CELLULAR INJURY *IN VITRO:* PHASE CONTRAST STUDIES ON INJURED CYTOPLASM

I. K. BUCKLEY, M.B., B.S.

From the Department of Pathology, University of Melbourne, Australia

ABSTRACT

Unfixed, compressed acinar cells of rat pancreas, isolated by mechanical and enzymatic means, were examined by phase microscopy and photomicrographed using 35 mm film and electronic flash illumination. Similarly, observations were made on Walker carcinoma cells; in addition, these cells were treated with solutions containing either phosphatidase A or enzyme inhibitors. Acinar cells contained, besides nuclei, perinuclear droplets and secretion granules, various membranous and vacuolar structures. The basal cytoplasm showed parallel dark lines interpreted as endoplasmic reticulum. In some cells, fragmentation of the reticulum was followed by the direct incorporation of fragments into simple myelin figures. In other cells it appeared that phase-lucent linear structures and vacuoles were derived by dilatation of cisternae of the endoplasmic reticulum. Perinuclear fluid collections arose either by dilation of the perinuclear cisternae of the endoplasmic reticulum or by fluid dilatation of the nuclear envelope. Phosphatidase A disrupted early vacuoles of Walker carcinoma cells. From this and the direct involvement of elements of the endoplasmic reticulum in myelin figures, it was concluded that the membranes limiting the endoplasmic reticulum incorporate phosphatides in continuous layers. While many severely injured cells formed large vacuoles, others developed concentrically laminated myelin figures; it was concluded that both types of structure derived from phosphatides liberated intracellularly, the vacuoles by vesicular myelin figure formation.

INTRODUCTION

Cytoplasmic vacuoles, first noted by Leeuwenhoek over 200 years ago (1), are among the most frequently encountered structural changes associated with cell injury (2–9). In spite of the large number of observations, the processes responsible for their formation have remained obscure (9, 10). Nevertheless it is well established that vacuole formation follows many different types of injury (5–7). Experimentally, it shows two striking features: first, it is reversible (11) and, secondly, it may occur within 15 seconds or less (12). These fingings support the view that pathological vacuole formation may best be interpreted in physical-chemical terms (13–17).

The present study is primarily concerned with the morphology and mechanisms of vacuole formation. With the use of combined phase microscopic and enzymatic methods to study rat pancreatic acinar and Walker carcinoma cells, an attempt has been made to determine the earliest stages of vacuole formation in terms of changed anatomy of the cell. In this task phase microscopy has proved a most useful tool, since, in spite of its limited resolution, it has allowed direct observation of whole unfixed cells from the time of their initial isolation until their ultimate breakdown. It is concluded that the earliest cell vacuoles result from fluid dilatation of the endoplasmic

reticulum and that later vacuoles derive, by vesicular myelin figure formation, from intracellularly liberated phosphatides.

MATERIALS AND METHODS

In outline, the present study involved the microscopic examination and photomicrography of freshly isolated rat pancreatic acinar and Walker carcinoma cells mounted in isotonic saline or treated with media containing either phosphatidase "A," enzyme inhibitors, oxalate or ethylenediaminetetraacetic acid (EDTA). In all, cells from 23 rat pancreases and 27 Walker tumours were examined.

Cell Preparations

PANCREATIC ACINAR CELLS: Rat (Sprague-Dawley or Wistar) pancreas was finely minced with scissors; the tissue fragments were treated with 0.1 per cent trypsin (Trypure Novo Industri, Copenhagen A/S) in 0.9 per cent sodium chloride for 30 minutes at 25°C, washed three times with 0.9 per cent sodium chloride, and the residual trypsin inactivated with autologous serum. Then a droplet containing tiny tissue fragments was transferred to a glass slide and gently compressed with a no. 1 cover glass. This operation, carefully performed, separated intact acini and single acinar cells from the trypsinized fragments.

WALKER CARCINOMA CELLS: Single cell suspensions of rat Walker carcinoma were produced by mincing the tumour with scissors, adding a little 0.9 per cent sodium chloride solution and, to separate cell clumps, passing the suspension through a plastic foam filter. Cell preparations were made by transferring a tiny drop of the filtrate to a glass slide and covering with a no. 1 coverglass.

