STUDIES ON DISPERSED PANCREATIC EXOCRINE CELLS

I. Dissociation Technique and Morphologic Characteristics of Separated Cells

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ABSTRACT

A procedure for dissociation of the guinea pig pancreas into individual cells is described which employs enzymatic digestion with pure collagenase, chymotrypsin, and hyaluronidase, utilizes an interposed chelation of divalent cations by EDTA, and is terminated by gentle shearing. Yields of cells are 50–60%, based on DNA recovered. The population comprises ~95% exocrine cells, the remainder consisting of endocrine, duct, and vascular endothelial cells. The exocrine cells, though spherical, retain the structural attributes of their *in situ* counterparts, including differentiation of the plasmalemma into zones corresponding to the former apical and basal plasmalemma, polarized distribution of organelles indicated by fields of zymogen granules in the cytoplasm underlying the former apex, central location of the Golgi complex, and placement of the rough endoplasmic reticulum and nucleus in the former basal pole of the cell. Electron microscope study of the effects of individual treatments used during dissociation indicates that digestion of basement membrane and collagen is solely due to collagenase activity and that separation of desmosomes (and possibly of zonulae adherentes) results only from exposure to low [Ca^{++}] and EDTA and is not effected by the enzymes used. Gap junctions are resistant to enzymes and EDTA; tight junctions resist enzyme treatment but undergo rearrangement upon exposure to EDTA. Both junctions require mechanical shear for complete cell separation. Neither chymotrypsin nor hyaluronidase produces visible alterations in stromal or junctional elements. Dissociation requires the concerted action of enzymes, chelation of divalent cations, and mechanical shear, since the individual treatments are alone ineffective.

The secretory process in the exocrine pancreas has been analyzed in the past at the level of cells *in situ*, either in the intact animal (1) or more recently in vitro, using a slice (2) or lobule (cf. 20) system. The data from these studies pertain to the average events occurring in the total cell population. A further simplification of the system to the level of individual exocrine cells offers several advantages: it increases the homogeneity of the exocrine cell population; it improves experimental conditions since the cells can be manipulated as a suspension of individual units; and it allows for even access to

all domains of the plasmalemma which cannot be obtained *in situ*. With regard to the last point, suspensions of isolated cells should provide an opportunity to examine problems relating to the surface properties of the cell including the location of secretogogue receptors, molecular organization of the cell surface as it relates to regional differentiation of the plasmalemma, and dynamics of the plasmalemma during exocytosis of zymogen granules.

Recently we have described a procedure for the preparation of a population of isolated exocrine cells from the guinea pig pancreas (3). The technique, which utilized enzymatic digestion with crude collagenase and hyaluronidase, short exposure to divalent cation chelators, and mild shearing forces, was based on procedures developed by Howard et al. (4) and Berry and Friend (5) to obtain populations of hepatic parenchymal cells. While exocrine cell populations obtained with this procedure maintained the main functional and structural characteristics of exocrine cells *in situ*, the procedure was not satisfactorily reproducible. This was traced to batch variations in commercial preparations of collagenase which possess variable levels of nonspecific proteases (3). Low levels of proteases led to abnormally long dissociation times, and the procedure had to be terminated by vigorous shearing which resulted in lowered cell yields. High levels of protease activity, on the other hand, caused either uncontrollable dissociation which was often accompanied by cell lysis or produced cell populations with decreased functional abilities, particularly secretogogue responsiveness.

To circumvent these problems, we describe here an improved tissue dissociation procedure[1] which utilizes in the enzyme digestion chromatographically purified collagenase, hyaluronidase, and protease activity supplied as α-chymotrypsin. As will be noted later, dissociation requires protease and collagenase activity since pure collagenase and chymotrypsin alone are ineffective. The approach used in developing this procedure was to determine, first, the general protease activity present in the best batch of crude collagenase we had used and, then, to add to pure collagenase an equivalent amount of protease activity in the form of chymotrypsin. Chymotrypsin was chosen since its action is well known, it is available in highly purified form, and it will not activate zymogens released during the procedure. For this last reason the use of trypsin is precluded.

During the course of developing the dissociation procedure, structural and functional criteria were used to evaluate the quality of each cell preparation. This paper deals with the techniques and structural aspects of the dissociation procedure, while the accompanying one (7) describes the function of the separated cells. The approach to the final procedure was, however, based on both criteria.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: chromatographically purified collagenase (*Clostridium histolyticum*) 250–600 U/mg; crude bovine testis hyaluronidase ~470 U.S.P. U/mg (free of proteolytic activity as tested against Azocoll); purified α-chymotrypsin 46–50 U/mg; and chromatrographically purified soybean trypsin inhibitor (STI)[2], Worthington Biochemical Corp., Freehold, N. J.; bovine plasma albumin (BPA), fraction V, Miles Laboratories, Inc., Kankakee, Ill.; Azocoll, Calbiochem, San Diego, Calif. All other chemicals were reagent grade. Male albino guinea pigs 350–400 g (8–9-wk old) were obtained from the Rockefeller University colony. They were starved for 18–22 h with water given ad libitum, stunned by a blow to the head, and exsanguinated intrathoracically before removal of the pancreas.

Incubation Media

All dissociation and incubation media consisted of Krebs-Ringer bicarbonate solution (KRB) equilibrated with 95% O_2 and 5% CO_2 (to pH 7.4), and contained a complete amino acid supplement (8), 14 mM glucose, 0.1 mg/ml STI to prevent tryptic activation of zymogens and, where required, 4% or 1% BPA. STI does not affect the activity of collagenase although it decreases the activity of α-chymotrypsin (measured by the Azocoll assay) by ~30% when the ratio of chymotrypsin/STI is 2 (cf. reference 9). Ca^{++} and Mg^{++} concentrations were adjusted as required and are indicated in the text. BPA was dissolved in and dialyzed against KRB (containing 1.0 mM Ca^{++}; 1.2 mM Mg^{++}) without amino acids or glucose at concentrations of 20–35%, and stored at −20°C. This stock BPA solution was diluted into KRB at which time amino acids, glucose, and Ca^{++} were

[1] A brief account of this procedure was published previously (6).

[2] *Abbreviations used in this paper:* BPA, bovine plasma albumin; DNA, deoxyribonucleic acid; EDTA, ethylenediamine tetracetate; STI, soybean trypsin inhibitor; KRB, Krebs-Ringer bicarbonate solution; RER, rough endoplasmic reticulum.

added at levels indicated. Pure collagenase, hyaluronidase, STI, and α-chymotrypsin were added as salt-free powders. Media used for incubation of cells after dissociation contained 100 U/ml K penicillin G and 50 μg/ml streptomycin SO_4. All glassware was siliconized.

Analytic Procedures

DNA was measured according to the method of Burton (10). General protease activity was assayed against Azocoll (11).

Light and Electron Microscope Procedures

Intact pancreas or tissue pieces obtained during dissociation were immersed in fixative, diced into ~0.5-mm cubes, and further fixed for 1–4 h at 25°C with 1% formaldehyde-2% glutaraldehyde (12) in 0.1 M Na cacodylate, pH 7.4. Separated cells were fixed by mixing 1 vol of cell suspension in KRB-1% BPA with 2 vol of the above fixative. After fixing for 1 h at 25°C, the suspensions were centrifuged in polyethylene tubes (Microfuge 152, Beckman Instruments, Inc., Fullerton, Calif.) at 10,000 *g* for 5 min. Thin disks were then cut from the tubes with a razor blade (13). Disks of cells and tissue blocks were postfixed for 1–2 h at 4°C with 1% OsO_4 in 0.1 M Na cacodylate, pH 7.4, washed once with Veronal-acetate buffer, pH 7.4, and stained in block for 1 h at 23°C with 0.5% uranyl acetate in Veronal-acetate buffer, pH ~5 (14).

In some experiments tissues or cells were fixed in 1% OsO_4 alone. 1 vol of cell suspension in KRB-1% BPA was mixed with 3 vol of 1.33% OsO_4 in 0.1 M Na cacodylate, pH 7.4. To obtain comparable fixation conditions for tissue, 1 vol of KRB-1% BPA was added to 3 vol of the same OsO_4 solution before dicing. Fixation was carried out for 1–1.5 h at 4°C.

Tissue blocks and cell pellets were dehydrated in ethanol and propylene oxide and embedded in Epon (15). Cell preparations fixed with OsO_4 alone did not form coherent pellets in Microfuge tubes and were processed in suspension with centrifugation at 50 *g* for 5 min between dehydration steps. For light microscopy, 0.5-μm Epon sections were stained with 1% methylene blue in 1% Na borate (16), and photographed with a Zeiss Photomicroscope II (Carl Zeiss, Inc., New York). Darkfield, phase-contrast, and differential interference-contrast microscopy of living cells was also performed with this microscope, using slides and cover slips dipped in 1% BPA and air dried. For electron microscopy, thin sections were doubly stained with uranyl acetate and lead citrate (17) and examined and photographed in a Siemens Elmiskop 101 or an Hitachi 11 B electron microscope.

Morphometric Analysis of Exocrine Cells

Point counting analysis of the fractional volume occupied by organelles in intact and isolated exocrine cells was performed according to Weibel (18). The cells and tissue were fixed with OsO_4 alone as described above since this was found to produce less shrinkage than double aldehyde fixation, as determined by observations of unfixed and fixed isolated cells by Nomarski optics. Measurements were made on prints with final magnifications of × 6,000–12000 using a transparent overlay carrying a quadratic lattice with 1-cm spacing. 20 micrographs each of random sections of intact tissue and of sets of isolated cells were analyzed.

