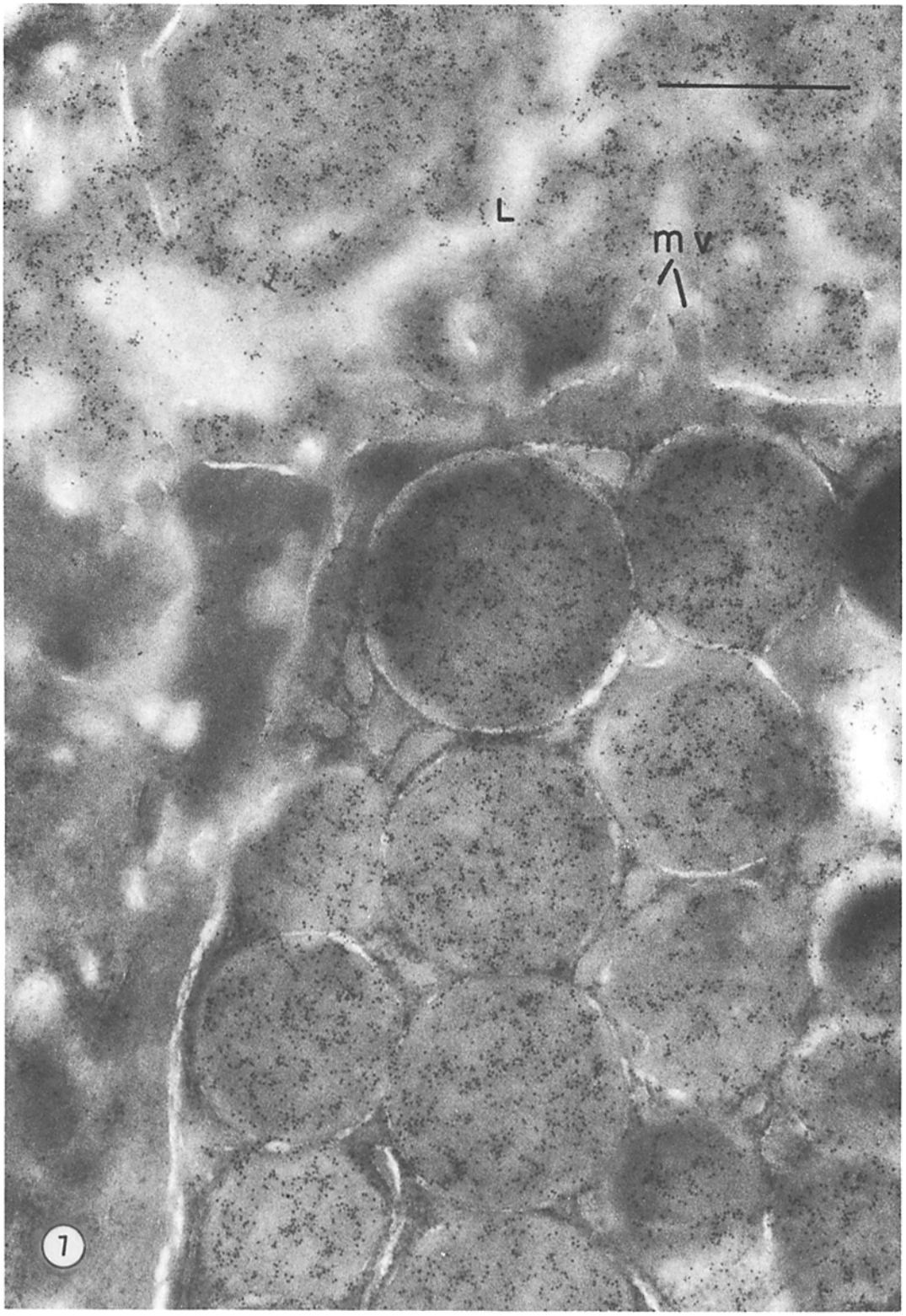

FIGURE 7 As in Fig. 5. Note ferritin staining of the content of the acinar lumen (*L*); microvilli (*mv*) on the apical plasmalemma. × 60,000. Bar, 0.5 μm.