Cell preparations were protected from drying by sealing the coverglass to the slide with clear nail varnish.

Explanation of Figures

All figures are phase-contrast photomicrographs of unfixed, unstained surviving cells injured in various ways: isolation from the parent tissue was followed by immersion in 0.9 per cent saline and, during examination and serial photomicrography, compression. Figs. 6, 7; 14, 15; 16, 17; 18, 19; 26 to 28, and 29 to 31 are taken from time-lapse series of photomicrographs.

Key to Abbreviations

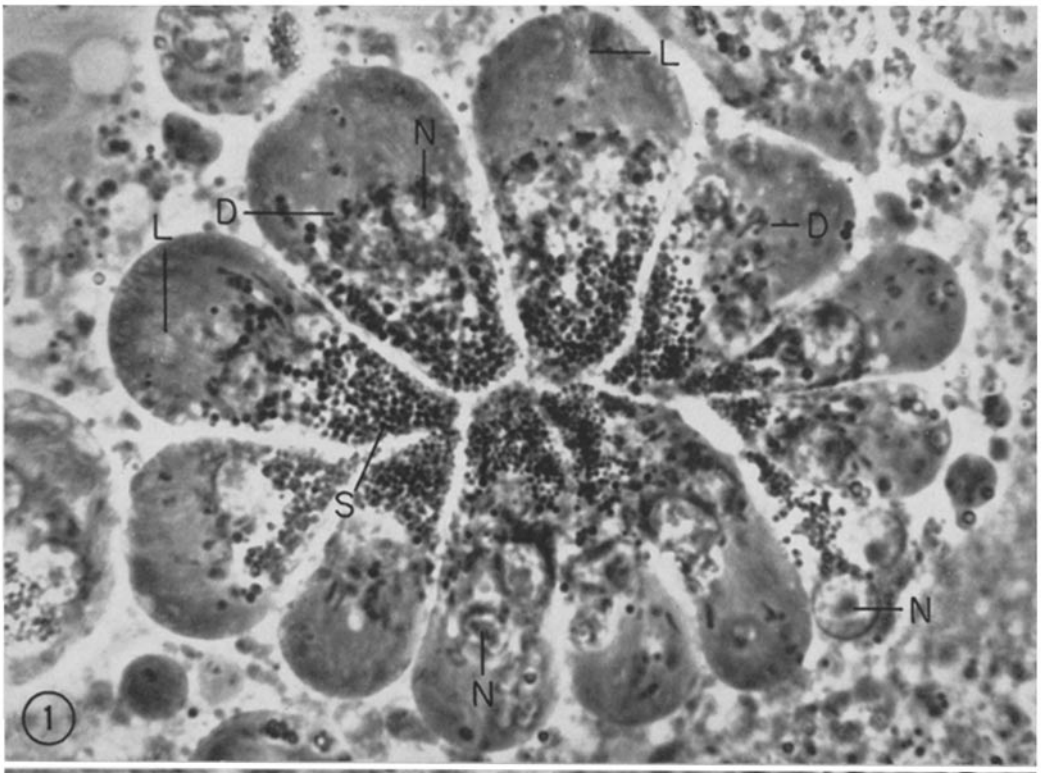
L, phase-lucent zones
V, vacuole
ER, endoplasmic reticulum
D, perinuclear droplets
MF, myelin figure
N, nucleus
PS, perinuclear sacs
S, secretion granules
LD, lipid droplets
NM, nuclear membrane
PV, paranuclear vauole
T, indicates the time interval between animal sacrifice and taking the photomicrograph
t, indicates the time interval between making the slide preparation and taking the photomicrograph.