RESULTS

Dissociation Protocol

The technique for dissociation of the pancreas into isolated exocrine cells using pure collagenase, hyaluronidase, and α-chymotrypsin was essentially the same as that already described by us (3) for the system in which crude collagenase was employed. The procedure is summarized in Table I and described in detail below. The progress of each step was monitored under low magnification darkfield or Nomarski optics.

After collection of the gland in KRB (step 1), the procedure was initiated by injecting 5 ml of the enzyme mix at 23°C into the interstitium of the gland (0.8–0.9 g wet weight) with a no. 26 needle attached to a syringe (step 2). This distended the gland due to interstitial edema and rapidly exposed the majority of the acini to enzyme. The distended gland and residual enzyme were transferred to a 25-ml Erlenmeyer flask and incubated at 37°C under 95% O_2/5% CO_2.

During the first digestion (step 3) single cells were not liberated, although the gland loosened and appeared feathery due to digestion of tissue stroma.

Upon exposure to EDTA, single cells began to be liberated spontaneously, although the bulk of the tissue remained intact (step 4). Divalent cations were reintroduced (step 5) before the second enzyme digestion.

During the second digestion (step 6) tissue organization was progressively lost and a fine sandlike suspension of lobules, groups of acini, and many single cells resulted. The end point of the enzymatic treatment was indicated by the appearance of rounded-up cells within the remaining tissue pieces and by movement of cells within these pieces upon gentle compression of the cover slip. This was reached after ~70 min from the onset of incubation with variations among experiments of ± 5 min.

TABLE I

Protocol for Dissociation of the Pancreas into Individual Cells

Treatment	Medium composition*	Time	Temp	Morphologic change
		min	*°C*	
1. Collection of the gland	KRB, 0.1 mM Ca^{++}, 1.2 mM Mg^{++}	~2	24	
2. Injection to the gland interstitium of enzymes	KRB, 0.1 mM Ca^{++}, 1.2 mM Mg^{++} 200 U/ml collagenase, 0.2 mg/ml chymotrypsin, 2 mg/ml hyaluronidase	~3	24	Distention of extracellular spaces
3. First enzymatic digestion	As in step 2.	12	37‡	Digestion of basement membrane and collagen begins; initiation of desmosomal separation
4. Chelation of divalent cations	KRB, 2mM EDTA (no Ca^{++} or Mg^{++})	10	37	Separation of desmosomes completed
5. Replacement of divalent cations	KRB, 0.1 mM Ca^{++} 1.2 mM Mg^{++}	3–5	37	
6. Second enzymatic digestion	As in step 2.	40–50	37	Completion of stromal digestion
7. Pipeting	As in step 2.	~3	24	Separation of tight and gap junctions
8. Washes	KRB, 1.0 mM Ca^{++}, 1.2 mM Mg^{++}, 4% BPA	~10	24	
9. Final cell suspension	KRB, 2.5 mM Ca^{++}, 1.2 mM Mg^{++}, 1% BPA		37§	

* All media consisted of KRB supplemented with amino acids and glucose (see Materials and Methods) and contained 0.1 mg/ml STI. Only relevant additions to media are given.

‡ Steps 3–6 were carried out in a water bath at 37°C with agitation at 130 oscillations/min.

§ Cell suspension was incubated with agitation at 60 oscillations/min.

At termination of digestion, the suspension of lobules was pipeted up and down five times through pipets with tip diameters of 1.3 and 0.9 mm (step 7) which resulted in a suspension of separated cells. Any undissociated tissue or cell clumps were removed by filtration through 20-μm mesh nylon gauze.

To separate the intact cells from enzymes, cell debris, and damaged cells, equal halves of the suspension were layered over duplicate 8-ml cushions of 4% BPA in KRB containing 1.0 mM Ca^{++} and 1.2 mM Mg^{++} (step 8). The calcium ion concentration was elevated at this point to stabilize the membranes of the fragile, separated cells. After centrifugation at 50 *g* for 5 min, the intact cells formed a well packed tan pellet, whereas the contaminants remained in the supernate which was discarded. The pooled pellets were washed two times by resuspension and recentrifugation in 8 ml of 4% BPA in the same KRB and were finally suspended in KRB containing 1% BPA[3] and normal levels of Ca^{++} (2.5 mM) and Mg^{++} (1.2 mM) (step 9). The efficiency of the washes was indicated by the absence of cell debris and of detectable activity against Azocoll in the final preparation.

The yield of washed cells was 50–60% based on DNA content as compared to that in the starting tissue. This corresponded to ~2 × 10^8 cells. The population consisted of ~95% intact single exocrine cells.

Structural Aspects of the Dissociation Sequence

LIGHT MICROSCOPE OBSERVATIONS: The appearance of the normal pancreas before injection of the enzymes is shown in Fig. 1. Note the narrow intercellular spaces surrounding acini and the pyramidal shape of the exocrine cells. After injection of the enzyme mixture, the intercellular space was distended and became more loosened in appearance during the first digestion period (Fig. 2). At this time the exocrine cells, still organized in acini, appeared more rounded in profile although still closely apposed laterally.

After EDTA treatment the tissue organization was clearly disrupted (Fig. 3). Most of the exocrine

[3] After dissociation, it is imperative to have BPA or another source of macromolecule in the various wash solutions and incubation media in order to prevent cells from sticking to the glassware and lysing. Before pipeting, the cells appear to be protected by proteins added in the enzyme mix and liberated from damaged cells.

cells were rounded up and separated into clusters of varying numbers which were held together through contacts on their apical or lateral surfaces. Some of the cells were already separated.

At the end of the second digestion, the tissue organization had disintegrated: many exocrine cells were seen to be free, the others remaining in clusters of two to three (Fig. 4). The cells possessed generally rounded profiles with small cytoplasmic blebs protruding from the surfaces of some of them. Debris consisting of organelles and cytoplasmic blebs from disrupted cells was seen in the background as were islet cells and segments of small ducts and blood vessels.

After pipeting and three washes with 4% BPA-KRB, the preparation consisted primarily (~95%) of intact exocrine cells (Fig. 5), the remainder comprising islet cells, a few duct cells, small blood vessels, and damaged unidentifiable cells. A small number of the exocrine cells was still connected through apical-apical contacts forming dimers or trimers. Other exocrine cells occasionally appeared to be in random contact due, most likely, to the pelleting. Cell debris was almost completely absent, indicating the efficiency of the washes.

ELECTRON MICROSCOPE OBSERVATIONS: The fine structure of the exocrine pancreas has been well described in the past (1). In the description below we will concentrate on those aspects of acinar organization which can be expected to be affected by tissue dissociation.

As is typical of glandular epithelia (19), the exocrine cells of the guinea pig pancreas were joined laterally by junctional complexes which comprised, in the apical-basal direction, tight junctions, zonulae adherentes, and desmosomes with a well demarcated central dense plaque located in the intercellular space (Fig. 6). A large (0.5 μm in diameter) gap junction (21) was frequently found on the lateral plasmalemma about two-thirds of the distance from the apex to the base (Fig. 7).

Tight junctions, zonulae adherentes, and desmosomes also connected centroacinar and exocrine cells. Desmosomes were particularly numerous in these types of contacts. Gap junctions between centroacinar and exocrine cells have not yet been noted.

The basal plasmalemma of the exocrine cells was lined with a typical basement membrane which bridged but did not enter the lateral intercellular space (Fig. 8). Ducts, capillaries, and islet cells were also surrounded by basement membrane. In young animals the extracellular spaces surrounding the acini contained a loose network of collagen fibrils. Elastic fibrils were infrequently seen.