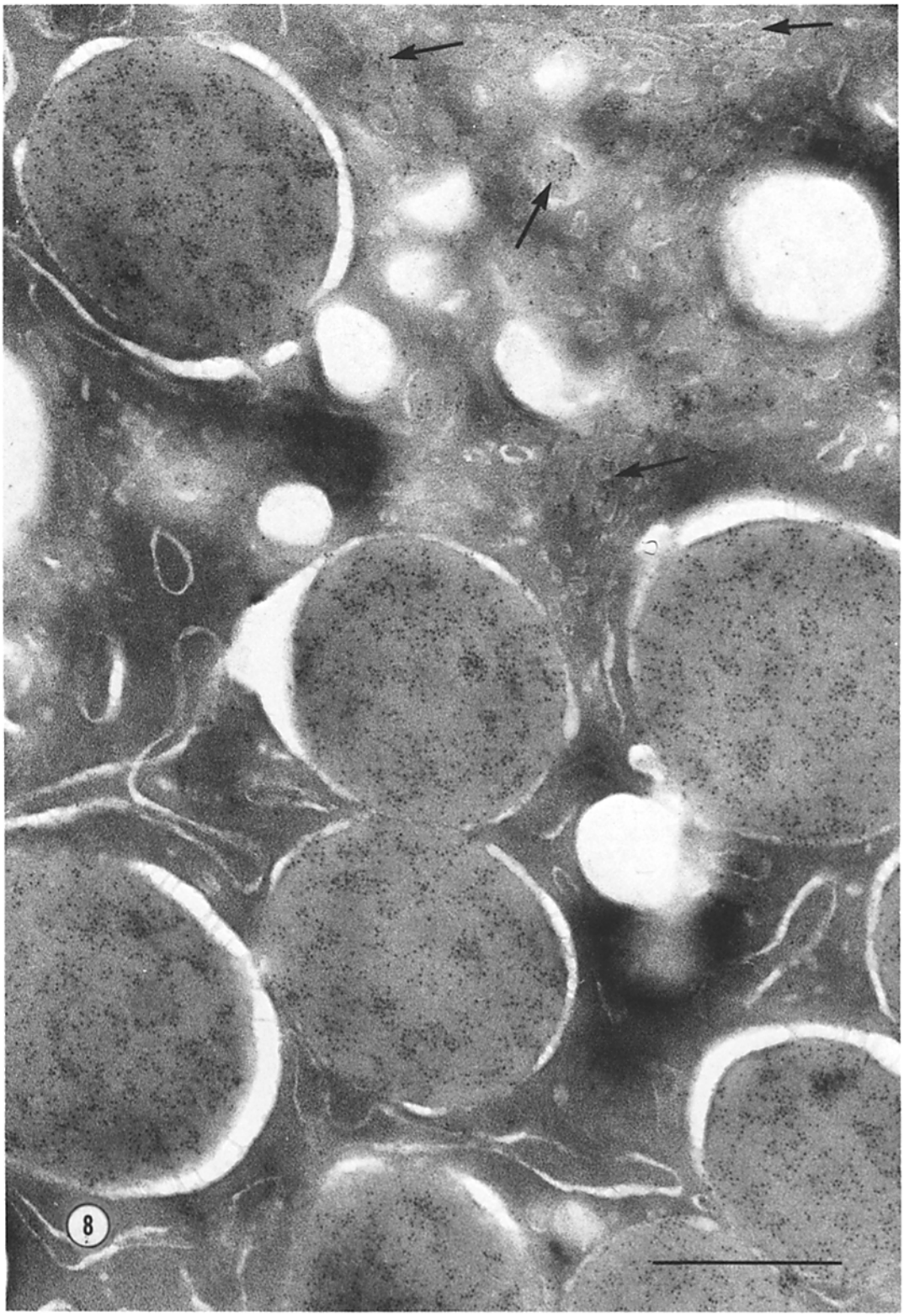

FIGURE 8 Thin section of BSA-embedded bovine pancreas reacted in the first step with 1 mg/ml $F(ab')_2$ antitrypsinogen. Note staining of vesicles and cisternae of the Golgi complex (arrows). × 60,000. Bar, 0.5 μm.