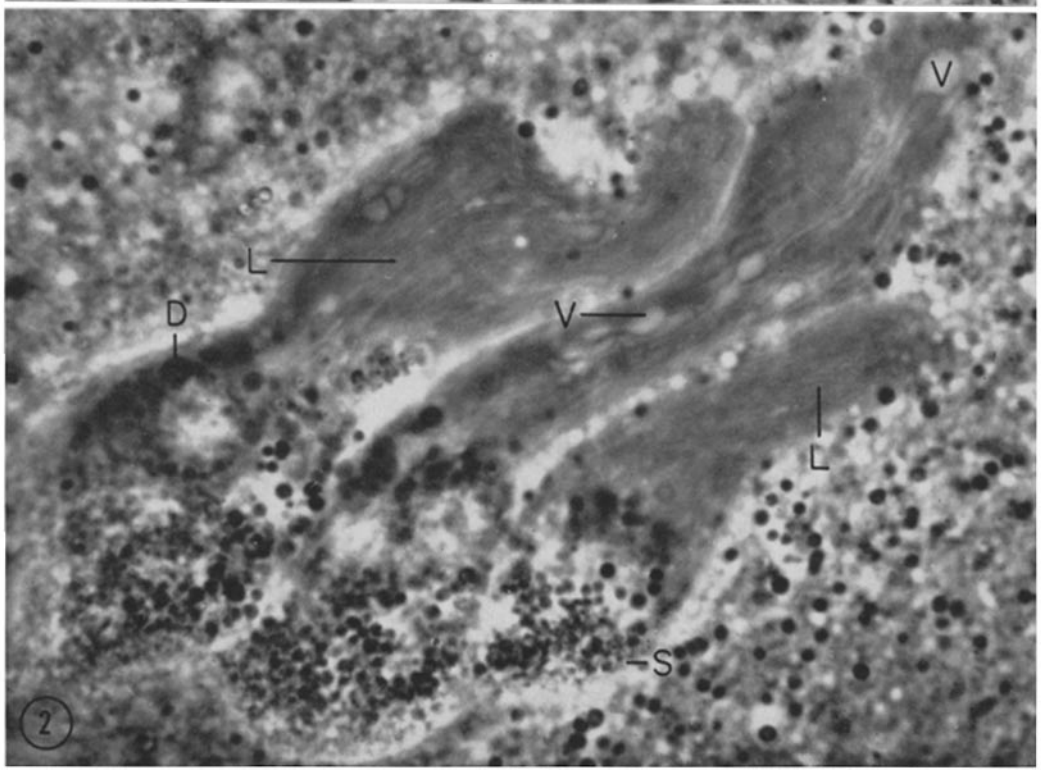
FIGURE 1

Unfixed, compressed acinar cells of the rat pancreas with acinar arrangement preserved. Shown are secretion granules (*S*) filling the cell apices, nuclei (*N*), perinuclear droplets (*D*), and, in the basal cytoplasm, elongate phase-lucent structures (*L*) which may represent dilated endoplasmic reticulum. Some perinuclear droplets, displaced into the basal cytoplasm by cell compression, have become elongate and even branched. T, 1½ hours; t, 5 minutes. × 1350.

FIGURE 2

Unfixed, compressed pancreatic acinar cells showing, in the spread out basal cytoplasm, parallel arrays of elongate phase-lucent structures (*L*) whose general disposition suggests a possible origin from endoplasmic reticulum. Some of these structures show localized spheroidal expansions resulting in the formation of small vacuoles (*V*). In addition these cells show apical secretion granules (*S*) and perinuclear droplets (*D*). T, 5½ hours; t, 30 minutes. × 1950.


L
N
D
L
D
S
N
N
1


V
L
D
V
L
S
2

Method of Examination

Microscopy was carried out with a Leitz (stand B) microscope fitted with Zeiss phase contrast equipment; photomicrographs were made using a Leica 1F camera with "Mikas" attachment, high contrast fine grain film and electronic flash illumination (18). Series of photomicrographs were used to record changes in individual cells. Cell compression, necessary for detailed observations, was effected by a simple microcompressor attached to the mechanical stage.

Enzyme and Enzyme-inhibitor Studies

PHOSPHATIDASE A: A drop of saline suspension of Walker carcinoma cells was mixed on a clean glass slide with a drop of a 1 mg per ml solution of phosphatidase A fraction of black snake (*Pseudechis porphyriacus*) venom (19) made up in 0.9 per cent saline. This preparation was quickly covered with a no. 1 coverslip, then placed on the warm stage of the microscope; cell changes were recorded photomicrographically.