Changes after the First Enzymatic Digestion

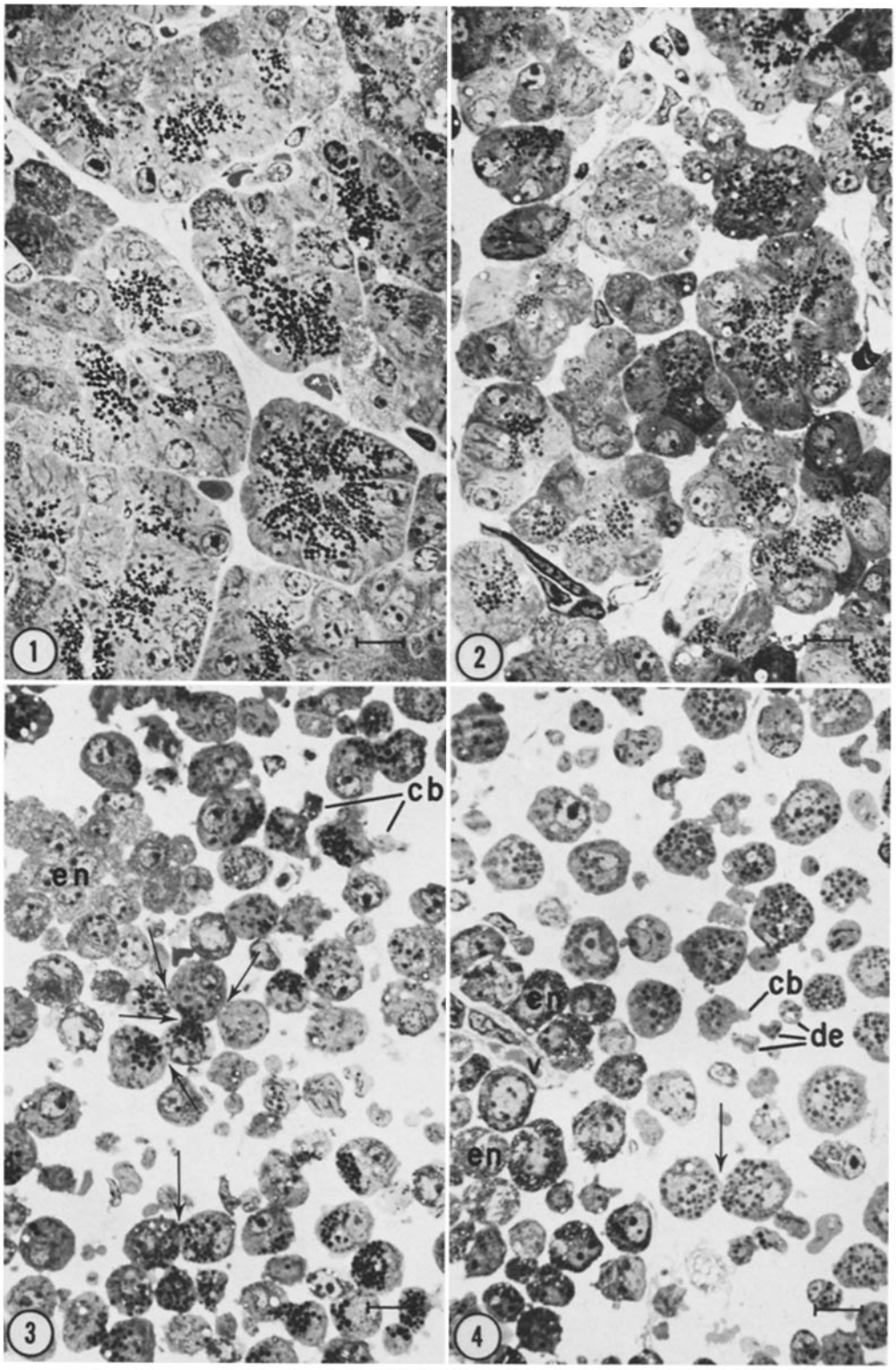
Most apparent at this stage was the uniform absence of the central dense plaque of the desmosomes in all exocrine-exocrine and exocrine-centroacinar cell contacts (Figs. 9, 10). Although the hemidesmosomes remained paired, the extracellular space between them was clearly widened. The cytoplasmic fibrils of the desmosomes were unaltered. Tight and gap junctions remained intact. Zonulae adherentes were usually unaffected, although some widening of the enclosed intercellular space was occasionally seen.

At the basal pole of the cell the basement membrane had largely disappeared, except for a few small loose patches (Fig. 11). Collagen fibrils were still visible although reduced in number.

Changes after EDTA Treatment

After EDTA exposure, many exocrine cells were widely separated from their neighbors along large stretches of the plasmalemma. While gap junctions remained intact, many tight junctions and zonulae adherentes had parted, exposing the acinar lumen to the medium. Hemidesmosomes were often seen to be pulled widely apart (Fig. 12). Unpaired hemidesmosomes were frequently present even though the adjacent plasmalemmas were still closely apposed. Images of hemidesmosomes, apparently engulfed as a plaque on the wall of an endocytic vacuole (Fig. 13), suggested that their partners had probably been internalized by phagocytosis. Such images comprising part of the wall of a detached vacuole in the apical cytoplasm were common (Figs. 14, 15).

At this stage, some of the exocrine cells were completely separated from the tissue. Near the former apical pole, they often carried cytoplasmic blebs derived from neighboring cells and attached through remnants of tight junctions and zonulae adherentes; such blebs were also seen on the former lateral plasmalemma attached by gap junctions. Hemidesmosomes were not seen on these separated cells and had probably already been internalized.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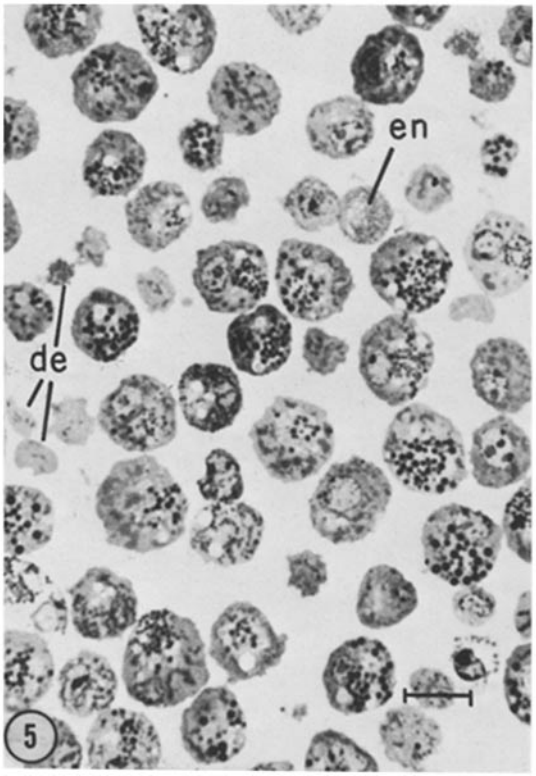


FIGURE 5 Final washed cell preparation consisting mainly of exocrine cells and, in this field, an endocrine cell (*en*). *de*, residual cytoplasmic debris. × 840.

Appearance at the End of the Second Enzymatic Digestion

Many single exocrine cells were spontaneously liberated from the tissue before pipeting. Those cells which were still in contact were held together primarily through gap and tight junctions.

At this stage, an increase in the number of condensing vacuoles containing loosely packed flocculent material was evident in both the separated cells (e.g. Figs. 16, 19) and in the remaining undissociated tissue. Hemidesmosomes were rarely seen, although images of these structures internalized in cytoplasmic vacuoles were common. Zonulae adherentes, when present, were ill defined. Collagen and basement membrane had completely disappeared by now.

Fine Structure of the Isolated Cells

Electron microscopic studies were made on thin sections of separated cells immediately after isolation and after incubation for up to 4 h at 37°C. The description will be restricted to exocrine cells, the major cell type in the population.

Observations on freshly isolated cells showed that, although they were rounded up, they retained their *in situ* anisotropy with regard to surface specialization of the plasmalemma and distribution of cytoplasmic organelles (Fig. 16). The former apical plasmalemma, identifiable by its associated microvilli, overlay an ectoplasmic layer comprising filamentous and amorphous material which, as in the intact tissue, excluded cellular organelles (Fig. 17). This layer, in turn, overlay a region of the cytoplasm containing the zymogen granule population. The apical plasmalemma was frequently delineated laterally by remnants of tight junctions and zonulae adherentes that were attached to swollen cytoplasmic blebs derived from neighboring cells (Fig. 17). Hemidesmosomes were absent from the cell surface but could be recognized on the wall of vacuoles in the apical cytoplasm.

The remainder of the plasmalemma consisted of a unit membrane free of basement membrane except for a few small patches (Fig. 16). Cytoplasmic blebs were occasionally seen attached through

FIGURES 1–5 Light micrographs of dissociation sequence. 0.5-μm thick Epon sections stained with alkaline methylene blue. Scale marker = 10 μm. All are × 680, except for Fig. 5 which is × 840.

FIGURE 1 Fresh pancreas.

FIGURE 2 Tissue at end of first 15-min digestion. Extracellular space is widened and exocrine cells are beginning to round up.

FIGURE 3 After EDTA treatment. Arrows indicate exocrine cells still attached to each other. *en*, endocrine cells; *cb*, cytoplasmic blebs on cells.

FIGURE 4 After second digestion but before pipeting. A few cells remain attached at their apices (arrow); *cb*, cytoplasmic bleb; *v*, vascular endothelium; *en*, endocrine cells; *de*, cell debris.