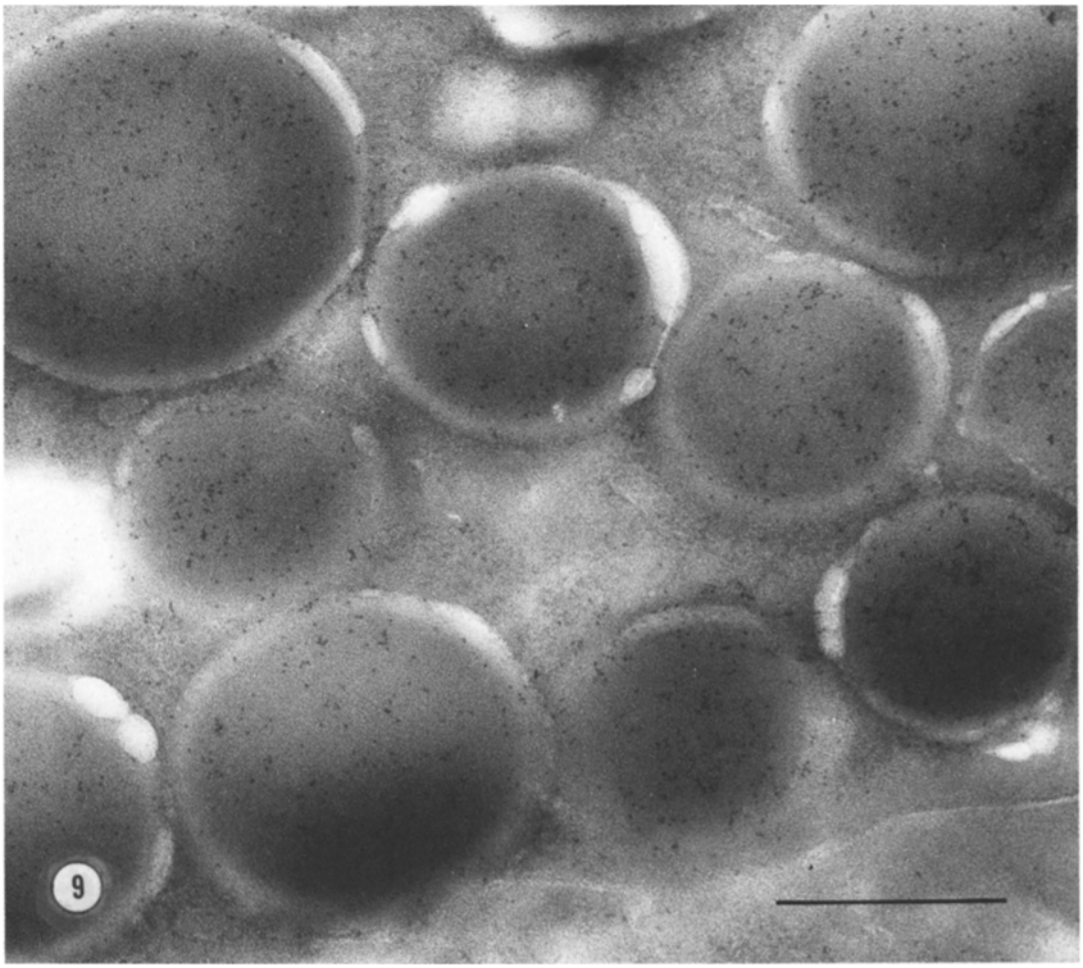

FIGURE 9 As in Fig. 8, but reacted in the first step with $F(ab')_2$-anti RNase, 1 mg/ml. × 60,000. Bar, 0.5 μm.

tion in Fig. 6, where it is evident that all zymogen granules in the field are positive for chymotrypsinogen. The absence of nonspecific staining of the nucleus and of collagen in the extracellular space is also evident in contrast to our previous results (18). In this and the following figures, the cisternal contents of the rough endoplasmic reticulum are not stained for reasons discussed later. Fig. 7 shows the apex of an exocrine cell with chymotrypsinogen-positive zymogen granules and acinar lumen content. Fig. 8, a thin section including vesicles and cisternae of the Golgi complex, has been reacted with $F(ab')_2$ antitrypsinogen and shows again uniform staining of zymogen granules as well as staining of the Golgi elements. Rough endoplasmic reticulum content and the cytosol are again not stained with ferritin. Fig. 9 is a thin section reacted with $F(ab')_2$ anti-RNase. Note that while all zymogen granules in the field are positively stained, the intensity of the reaction is less than that observed for trypsinogen or chymotrypsinogen which might be expected in view of the lower relative proportion of RNase in bovine pancreatic juice (approx. 3%) compared to the former two zymogens (approx. 23% and 20%, respectively) (15, 9), although preferential extraction of RNase or lower numbers of antigenic sites per molecule could as well explain the differences in staining. Though not shown, DNase and carboxypeptidase A localized to all zymogen granules in all cells examined. Fig. 10 is a control in which the first-step reagent ($F(ab')_2$ antichymotrypsino-