FLUORIDE AND CYANIDE: Saline - washed Walker carcinoma cells were resuspended in a solution containing sodium cyanide (0.136 M) and sodium fluoride (0.042 M) brought to pH 7.5 with hydrochloric acid (0.062 M), making the total concentration 0.24 M. Slide preparations, sealed to prevent drying, were examined microscopically as described above.

OXALATE AND EDTA: Saline - washed Walker carcinoma cells were resuspended either in 0.3 per cent sodium oxalate, made up with 0.9 per cent sodium chloride, or in 0.02 per cent ethylenediaminetetraacetic acid, made up with saline. Resuspended cells were studied in the usual manner.

RESULTS

Pancreatic Acinar Cells

Freshly isolated, compressed acinar cells of the rat pancreas appeared as mono- or bi-nucleate bipolar cells, occurring either singly or in acini (Fig. 1). Dense secretion granules filled the apices of the cells. Merging with these granules and closely surrounding the nuclei were less dense, readily deformable bodies, hereafter referred to as perinuclear droplets. Highly refractile lipid droplets were seen in the basal cytoplasm of some cells (Figs. 14 and 15). With the present method mitochondria were not positively identified in any cells. In many cells the basal cytoplasm contained arrays of linear phase-lucent structures.

For closer study the cells were compressed to the point of rupture and detailed observations of the various cell structures made subsequently. In order to systematize the description, observations are presented under the following headings: vacuoles; endoplasmic reticulum; intracellular myelin figures; perinuclear sacs; nuclear membranes; secretion granules, perinuclear droplets, and mitochondria.

VACUOLES

The basal cytoplasm of many cells revealed parallel arrays of fine, sometimes branching, phase-lucent linear images of fairly uniform calibre (0.25 μ–0.5 μ) separated from one another by phase-dark areas generally aligned parallel to the cell's major axis (Figs. 1 and 2). Some of the phase-lucent linear structures showed local saccular or spherical expansions, sometimes in series, along their length (Fig. 2). Such expansions exhibited varying degrees of phase lucency; usually the greater the degree of expansion, the greater the phase lucency. The more phase-lucent spheroidal areas appeared as typical cytoplasmic vacuoles, similar to those (seen in non-compressed cells) having no demonstrable connection with phase-lucent linear structures. Some cells contained a number of small vacuoles, which, although not demonstrably connected with any phase-lucent linear structures, were arranged close to one another in linear series, suggesting a possible origin from some linear structure (Fig. 3).

A feature of the various phase-lucent structures described was their extreme lability. With the passage of time they slowly changed in size and shape and in the degree of phase lucency. Decreases as well as increases in size and in the degree of phase lucency occurred so that whereas some vacuoles became more prominent, others gradually faded from view (Figs. 18 and 19).

Many severely injured cells showed, besides those vacuoles connected with phase-lucent linear structures, large peripheral vacuoles having no demonstrable connection with these structures (Figs. 4 and 5). Such vacuoles, gradually increasing in number and size, eventually came to occupy most of the cytoplasm, and this change was accompanied by considerable cellular swelling. Following compression-induced cell rupture, many of these larger vacuoles remained intact, surviving as elastically deformable phase-lucent membrane-limited spheres freely floating in the extracellular fluid (Figs. 5, 8, and 9). In this situation vacuoles closely resembled the membrane-limited transparent globular bodies formed