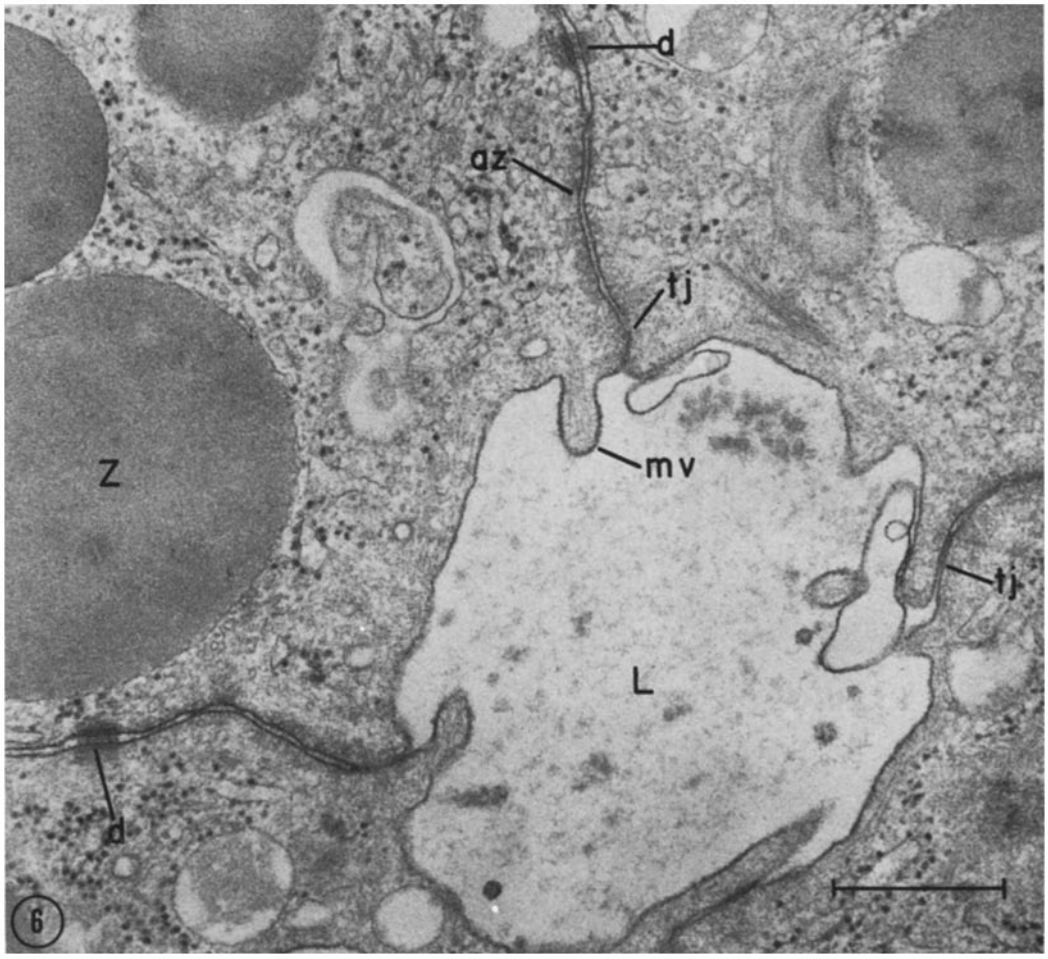


FIGURE 6 Transection through apical portions of four exocrine cells before dissociation. Junctional complex comprising tight junctions (*tj*), adhering zonulaes (*az*), and desmosomes (*d*) is shown. *Z*, zymogen granule; *L*,acinar lumen; *mv*, microvilli on apical plasmalemma. Scale marker = 0.5 μm. × 44,000.

gap junctions to that portion of the plasmalemma corresponding to the former lateral surface (Fig. 18). These blebs were not swollen, suggesting that the gap junction still allowed for equilibration across it of water and small solutes. Invaginations reminiscent of pinocytotic pits were frequently seen along the former basal plasmalemma. Pinocytotic pits in this region are characteristic features of exocrine cells *in situ* (e.g. Fig. 8).

In addition to the placement of zymogen granules, other organelles characteristic of exocrine cells also retained their *in situ* polarity in the isolated cells. Thus, the elements of the Golgi complex were centrally located between the nucleus and the former apical region, and the nucleus was disposed in the former basal pole which contained the majority of the rough endoplasmic reticulum (RER). The increase in condensing vacuoles containing loosely packed material persisted (e.g. Fig. 16). Autophagic vacuoles containing ribosomes, RER, and unidentified membranes were commonly seen (see Fig. 7 of reference 7) and were located near the Golgi region or laterally in the cell.

After 4-h incubation in KRB-1% BPA, the isolated cells retained their polarity and were well preserved. Some additional increase in the proportion of condensing vacuoles and autophagic vacuoles was detected and quantitated. Cytoplasmic blebs at the former apical and lateral sides of the cell had disappeared. Hemidesmosomes in cytoplasmic vacuoles were no longer detectable.

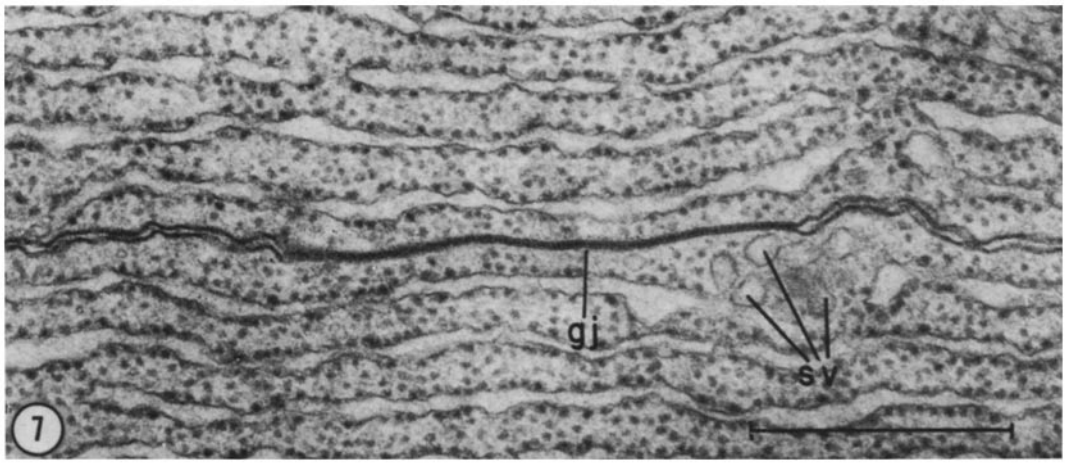


FIGURE 7 Gap junction (*gj*) in lateral intercellular space between exocrine cells. A cluster of smooth vesicles (*sv*) is shown near the end of the junction in the cytoplasm. Scale marker = 0.5 μm. × 68,000.

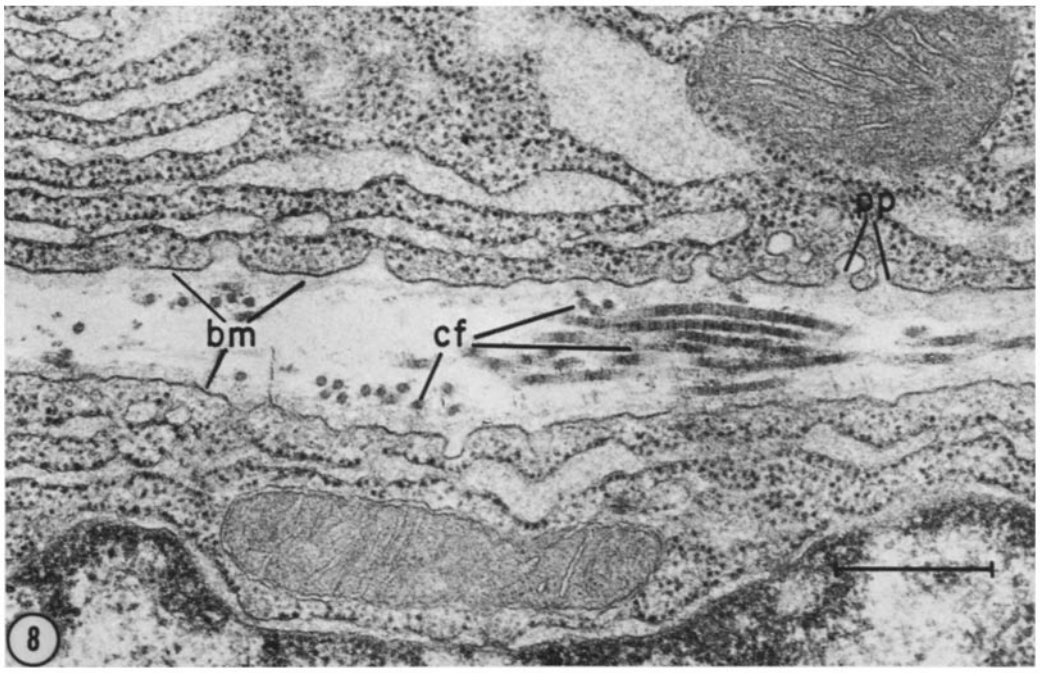


FIGURE 8 Extracellular space at the acinar base in fresh tissue. Basement membrane (*bm*) and collagen fibers (*cf*) are indicated as are pinocytotic pits (*p*). Scale marker = 0.5 μm. × 39,000.

Morphometic Analysis

To determine if changes occur in the relative volumes of cytoplasmic constituents of exocrine cells after tissue dissociation, morphometric measurements were done on cells in intact tissue, freshly isolated cells, and cells incubated over 4 h.

The results are given in Table II. Immediately after dissociation and for up to 4-h incubation, the nuclear-to-cytoplasmic volume ratios of isolated cells were identical to those of exocrine cells *in situ*, suggesting that the overall volume of the isolated cells was unaffected by dissociation and subsequent incubation. This conclusion is based on

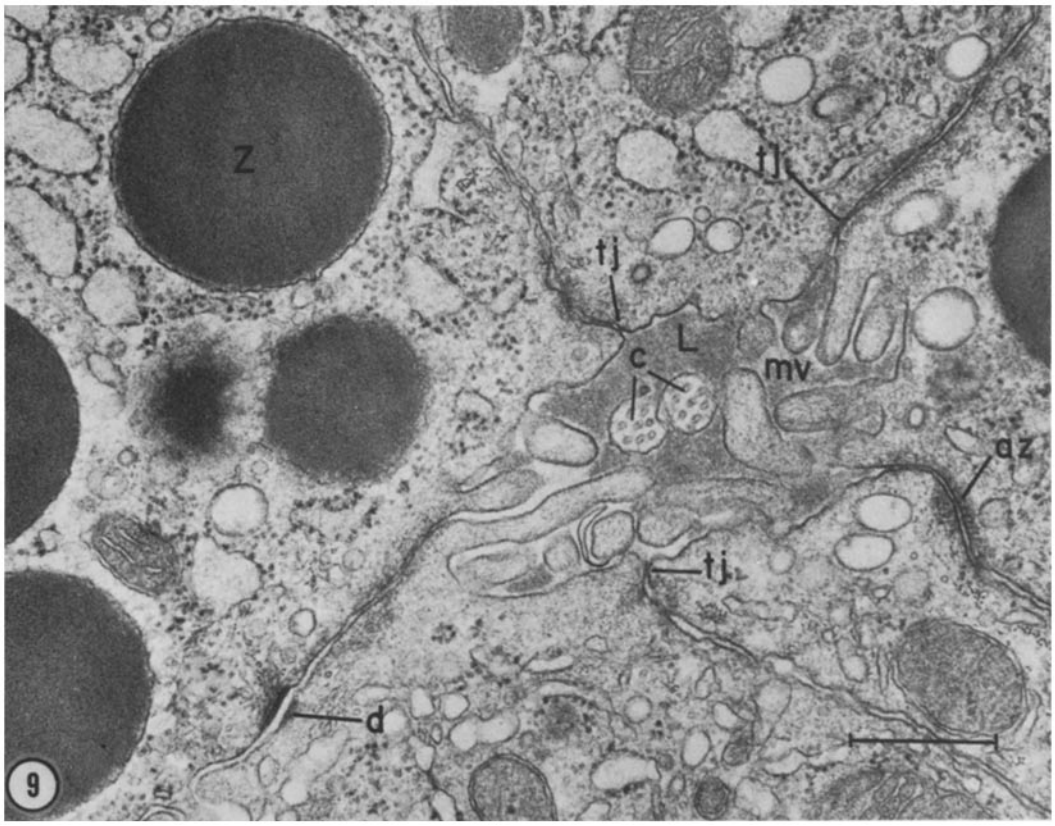


FIGURE 9 Transection through the apical zone at end of first digestion period. Central plaque of the desmosome is absent. Tight junctions (*tj*) and adhering zonules (*az*) remain intact. *L*, acinar lumen; *mv*, apical microvilli; *c*, cilia probably originating from centroacinar cells not included in the section; *Z*, zymogen granule. Scale marker = 0.5 μm. × 38,000.