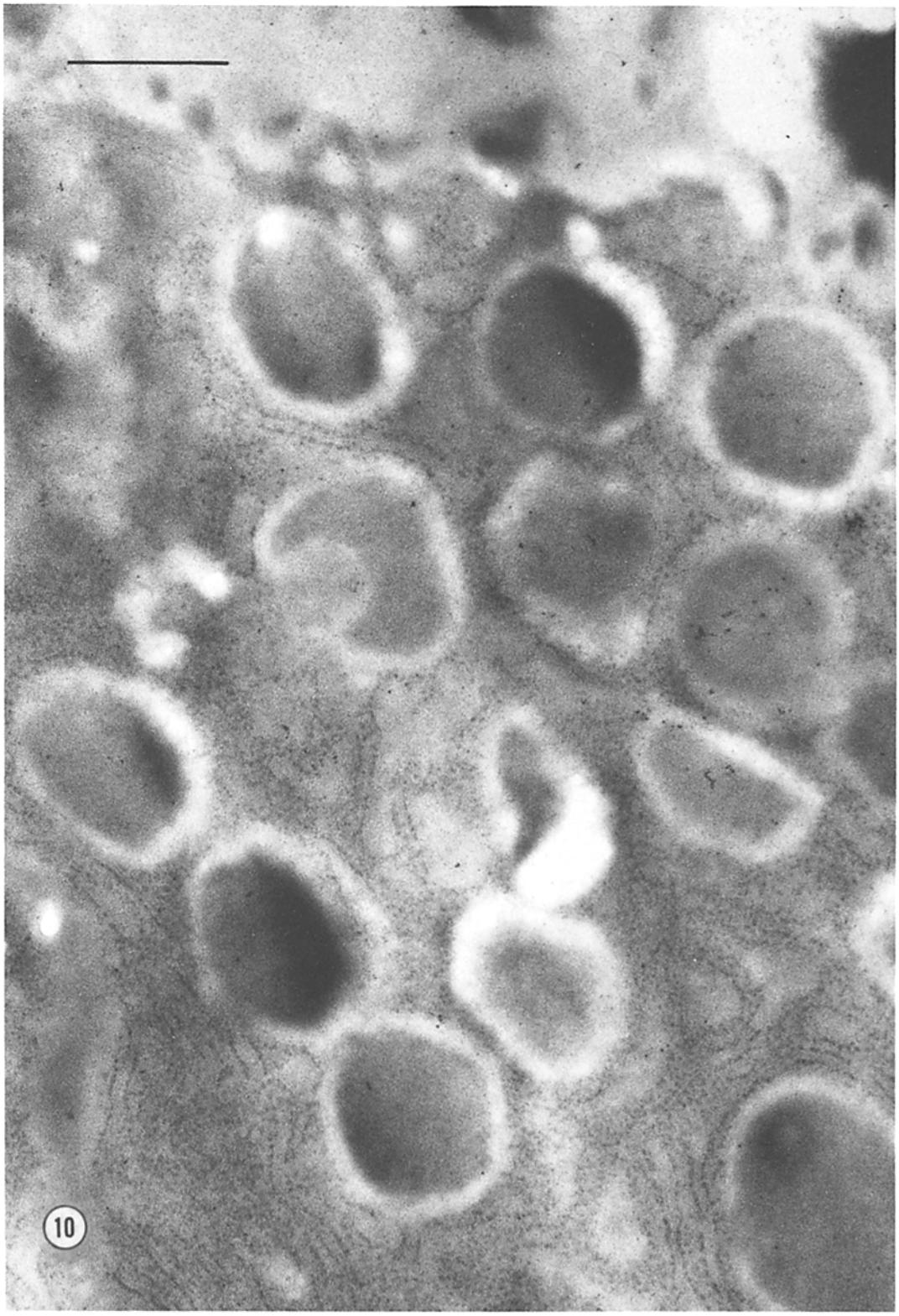

FIGURE 10 BSA thin section of bovine pancreas reacted in first step with $F(ab')_2$ antichymotrypsinogen previously adsorbed with insolubilized chymotrypsinogen and applied at 10 mg/ml. Note absence of staining of zymogen granules. × 55,000. Bar, 0.5 μm.

gen) was adsorbed by agarose insolubilized antigen then applied at 10 × concentration before staining with the second-step conjugate. Note the low background staining (cf. Fig. 7).

LOCALIZATION OF SECRETORY PROTEINS ON THIN SECTIONS OF PROTEIN-EMBEDDED PANCREATIC ZYMOGEN GRANULE FRACTIONS: Even though care was taken to obtain random tissue blocks from three regions of the gland, in view of the size of the gland we nonetheless examined only a relatively very small number of cells from each gland for each antigen. To provide a more complete sampling of the gland, we therefore collected equal weights of tissue from each region of the gland (representing in total approx. 25% of the weight of the gland), and isolated from pooled homogenates of the tissue total zymogen granule fractions according to standard techniques (36). After fixation and embedment in BSA exactly as for tissue, each $F(ab')_2$ fraction used on thin sections of cells was applied to thin sections of the pellets. Fig. 11 is a typical example in which the distribution of trypsinogen is shown. Note that all granules in the field are stained, and note the low level of nonspecific staining over the protein matrix and contaminating organelles such as mitochondria (Fig. 11, inset). Qualitatively identical results were obtained with each of the other $F(ab')_2$-specific antibodies. Controls, as for tissue sections, were negative (not shown).

DISCUSSION

The main conclusion of this study is that all cells of the bovine pancreas and all zymogen granules within any one cell are qualitatively alike with regard to their complement of the five secretory proteins localized by electron microscope immunocytochemistry. These data complement and extend our earlier observations on the localization of trypsinogen in the bovine pancreas (18) and those of Painter et al. (26) for RNase in the same tissue. While the list of secretory proteins examined in our study is not inclusive, there being from 9–11 different enzyme activities reported in bovine pancreatic juice (32), nevertheless the five proteins (trypsinogen, chymotrypsinogen A, carboxypeptidase A, RNase, and DNase I) comprise approx. 60% of the protein mass in bovine pancreatic juice and zymogen granules (15, 9). In the most extensively studied case to date, the proteins discharged by the guinea pig pancreas, at least 19 unique peptide species have been identified, and of these 13 have been assigned enzymatic function (31). Our series encompasses proteins ranging in molecular weight from approx. 13,000 (RNase) to approx. 45,000 for carboxypeptidase A, possessing isoelectric points ranging from basic (RNase, trypsinogen, and chymotrypsinogen) to acidic (carboxypeptidase A), and consisting in part of glycoproteins (DNase (29) and RNase B (28)). Thus, the proteins localized in this study also represent qualitatively the spectrum of proteins secreted by the bovine pancreas.