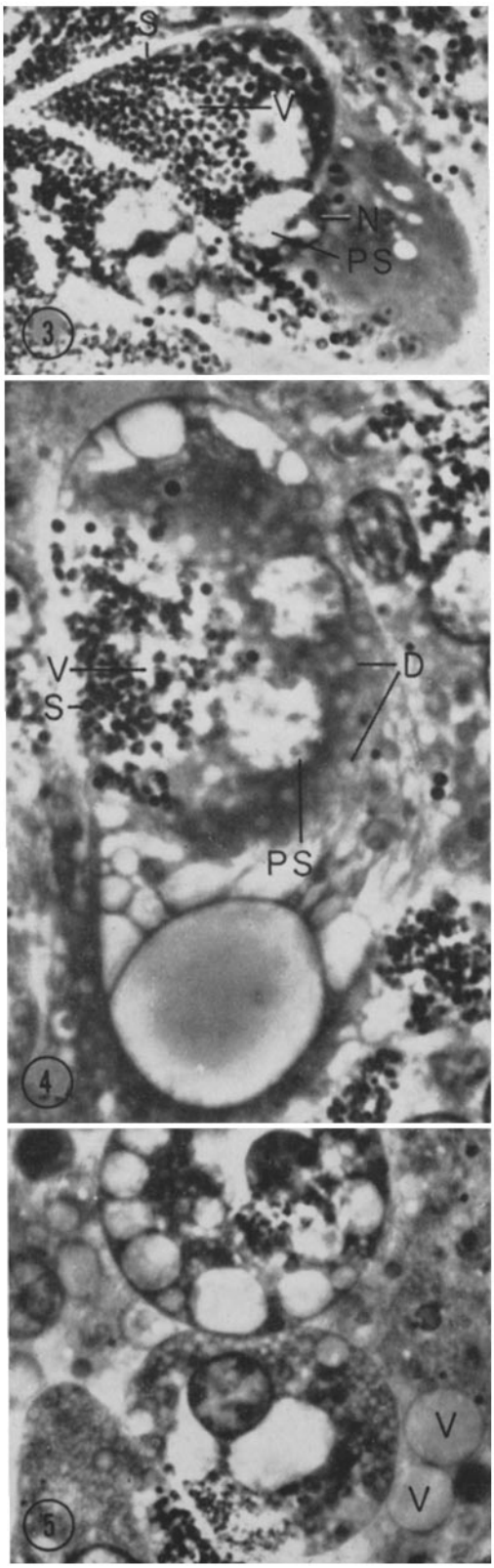


FIGURE 3

Rat pancreatic acinar cell containing, in the basal cytoplasm, several series of vacuoles whose linear arrangement suggests a possible origin from endoplasmic reticulum (*cf.* Fig. 2). To a large extent, phase-lucent structures, the perinuclear sacs (*PS*), camouflage the otherwise dark nuclei (*N*). Secretion granules (*S*) are closely associated with a phase-lucent vacuolar system (*V*) which extends throughout the cell apex. T, 2 hours; t, 15 minutes. × 1500.

FIGURE 4

Compressed binucleate pancreatic acinar cell containing, in addition to elongate, irregularly expanded phase-lucent zones, large peripheral totally enclosed vacuoles unconnected with the elongate phase-lucent structures. Also shown in this cell are highly phase-lucent perinuclear sacs (*PS*), vacuolated perinuclear droplets (*D*), secretion granules (*S*), and, associated with these, an extensive system of vacuoles (*V*). T, 6 hours; t, 2½ hours. × 2400.

FIGURE 5

Severely iniured pancreatic acinar cells containing, besides vacuolated perinuclear droplets, paranuclear vacuoles and numerous small vacuoles, numbers of large peripheral vacuoles. Seen amongst the cellular debris surrounding these cells are membrane-limited vacuoles (*V*) which have survived cell rupture. T, 3 hours; t, 3 minutes. × 1500.

on injured fibroblasts *in vivo* (12) and on injured placental tissue *in vitro* (20).

Endoplasmic Reticulum

UNRUPTURED CELLS: Photomicrographs of the cytoplasm of some cells, spread very thinly as a result of compression, revealed a peripherally extending parallel array of fairly uniform fine dark lines (Fig. 6). These measured approximately 0.16 μ in width and were spaced at intervals of about 0.25 μ. Further spreading of the cytoplasm demonstrated these dark linear structures more clearly (Fig. 7). By this stage however they had lost their parallel orientation, were less uniform in width, and appeared generally thicker (up to 0.5 μ).

These dark lines are interpreted as swollen elements of the endoplasmic reticulum.