the assumption that the nuclear volume is the same in isolated cells as those *in situ*, an assumption confirmed by light microscopy. With the exception of mitrochondria and zymogen granules, alterations were noted in the other cell compartments. In freshly isolated cells the relative volume of the RER cisternal space was similar to that of exocrine cells *in situ;* this volume decreased slightly over 4-h incubation. On the other hand, in freshly isolated cells the volume of the Golgi region was approximately two times greater than that of intact tissue, although the volume tended to return to normal after 1–2-h incubation, indicating that the initial increase was due to a generalized swelling of Golgi elements. The total volume of the condensing vacuole population was approximately four times greater than in the intact tissue and was accounted for mainly by an increase of loose condensing vacuoles. Between 2 and 4 h of incubation, the volume of loose condensing vacuoles doubled over that found immediately after dissociation. Finally, the volume of lysosome-like (autophagic) bodies, while initially the same in isolated cells and cells *in situ*, progressively increased approximately seven-fold during 4-h incubation. The increase in fractional volume of the Golgi zone, condensing vacuoles, and autophagic vacuoles appeared to result from a commensurate and equivalent decrease in the volume of the RER and cytoplasmic matrix.

Effect of Individual Treatments on Tissue Integrity

During dissociation, several different agents were used in combination (during enzyme treatment: low [Ca^{++}], collagenase, chymotrypsin, and hyaluronidase) or alone (EDTA), and it was

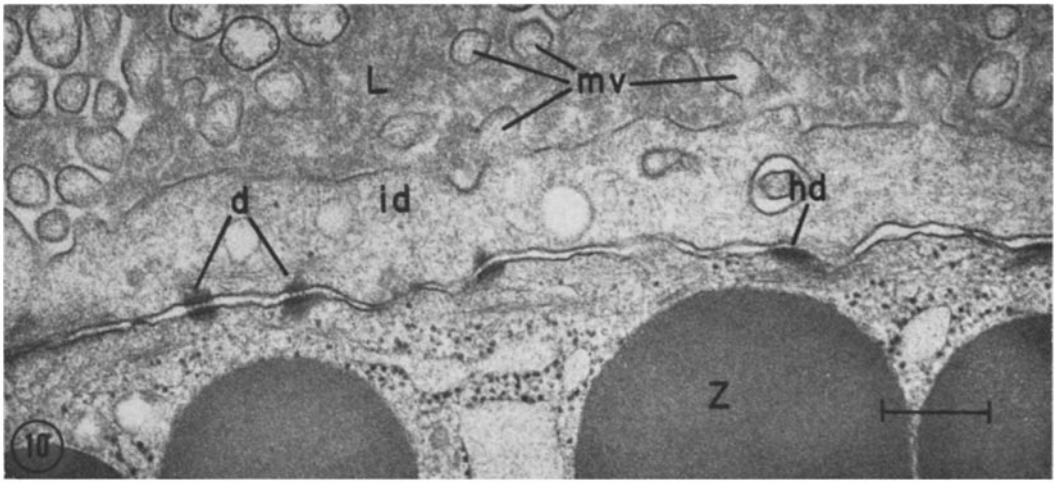


FIGURE 10 Junction between exocrine cell identified by zymogen granule content (*Z*) and an intercalated duct cell (*id*) at end of first digestion period. Several desmosomes (*d*) lacking their central dense plaque and a hemidesmosome (*hd*) are seen. *L*, duct lumen; *mv*, microvilli on centroacinar cell. Scale marker = 0.5 μm. × 38,000.

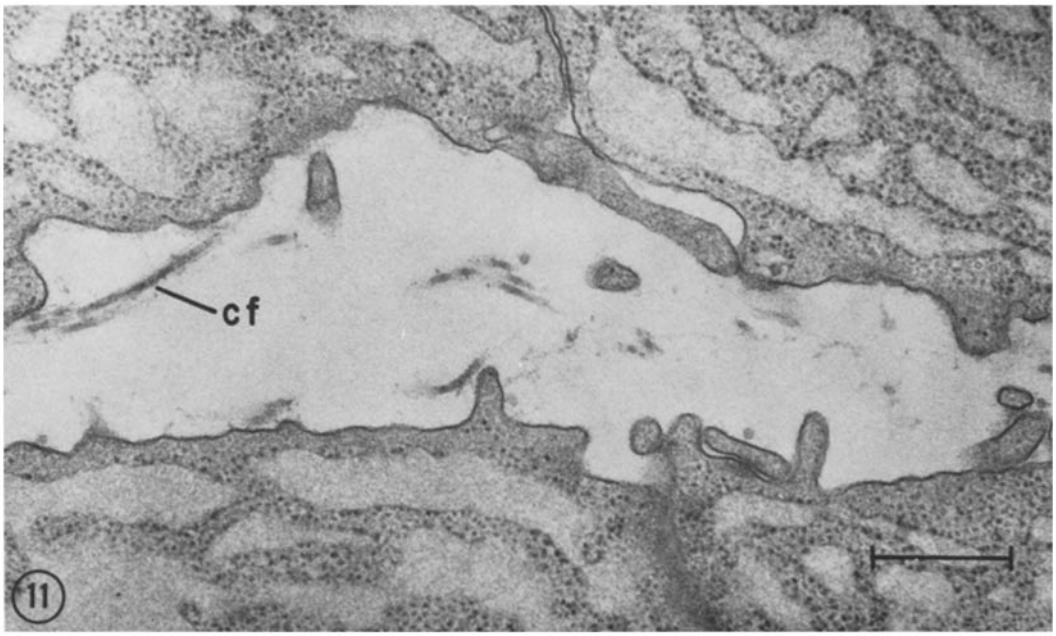


FIGURE 11 Basal extracellular space at end of first digestion period. Basement membrane has largely disappeared though a few collagen fibers (*cf*) remain. Scale marker = 0.5 μm. × 37,000.

important to assess each in effecting dissociation. To this end the gland was distended with and incubated in each of these agents for the indicated times, and morphologic studies were done.

LOW [Ca^{++}]: When KRB containing 0.1 mM Ca^{++} (with 1.2 mM Mg^{++}) was injected and used for incubation over 15 min, the only detectable change consisted of extraction of the central dense plaque and associated amorphous material in the intercellular space between desmosomes. Extrac-

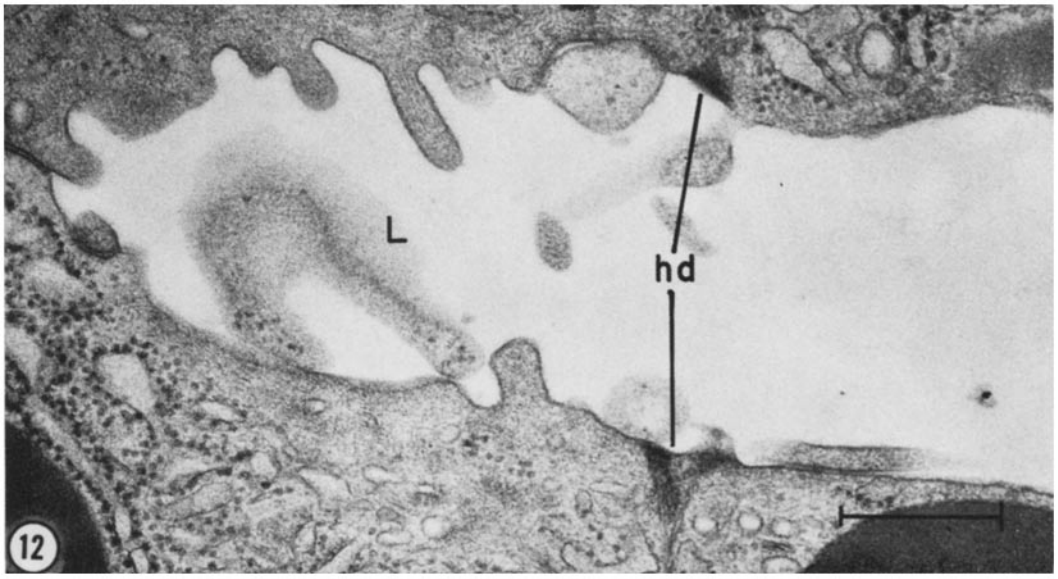
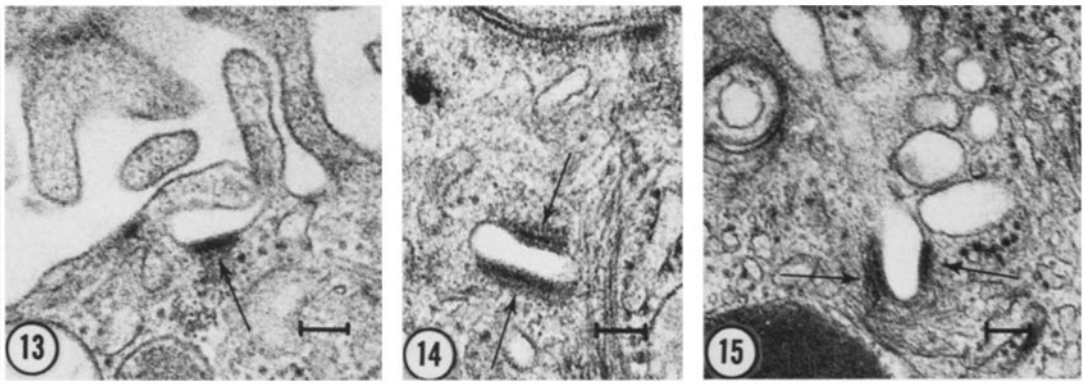