As the classic studies of Greene et al. (9) showed, the enzymatic composition of secretory proteins in bovine zymogen granules and resting pancreatic juice collected from the gland is quantitatively identical. Our studies, though qualitative in nature, enable us to reinterpret their data as indicating that the zymogen granules of all exocrine cells in the bovine gland likely contribute equally to the final makeup of the pancreatic juice. This interpretation is further strengthened by our observations that the distribution of secretory proteins appears to be uniform in each zymogen granule in a total zymogen granule fraction, which more uniformly samples the entire granule population of the gland compared to that obtained by sections of cells.

In addition, our localization studies have shown that all the secretory proteins we have examined can be detected in cisternae, vesicles, and condensing vacuoles of the Golgi complex, implying that mixing of secretory proteins before packaging into zymogen granules occurs either at the level of the Golgi complex or earlier in the secretory pathway within the cisternae of the rough endoplasmic reticulum. It is of interest to note that trypsinogen, chymotrypsinogen, and carboxypeptidase (none of which are glycoproteins) were detectable in the cisternal spaces of the Golgi complex along with known glycoproteins such as DNase and RNase, suggesting that passage through the Golgi complex represents an obligatory path for both classes of secretory proteins.

The recent observations of Devillers-Thiery et al. (7) indicate that canine pancreatic secretory proteins, including trypsinogen (and likely exportable proteins in general), are endowed with an amino terminal extension which signals attachment of polysomes to the membrane of the rough endoplasmic reticulum and initiates transfer of the nascent peptide across the endoplasmic reticulum membrane into its cisternal space. The findings of Devillers-Thiery et al. (7) also suggest that we