RUPTURED CELLS: Although the endoplasmic reticulum was still identifiable following rupture of the plasma membrane, direct contact with the surrounding saline medium resulted in its gradual dispersion and loss of ordered arrangement Figs. 8 and 9). Individual components of the endoplasmic reticulum appeared as unevenly thickened, branching dark lines, some of which appeared to contact displaced perinuclear droplets (Fig. 9).

Eventually the endoplasmic reticulum broke up into phase-dark fragments of varying shapes and sizes (Fig. 10), and in many instances these gave rise to a variety of curved, ovoid, and spherical membranous structures (Fig. 11). However it was not possible to analyse details of these changes fully because the structure of the smallest membrane fragments could be observed only indirectly, that is, through the study of photographic prints.

In some preparations, relatively clear spaces, apparently lacking in any limiting membrane, were observed in the midst of dense concentrations of ER fragments (Fig. 11). These spaces contained either a very low concentration of reticulum fragments or none at all; usually such spaces were not so phase lucent as membrane-limited vacuoles.

Not infrequently, complex curvilinear structures resembling myelin figures were observed floating among the fragments of the endoplasmic reticulum (Figs. 12 and 13). Some of these curvilinear structures lacked the structure of typical myelin figures in that they comprised rather unusual configurations of unevenly thickened dense lines, thus suggesting that they were made up of more or less intact elements of the endoplasmic reticulum (Fig. 12). Others, consisting of more regularly disposed fine dark lines arranged as concentric laminations, had a structure typical of the myelin figures derived from extracted phosphatides (Fig. 13).

Intracellular Myelin Figures

Myelin figure formation was frequently noted within intact injured cells. Appearing as round or oval bodies of concentrically variable density, intracellular myelin figures (MF) gradually increased in size within apparently homogeneous cytoplasm (Fig. 14); however when the cell cytoplasm had spread into a very thin sheet, it became apparent that the myelin figures (MF) were surrounded by the endoplasmic reticulum (ER) (Fig. 15). Such concentrically laminated myelin figures were never seen closely associated with cytoplasmic vacuoles.

In one instance the rearrangement of segments of the endoplasmic reticulum to form structures resembling simple myelin figures was observed within an intact cell. The cell, a grossly swollen