FIGURE 12 Apical zone after EDTA treatment. The acinar lumen (*L*) is now open to the medium after separation of desmosomes indicated by remaining hemidesmosomes (*hd*). Scale marker = 0.5 μm. × 45,000.



FIGURES 13–15 Selected views suggesting internalization of desmosomes as part of the wall of endocytic vacuoles near the cell apex. End of EDTA treatment. Fig. 13, × 70,000. Scale marker = 0.1 μm. Fig. 14, × 75,000. Scale marker = 0.1 μm. Fig. 15, × 60,000. Scale marker = 0.1 μm.

tion was more pronounced in desmosomes located basally and was more efficient after 1-h incubation, indicating that outward diffusion of Ca^{++} may be limiting. After 1-h incubation, an increase in the number of condensing vacuoles, especially those of the loose variety, was evident (as in Fig. 19). Gap and tight junctions and zonulae adherentes remained intact.

These effects appeared to be due solely to low Ca^{++}, since incubation in KRB containing 2.5 mM Ca^{++} with or without 1.2 mM Mg^{++} resulted in no changes. However, similar alterations were noted when KRB was used which lacked Ca^{++} but contained an equivalent concentration of divalent cations as Mg^{++} (3.7 mM).

EDTA: After 15 min of incubation with 2 mM EDTA in the absence of divalent cations, all the changes noted after 1-h incubation in low (0.1 mM) Ca^{++}-containing medium were present (Fig. 19). At the base of the cell, the plasmalemma was thrown into folds over which the basement membrane was elevated (Fig. 20). The basally located

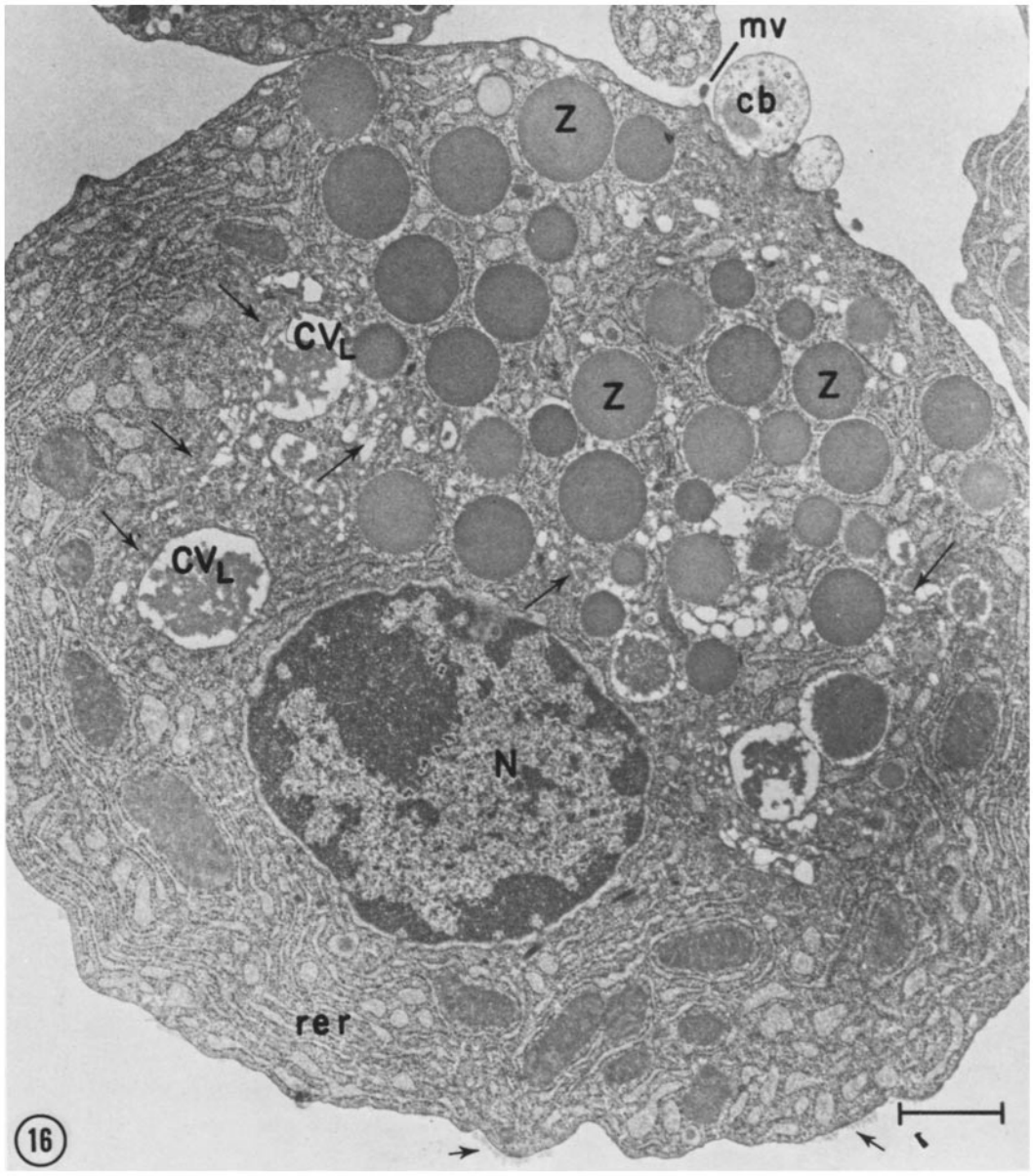


FIGURE 16 Low-power electron micrograph of an isolated exocrine cell immediately after completion of tissue dissociation. The former apical plasmalemma is identified by associated microvilli (*mv*); cytoplasmic blebs (*cb*) derived from neighboring cells are commonly located here. The remaining plasmalemma is a simple membrane devoid of basement membrane but for a few small patches (short arrows). Polarity of organelle distribution is indicated by the zymogen granule (*Z*) field in the former apical zone; central location of Golgi elements (arrows) including loose condensing vaculoes (CV_L), and basal location of the nucleus (*N*) and surrounding fields of rough endoplasmic reticulum (*rer*). Scale marker = 1 μm. × 14,000.

RER cisternae were somewhat dilated. Collagen fibrils and tight and gap junctions remained intact, although the intercellular space between zonulae adherentes was occasionally enlarged.

After 1-h incubation with EDTA, no further changes in cell junctions or matrix elements were noted, although the cells were damaged, as indicated by massive vesiculation of the RER with

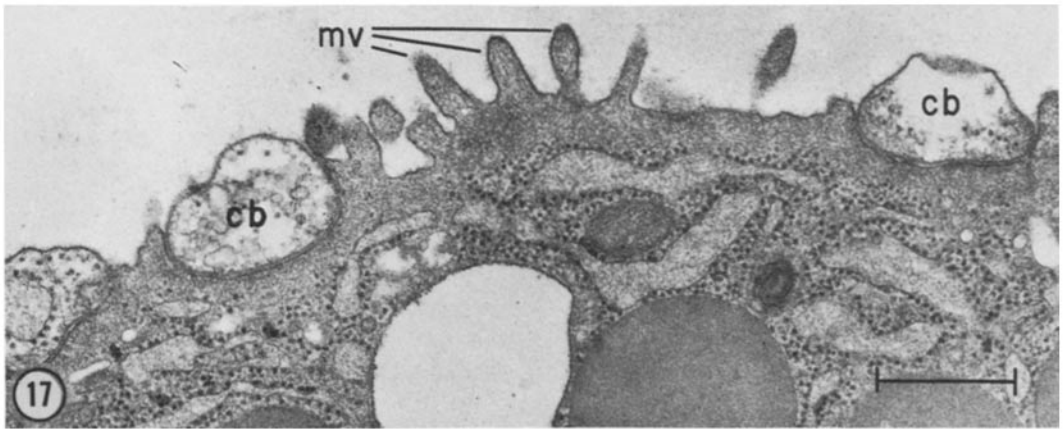


FIGURE 17 Higher magnification view of the former apical plasmalemma on an isolated exocrine cell. Associated microvilli (*mv*) are indicated as are cytoplasmic blebs (*cb*) torn from adjacent cells during dissociation. These are attached by tight junctions and possibly adhering zonules. Scale marker = 0.5 μm. × 36,000.