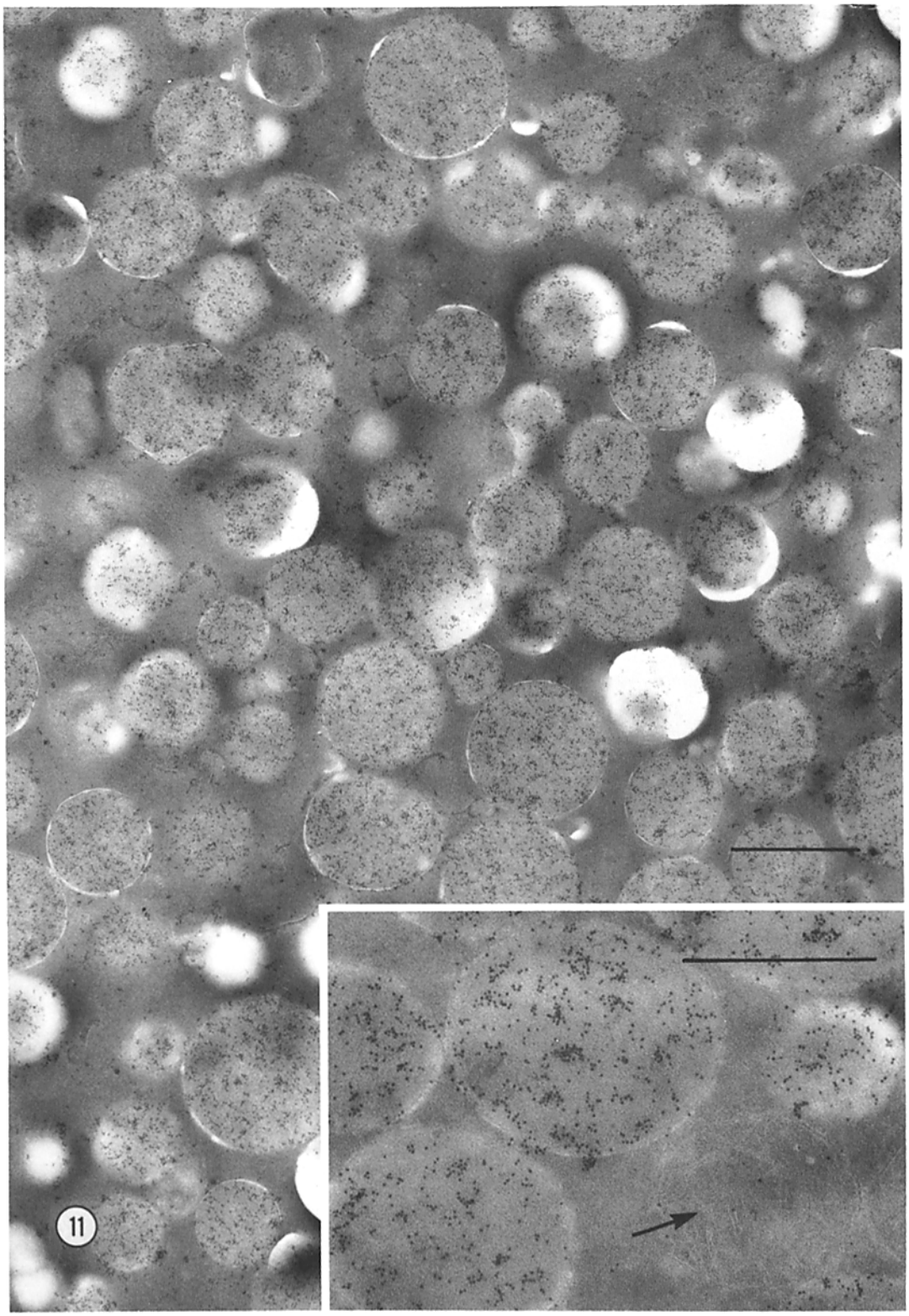

FIGURE 11 Thin section of BSA-embedded zymogen granule fraction reacted in first step with 0.1 mg/ml of $F(ab')_2$ antitrypsinogen. Note uniform staining of all zymogen granules. × 20,500. Bar, 1 μm. *Inset* to Fig. 11 is a higher magnification field from a similar pellet reacted with $F(ab')_2$ antitrypsinogen. Arrow shows absence of staining over mitochondrion. × 60,000. Bar, 0.5 μm.

should have detected secretory proteins in the cisternae of the rough endoplasmic reticulum by means of electron microscope immunochemistry on thin sections of protein-embedded tissue. Painter et al. (26) have, however, observed RNase in this compartment by immunoelectron-microscopy on frozen thin sections of bovine pancreas. This result suggests that the mild fixation conditions we use for preparation of thin sections of protein-embedded tissue may lead to extraction of proteins from the cisternae of the rough endoplasmic reticulum, although other factors such as masking of antigenic sites are not excluded in our study.

Our findings on the uniform content of secretory proteins in zymogen granules of the exocrine pancreatic cell are in accord with the results of Herzog and Miller (11, 12), who have shown by electron microscope cytochemistry that all secretory granules of all acinar cells of the rat parotid and lacrimal glands contain endogenous peroxidase. Their results also clearly show that all elements of the rough endoplasmic reticulum and some vesicles and cisternae of the Golgi complex are active in the synthesis and packaging of this enzyme, again pointing to the relative insensitivity of localization techniques such as we have used which do not rely on detection amplification provided by enzyme activity.

In contrast to the exocrine pancreas, most endocrine glands producing several secretory products, e.g., the islets of Langerhans and anterior pituitary (8), accomplish this by virtue of individual cells synthesizing a single product. Further specialization of product synthesis and packaging occurs in polymorphonuclear leucocytes where separate populations of storage granules with different contents are sequentially produced within the same cell (3).

According to the studies of Rothman and colleagues (summarized in reference 30), the pancreas of certain species can acutely vary its output of individual secretory proteins (specifically, potential enzyme activity) depending on the nature of the discharge stimulant. Our data, although strictly pertaining to the bovine pancreas and qualitative, would suggest that regulation of output of individual proteins likely does not occur by selective release of one population of zymogen granules containing a specific secretory protein(s). This conclusion is also consistent with observations on the apparent parallel intracellular transport and discharge of secretory proteins in both the stimulated and unstimulated guinea pig pancreas (35, 37). As mentioned before, earlier biochemical studies on the bovine pancreas indicated quantitative identity of secretory proteins in unstimulated pancreatic juice and zymogen granules (9); recent studies (Fink, E., J. D. Jamieson, and L. J. Greene, unpublished) indicate parallel stimulated (carbamylcholine) discharge of amylase, secretory trypsin inhibitor, and total pulse labeled secretory proteins from slices of bovine pancreas incubated in vitro.

According to Rothman's recent hypothesis (30), however, nonparallel discharge of pancreatic secretory proteins does not require selective granule release, but, rather, is explained by selective transport directly across the plasmalemma of secretory proteins free in the cell sap; the composition of proteins in the cell sap, in addition, may be modified by exchanges of secretory proteins between intracellular membrane-bounded compartments and the cell sap. The absence of detectable secretory proteins in the cell sap as determined by immunocytochemistry (this study and reference 26) does not refute the above hypothesis since we know neither the concentration of secretory proteins which must be detected in this compartment nor the lower limit of sensitivity of current immunocytochemical techniques. The fact that cytochemical localization of peroxidase in other types of exocrine cells shows no detectable enzyme activity in the cytosol (11, 12) despite the considerable enzymatic amplification of reaction product tends, however, not to support this hypothesis. We should also keep in mind that reconciliation of apparent discrepancies of data must consider procedural differences and species variations. With regard to the latter, it is of interest to note that the content of secretory proteins in exocrine cells surrounding the islets of Langerhans differs quantitatively from that in noninsular regions of the rat pancreas so that the composition of the final secretion could reflect the output of these cells whose content of secretory proteins may be influenced by adjacent endocrine cells (22).