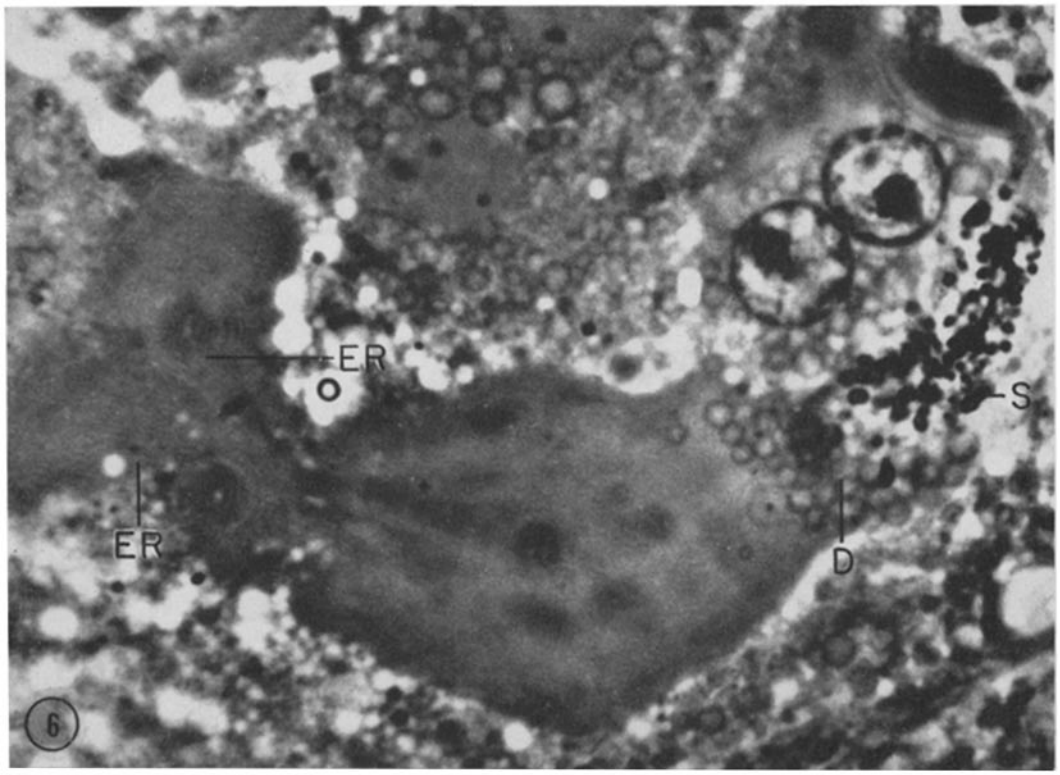
---

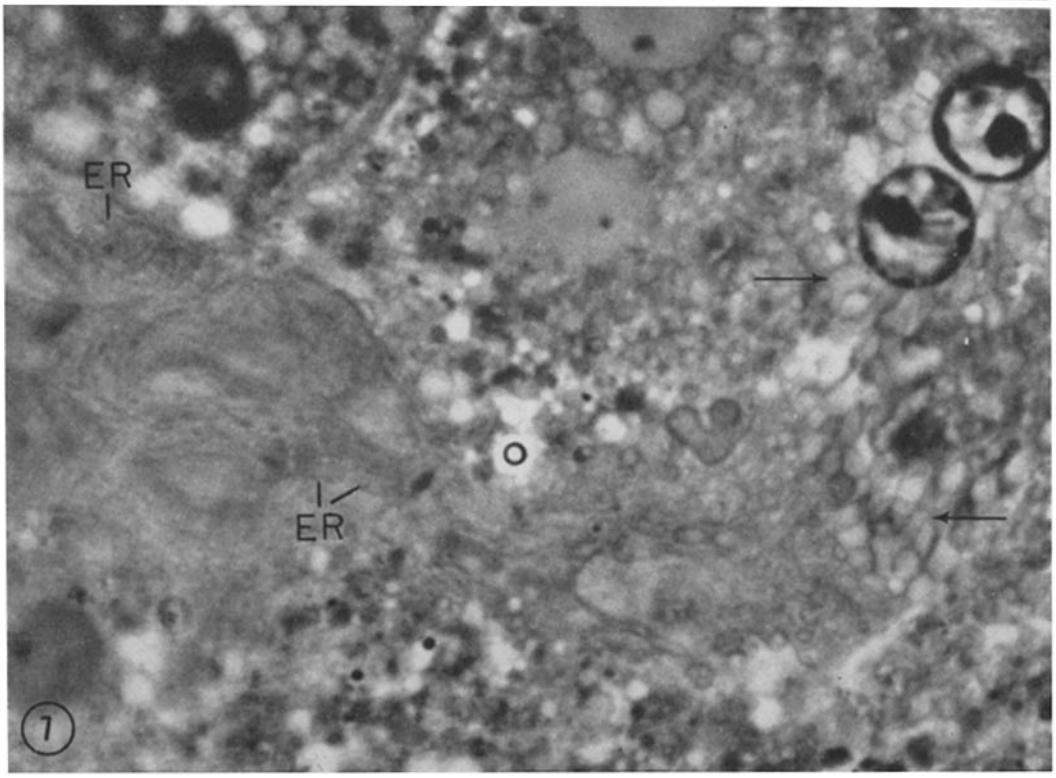
FIGURE 6

Greatly compressed binucleate pancreatic acinar cell showing in the basal cytoplasm a parallel array of fine dark lines interpreted as endoplasmic reticulum (*ER*). Secretion granules (*S*) and partially vacuolated perinuclear droplets (*D*) are seen near the nuclei. T, 3 hours 50 minutes; t, 2 hours 50 minutes. × 2100.

FIGURE 7

Twenty-five minutes later the basal cytoplasm of the same cell (see Fig. 6) shows more clearly the presence of endoplasmic reticulum (*ER*). Now however the reticulum is irregularly thickened and its general arrangement is more haphazard. Perinuclear droplets and secretion granules now show an extreme degree of vesiculation (see arrows). T, 4¼ hours; t, 3¼ hours. × 2100.