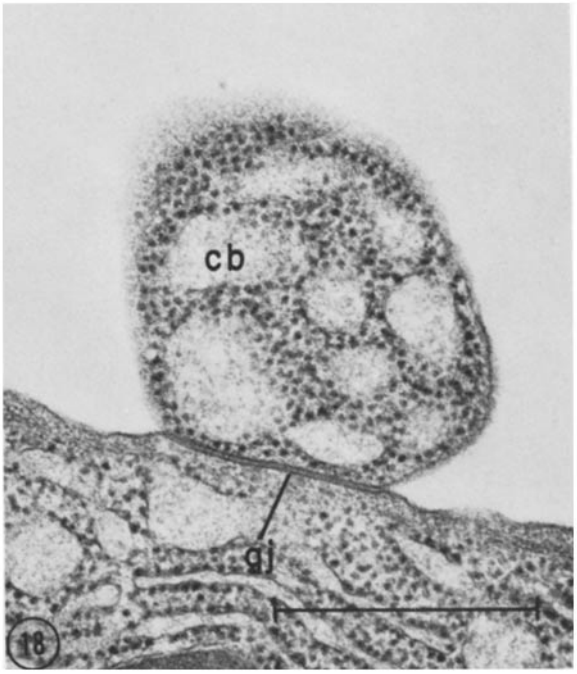


FIGURE 18 A cytoplasmic bleb (*cb*) containing cisternae of the rough endoplasmic reticulum is attached to the lateral side of a separated cell via a gap junction (*gj*). Scale marker = 0.5 μm. × 67,000.

ribosomal detachment, extraction or destruction of zymogen granules, and fragmentation of the Golgi complex.

COLLAGENASE: 1-h incubation in KRB containing 200 U/ml collagenase, 0.1 mM Ca^{++}, and 1.2 mM Mg^{++} led to the complete removal of basement membrane and collagen fibrils. Tight and gap junctions and zonulae adherentes were unaltered although the desmosmal central plaque was absent. Alteration of desmosomes was attrib-

TABLE II

Morphometric Analysis of In Situ and Isolated Pancreatic Exocrine Cells

Cell source‡	% Relative cytoplasmic volumes*								Volume nucleus / Volume cytoplasm
	RER cisternae	Golgi region	CV loose	CV tight	ZG	Autophagic vacuoles	Mitochondria	Cytoplasmic matrix	
									%
Intact tissue	19.4 ± 1.2	5.8 ± 0.8	-	1.7 ± 0.5	19.5 ± 1.8	0.2 ± 0.1	7.1 ± 0.7	46.2 ± 1.3	13.6 ± 2.1
Isolated cells 10-min-incubation	17.0 ± 1.5	10.5 ± 0.7	3.8 ± 0.4	3.1 ± 0.5	20.0 ± 1.6	0.2 ± 0.1	6.5 ± 0.5	39.9 ± 1.1	12.1 ± 1.2
30-min incubation	16.0 ± 0.9	12.5 ± 0.8	5.4 ± 0.6	1.8 ± 0.4	16.5 ± 1.0	0.3 ± 0.1	7.3 ± 0.5	40.4 ± 1.0	13.4 ± 2.0
1-h incubation	14.1 ± 1.0	10.3 ± 0.9	5.2 ± 0.8	2.7 ± 0.5	17.8 ± 1.2	1.0 ± 0.2	7.4 ± 0.6	43.1 ± 1.5	13.5 ± 2.0
2-h incubation	13.3 ± 0.8	7.8 ± 0.7	4.8 ± 0.7	2.8 ± 0.5	19.7 ± 1.5	1.1 ± 0.3	7.6 ± 0.7	43.1 ± 1.4	14.2 ± 2.2
4-h incubation	12.5 ± 0.9	8.4 ± 0.7	8.4 ± 0.8	2.8 ± 0.5	20.2 ± 1.4	1.4 ± 0.3	6.1 ± 0.5	40.4 ± 1.5	13.0 ± 2.1

* Data are given as percent relative to cytoplasmic volume (= 100%) ± SEM. Cytoplasmic volume = total cell volume-nuclear volume. The relative cytoplasmic volume of the Golgi complex refers to the region of the cell occupied by both membranous elements and associated cytoplasmic matrix. The cytoplasmic matrix refers to the volume remaining after formed elements, including the Golgi region, were subtracted from the cytoplasmic volume. The condensing vacuole population was subdivided into condensing vacuoles with a loosely packed content ("loose condensing vacuoles") and condensing vacuoles with a tightly packed content ("tight condensing vacuoles").

‡ 20 micrographs from intact tissue and from each sample of isolated incubated cells were analyzed.

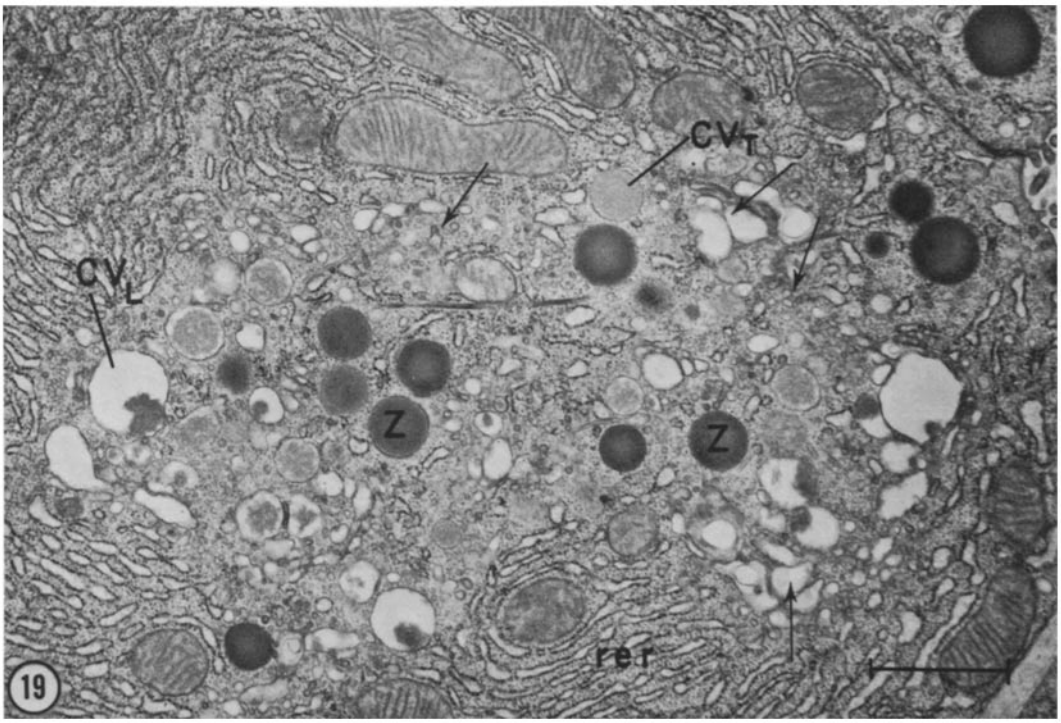


FIGURE 19 Low-magnification micrograph of an exocrine cell from tissue incubated 15 min in medium containing 2.0 mM EDTA. Note distention of Golgi vacuoles (arrows) and appearance of loose condensing vacuoles (CV_L). *Z*, zymogen granule; CV_T, tight condensing vacuole; *Z*, zymogen granule; *rer*, rough endoplasmic reticulum. Scale marker = 1 μm. × 17,000.

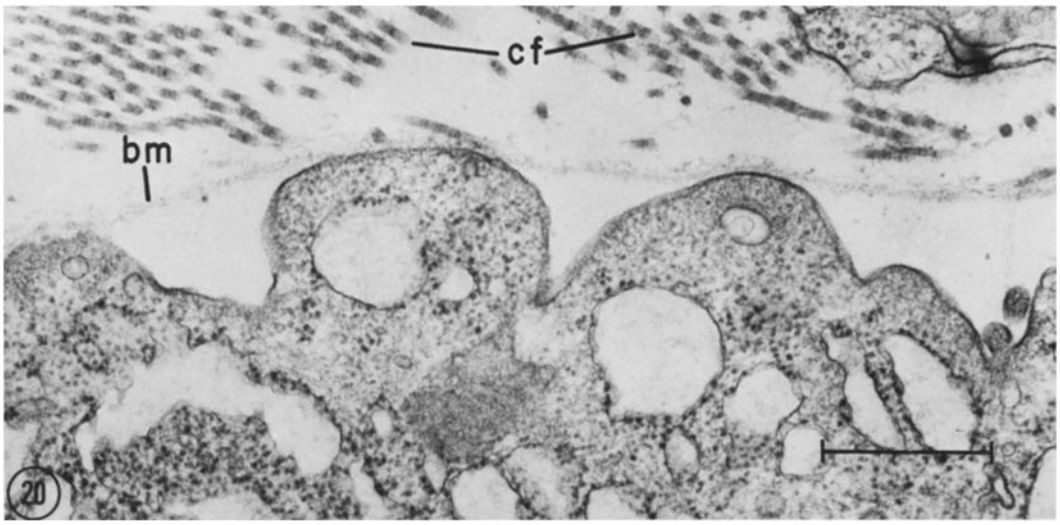


FIGURE 20 Tissue treated as in Fig. 19 and showing elevation of the basement membrane over infoldings of the basal plasmalemma. Scale marker = 0.5 μm. × 43,000.

utable to the low [Ca^{++}] since incubation with collagenase in medium containing 2.5 mM Ca^{++} and 1.2 mM Mg^{++} preserved the plaque. The latter conditions resulted in complete digestion of collagen fibrils but less complete removal of basement membrane. Incubation with collagenase alone did not lead to tissue dissociation.