The technique for surface localization of antigens on thin sections used in the present study has been improved over that used by us previously. With regard to the antibody used in the first step of the indirect localization sequence, it is evident that affinity purified specific $F(ab')_2$ antibodies have less tendency to aggregate than IgG, hence improving resolution, but, more importantly, do not interact with collagen, nuclei, and the embed-

ding matrix, thus lowering background and enhancing sensitivity. These improvements are likely attributable to removal of the Fc fragment of IgG by pepsin digestion.

With regard to the second-step reagent, we have used the same solid-phase coupling procedure as before to prepare ferritin conjugates, substituting affinity purified $F(ab')_2$ fragments for Fab used previously. The $F(ab')_2$ conjugates may remain in part bivalent, which should not only improve sensitivity for detection of the first step antibody but also facilitate checking of conjugates by immunoprecipitation which was not possible with Fab-ferritin conjugates of similar composition (J. D. Jamieson and J. P. Kraehenbuhl, unpublished observations (18)). Fractionation of the conjugate by ion exchange and gel chromatography rids it of unconjugated $F(ab')_2$ (which would otherwise compete for the conjugate in the detection step), eliminates immunologically detectable free ferritin (reducing the chances of nonspecific background), and insures that the conjugate is monomeric and free of aggregates. The yields of $F(ab')_2$-ferritin conjugates obtained exceed those of our previous Fab-ferritin conjugates (18), but are considerably lower than IgG-ferritin conjugates obtained by Kishida et al. (16).

Finally, though not shown here, we have attempted to localize secretory proteins in prefixed pancreas by using a diffusion localization procedure in which the detector is a Fab-hemeoctapeptide conjugate (17). The results, shown elsewhere (20), indicate that only a fraction of the granule population in a given cell is positive for a given secretory protein, which disagrees with the results obtained by surface localization. Hence, the diffusion localization technique is unsuitable for reliably detecting antigens compactly packaged in organelles due to the difficulty of overcoming penetration barriers. Further, in order to obtain any penetration of immunologic reagents into the tissue, one is limited to formaldehyde as a fixative, which, as we have already shown (19), does not prevent extraction of granule content, and is unacceptable with regard to preservation of fine structure. Given these considerations, results obtained with diffusion localization procedures should always be validated by surface localization.

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REFERENCES

1. Ainsworth, S. K., and M. J. Karnovsky. 1972. An ultrastructural staining method for enhancing the size and electron opacity of ferritin in thin sections. *J. Histochem. Cytochem.* **20:**225–229.
2. Amstay, M. S. 1967. Purification of ferritin-conjugated antibody by DEAE-cellulose chromatography. *J. Lab. Clin. Med.* **69:**997–1002.
3. Bainton, D. F., and M. G. Farquhar. 1968. Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. *J. Cell Biol.* **39:**299–317.
4. Borek, F., and M. Silverstein. 1961. Characterization and purification of ferritin-antibody globulin conjugates. *J. Immunol.* **87:**555–561.
5. Clemente, F., A. deCaro, and C. Figarello. 1972. Composition du suc pancréatique humain étude immunoenzymologique. *Eur. J. Biochem.* **31:**186–193.
6. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. *J. Biol. Chem.* **245:**3059–3065.
7. Devillers-Thiery, A., T. Kindt, G. Scheele, and G. Blobel. 1975. Homology in amino-terminal sequence of precursors to pancreatic secretory proteins. *Proc. Natl. Acad. Sci. U. S. A.* **72:**5016–5020.
8. Farquhar, M. G. 1971. Processing of secretory products by cells of the anterior pituitary gland. *In* Subcellular Structure and Function in Endocrine Organs. H. Heller and K. Lederis, editors. Cambridge University Press, New York. 79–122.
9. Greene, L. J., C. H. Hirs, and G. E. Palade. 1963. On the protein composition of bovine pancreatic zymogen granules. *J. Biol. Chem.* **238:**2054–2070.
10. Habeeb, A. F. S. A. 1969. A study of the antigenicity of formaldehyde- and glutaraldehyde-treated bovine serum albumin and ovalbumin-bovine serum albumin conjugate. *J. Immunol.* **102:**457–465.
11. Herzog, V., and F. Miller. 1970. Die lokalisation endogener peroxydase in der glandula parotis der ratte. *Z. Zellforsch. Mikrosk. Anat.* **107:**403–420.
12. Herzog, V., and F. Miller. 1976. The localization of endogenous peroxidase in the lacrimal gland of the rat during postnatal development. *J. Cell Biol.* **53:**662–680.
13. Hornick, L., and F. Karusch. 1972. Antibody affinity. III. The role of multivalence. *Immunochemistry.* **9:**325–340.
14. Jamieson, J. D. 1972. Transport and discharge of exportable proteins in pancreatic exocrine cells: *in vitro* studies. *Curr. Top. Membranes Transp.* **3:**273–338.
15. Keller, P. J., E. Cohen, and H. Neurath. 1958. The proteins of bovine pancreatic juice. *J. Biol. Chem.* **233:**344–349.
16. Kishida, Y., B. R. Olsen, A. Berg, and D. J.