α-CHYMOTRYPSIN AND HYALURONIDASE: Incubation of the gland with these enzymes alone for 1 h at concentrations used in the combined enzyme treatment resulted in no alterations of either cell junctions or matrix elements over those attributable to low [Ca^{++}].

DISCUSSION

Our results indicate that dissociation of the pancreas into a population of single cells depends on four factors: (*a*) collagenolytic and (*b*) proteolytic activity, (*c*) chelation of divalent cations with EDTA, and (*d*) shearing forces generated by agitation of the flask and pipeting. That these factors are necessary, but not sufficient, is indicated by the observation that each factor applied alone does not lead to dissociation.

Although the combination of treatments was arrived at empirically, and could be refined further, it satisfied our objectives: to obtain a population of viable exocrine cells in good yield. For practical reasons therefore only those agents which were cytotoxic (chymotrypsin and EDTA treatment) were examined in detail in order to determine the optimal procedure. Chymotrypsin at levels two times higher than used in the final procedure led to rapid but uncontrollable dissociation which often ended with cell lysis, while chymotrypsin at half the concentration used finally resulted in impractically long dissociation times. When chymotrypsin was applied separately, after collagenase treatment, it was less effective than combined digestion and resulted in a final preparation consisting of clusters of three to four cells. Omission of EDTA, reduction of its concentration to 0.1 mM, or substitution of a wash in low (0.1 mM) Ca^{++} or Ca^{++}-free medium, markedly reduced dissociation efficiency. EDTA at a concentration of 10 mM proved to be cytotoxic. Application of EDTA at the end of the procedure yielded cells with many cytoplasmic blebs and a tendency to lyse; inclusion of EDTA before enzymatic digestion was ineffective.

The amount of pure collagenase activity used was based on our previous studies (3). The use of collagenase with two times that activity did not improve dissociation; levels of < 200 U/ml were not tested, although they may be adequate since the morphologic effects are seen after only 15-min incubation.

Other proteases such as pronase, subtilisin, carboxypeptidase, and leucine aminopeptidase proved to be less effective to the extent that they were tested; for reasons mentioned earlier, trypsin

cannot be used for dissociation of the pancreas.

Although hyaluronidase was originally included because of its use for dissociation of the liver (5), we have found recently that omission of hyaluronidase does not alter our dissociation procedure so long as an equivalent amount of protein, supplied as BPA, is included during enzyme digestion. This added protein appears to protect those cells liberated spontaneously during the later stages of dissociation.

The rationale for the success of our procedure is supplied in part by morphologic observations on tissue exposed to the individual agents or treatments. Two observations are clear. First, disruption of desmosomes, as indicated by disappearance of the central dense plaque and concomitant separation of hemidesmosomes, is the result of exposure to low [Ca^{++}] including that produced by EDTA. Sensitivity of desmosomes to chelating agents in other tissues (5, 22, 23) has been reported, although Borysenko and Revel (24) have shown that desmosomes in rat pancreas, submandibular gland, and liver as well as those of stratified squamous epithelia are resistant to EDTA. Desmosomes in simple columnar epithelia, however, were found to be EDTA sensitive. In our study, desmosomes were resistant to pure collagenase and chymotrypsin when enzymatic treatment was in media containing 2.5 mM Ca^{++}; this may explain why dissociation is inefficient when carried out in media containing 1.0 or 2.5 mM Ca^{++}. Similarly, desmosomes in the rat pancreas are trypsin resistant (24), although resistance is dependent on tissue source (24). Overton (25) has reported that trypsin cleaves desmosomes in embryonic tissue although her experiments were carried out in Ca^{++}-free media. Second, dissolution of the basement membrane and collagen fibrils is effected by pure collagenase alone (26); collagenase is effective only so long as the medium contains Ca^{++} (unpublished observations and reference 27).

In contrast, chymotrypsin treatment alone does not result in any apparent change in fine structure, although it is required for dissociation. Possibly chymotrypsin cleaves exposed plasmalemmal proteins in a manner similar to its effect on red blood cells (28) and, in the process, removes groups which might directly or through ligands such as Ca^{++} be responsible for cell adhesion. The lack of effect of chymotrypsin on matrix collagen is consistent with its inability to attack triple helical collagen (29).

Both gap and tight junctions resist digestion by collagenase and chymotrypsin, either alone or in combination, while gap junctions survive prolonged exposure to EDTA which is consistent with their resistance to proteolysis and chelators of divalent cations (30). Tight junctions, however, undergo alterations by EDTA treatment as detected with freeze-fracture (Gilula, personal communication). Disruption of gap and tight junctions is aided by mechanical shear at the end of the procedure, although some succumb to flask agitation as indicated by the appearance of free cells during the final digestion step. During mechanical shearing, membrane bounded cytoplasmic blebs appear to be avulsed from neighboring cells to remain attached to the separated cell through remnants of tight or gap junctions. In view of the beltlike arrangement of tight junctions around the apices of exocrine cells *in situ*, it is evident that the plasma membrane of the cell from which these blebs were derived must reseal; otherwise, a much smaller yield of cells would be expected.

The fate of the zonulae adherentes is difficult to assess, although the impression is that separation of this junction follows separation of desmosomes, suggesting that it is either mechanically less adhesive than tight and gap junctions or is also disrupted by low [Ca^{++}].

The disposal of junctional elements during and after dissociation is partially understood. In the case of desmosomes, it appears that shortly after the loss of the central dense plaque, the separated hemidesmosomes are internalized on the wall of endocytic vacuoles. In freshly isolated cells internalized desmosomes are frequently seen, but during subsequent incubation all vestiges of desmosomes disappear possibly through lysosome-mediated digestion and depolymerization of associated fibrils in the cell sol. Similar images have been seen in other enzymatically dispersed cells (5, 25). Tight and gap junctions associated with cytoplasmic blebs are frequently seen on the surface of freshly dispersed cells but with time disappear. Whether the blebs lyse or are released to the medium, or whether they are internalized along with the junction cannot be ascertained from thin sections, although freeze-fracture studies underway suggest that the latter route is followed by some tight junctions. The disposal of separated zonulae adherentes is not understood.

In comparison with previously published dissociation procedures in which digestion with crude collagenase forms the basis (4, 5), our procedure

differs in three respects. First, collagenolytic and protease activities are present as pure enzymes whose activities are readily controlled. In the older procedures, these activities were present at variable levels depending on the batch of crude collagenase used. Second, enzyme digestion is performed in media containing low [Ca^{++}] which supports the collagenase activity, (27) yet permits desmosomal disruption. Many previous procedures, with the exception of a recent report by Howard et al. (32), have utilized Ca^{++} and Mg^{++}-free media during digestion with crude collagenase. Under these conditions, it is likely that tissue dissociation was due mainly to the proteases in crude collagenase. On the other hand, Ca^{++}-free media no doubt potentiated dissociation as a result of desmosomal cleavage. Third, our procedure employs early EDTA treatment which should accelerate desmosomal cleavage thus loosening the tissue and facilitating enzyme accessibility. In other procedures EDTA has usually been applied terminally (5) although it has been suggested (33) that early application of chelators shortens enzymatic dissociation of the liver possibly for the reasons mentioned.

The isolated cell population which we have obtained consists primarily of exocrine cells which are present in roughly the same proportion as found in the intact pancreas. The following comments will be limited to this cell type.

In its general organization the isolated exocrine cell retains the characteristic features of its counterpart *in situ* including in phase distribution of intracellular organelles and structural differentiation of the bounding plasmalemma into a former apical (lumenal) zone recognized on thin section by associated microvilli and a zone comprising the former lateral-basal plasmalemma. That these features are retained over several hours of incubation suggests that tissue organization per se is not necessarily the determinant of cellular polarity and that preexisting structural elements within the cell are probably the prime factors. This is of special importance in the case of the apical plasmalemma since, in view of the fluid nature of cell membranes (34) which allows for movement of membrane constituents in the plane of the membrane, it might have been anticipated that the apical domain with its microvilli would shift in position relative to the underlying cytoplasm. In the intact tissue, the apical plasmalemma is delineated circumferentially from the remainder of the plasmalemma by junctional complexes associated with neighboring cells. In the isolated cells, such contact points are lost, yet the apical plasmalemma remains as an identifiable region in register with underlying cytoplasmic organelles, principally the zymogen granule field, suggesting that this region is anchored with reference to the underlying cytoplasm, possibly through microfilaments (or other structures) associated with the microvillar core (35). The fate of specific membrane proteins on the surface of the isolated cells (e.g. hormone and lectin receptors) remains unknown but is under investigation. For the moment, however, freeze-fracture studies of isolated cells carried out with Dr. N. B. Gilula show that small "islands" and strands of tight junctions migrate from the former apical pole toward the lateral and basal sides of the cell where they become associated with remnants of gap junctions, suggesting lateral mobility of some membrane constituents.

The observation that the exocrine cells round up upon separation deserves comment. While it is generally accepted that microtubules (36), and possibly microfilaments (35, 37), are in part responsible for development of cell shape, their role in maintenance of cell shape is still open to some speculation. In the isolated exocrine cells, microfilaments and microtubules are still visible although a detailed morphologic examination is needed to determine if partial depolymerization and or redistribution of these structures can be correlated with the shape change of the separated cells.

Addendum

Bolender (*J. Cell Biol.* 1974 **61**:269) has recently reported stereological data on the normal guinea pig pancreas. With the exception of a lower value for zymogen granule volume (~7%), his values are generally similar to those reported by us using similar techniques.

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