PROCKOP. 1975. Two improved methods for preparing ferritin-protein conjugates for electron microscopy. *J. Cell Biol.* **64**:331-339.
17. KRAEHENBUHL, J. P., R. E. GALARDY, and J. D. JAMIESON. 1974. Preparation and characterization of an immunoelectron microscope tracer consisting of a heme-octapeptide coupled to Fab. *J. Exp. Med.* **139**:208-223.
18. KRAEHENBUHL, J. P., and J. D. JAMIESON. 1972. Solid phase conjugation of ferritin to Fab-fragments for use in antigen localization on thin sections. *Proc. Natl. Acad. Sci. U. S. A.* **69**:1771-1775.
19. KRAEHENBUHL, J. P., and J. D. JAMIESON. 1974. Localization of intracellular antigens by immunoelectron microscopy. *Int. Rev. Exp. Pathol.* **12**:1-53.
20. KRAEHENBUHL, J. P., L. RACINE, and R. E. GALARDY. 1976. Use of a heme-octapeptide as an electron microscope tracer. *In* Immunoenzymatic Techniques. Institute National de la Santé et de la Recherche Medicale, Vol. 2. Chapt. 7. G. Feldman, editor. Noord Hollandsche Uitg. Mij. Amsterdam.
21. LAURENT, C., and J. KILLANDER. 1964. A theory of gel filtration and its experimental verification. *J. Chromatogr.* **14**:317-330.
22. MALAISSE-LAGAE, F., M. RAVAZZOLA, P. ROBBERECHT, A. VANDERMEERS, W. J. MALAISSE, and L. ORCI. 1975. Exocrine pancreas: evidence for topographic partition of secretory function. *Science (Wash. D. C.).* **190**:795-797.
23. MARCHIS-MOUREN, G. 1965. Étude comparée de l'équipement enzymatique du suc pancréatique de diverses espèces. *Bull. Soc. Chim. Biol.* **47-II**:2207-2217.
24. MCLEAN, J. D., and S. J. SINGER. 1970. A general method for the specific staining of intracellular antigens with ferritin-antibody conjugates. *Proc. Natl. Acad. Sci. U. S. A.* **65**:122-128.
25. OUCHTERLONY, O. 1958. Diffusion-in-gel methods for immunological analysis. *Prog. Allergy.* **5**:1-78.
26. PAINTER, G., K. T. TOKUYASU, and S. J. SINGER. 1973. Immunoferritin localization of intracellular antigens: the use of ultracryotomy to obtain ultrathin sections suitable for direct immunoferritin staining. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1649-1653.
27. PALADE, G. 1975. Intracellular aspects of the process of protein secretion. *Science (Wash. D. C.).* **189**:347-358.
28. PLUMMER, T. H., JR. 1968. Glycoproteins of bovine pancreatic juice. *J. Biol. Chem.* **243**:5961-5966.
29. PRICE, A., T.-Y. LIU, W. H. STEIN, and S. MOORE. 1969. Properties of chromatographically purified bovine pancreatic deoxyribonuclease. *J. Biol. Chem.* **244**:917-923.
30. ROTHMAN, S. S. 1975. Protein transport by the pancreas. *Science (Wash. D. C.).* **190**:745-753.
31. SCHEELE, G. A. 1974. Two-dimensional gel analysis of soluble proteins. *J. Biol. Chem.* **250**:5375-5385.
32. SCHEELE, G. A. 1976. Secretory proteins of the exocrine pancreas. *In* Cell Biology. P. L. Altman and D. D. Katz, editors. Federation of American Societies for Experimental Biology. Biological Handbooks. Bethesda, Md. 334-336.
33. SCHEELE, G. A., and G. E. PALADE. 1975. Studies on the guinea pig pancreas. *J. Biol. Chem.* **250**:2660-2670.
34. SCHEIDEGGER, J. J. 1955. Une micro-méthode de l'immunelectrophorèse. *Int. Arch. Allergy Appl. Immunol.* **7**:103-110.
35. TARTAKOFF, A. M., L. J. GREENE, J. D. JAMIESON, and G. E. PALADE. 1974. Parallelism in the processing of pancreatic proteins. *In* Cytopharmacology of Secretion. B. Ceccarelli, J. Meldolesi, and F. Clementi, editors. Raven Press, New York. **2**:177-194.
36. TARTAKOFF, A. M., and J. D. JAMIESON. 1974. Subcellular fractionation of the pancreas. *In* Methods in Enzymology, Biomembranes Part A. S. Fleischer and L. Packer, editors. Academic Press, Inc., New York. **31**:41-59.
37. TARTAKOFF, A. M., J. D. JAMIESON, G. A. SCHEELE, and G. E. PALADE. 1975. Studies on the pancreas of the guinea pig. Parallel processing and discharge of exocrine proteins. *J. Biol. Chem.* **250**:2671-2677.