ER
S
ER
D
6


ER
ER
7

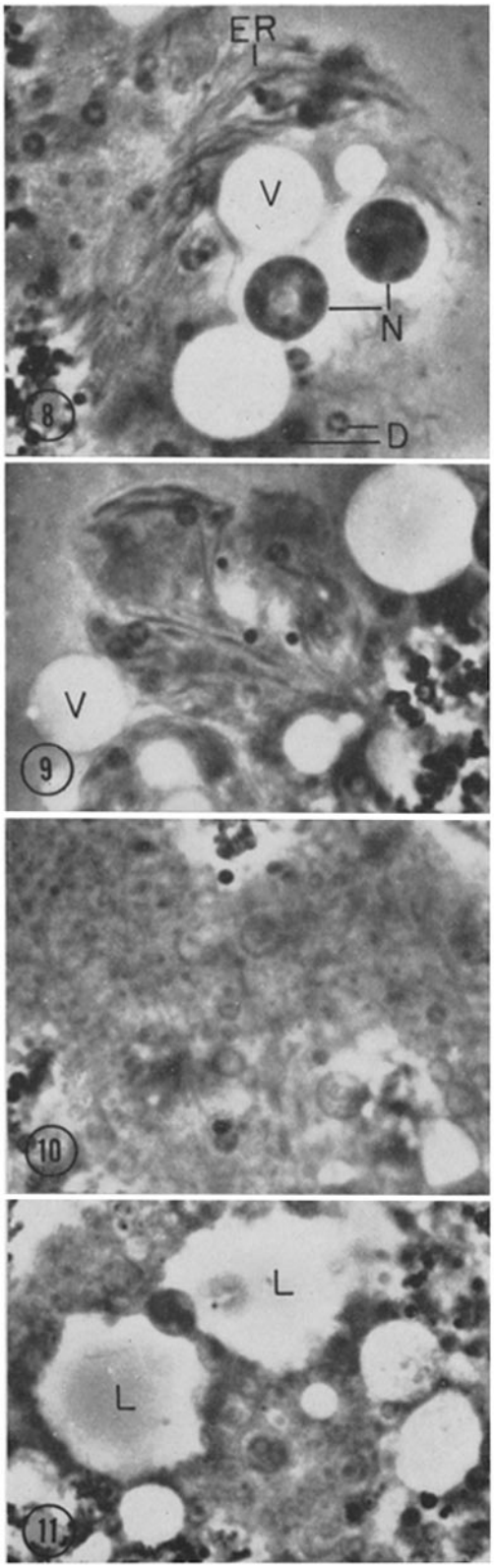


FIGURE 8

Cytoplasm of recently ruptured pancreatic acinar cell illustrating disorganization of the membranes of the endoplasmic reticulum (*ER*) as they contact the surrounding saline medium. Nuclei (*N*), partially vacuolated perinuclear droplets (*D*) and cytoplasmic vacuoles (*V*) are also shown. T, 1½ hours; t, 12 minutes. × 1950.

FIGURE 9

Membranes of the endoplasmic reticulum, cytoplasmic vacuoles, and perinuclear droplets from recently ruptured pancreatic acinar cell. One cytoplasmic vacuole (*V*) is surrounded by only a fine membrane but continues to exist, independent of the cytoplasmic mass, in direct contact with the extracellular medium. T, 1½ hours; t, 7 minutes. × 1950.

FIGURE 10

Short segments of endoplasmic reticulum (and other membranous structures) formed on contact with the surrounding saline medium following cell rupture. That these short segments of reticulum occur as individual entities can be fully appreciated only by direct observation which shows their independent movement due to Brownian buffetting or local currents. T, 1½ hours; t, 2 minutes. × 2100.

FIGURE 11

Various curved and spheroidal forms adopted by segments of the endoplasmic reticulum derived from ruptured pancreatic acinar cells. Seen also are somewhat irregular apparently non-membrane-limited phase-lucent spaces (*L*) amongst the membrane fragments. T, 3 hours 20 minutes; t, 2 hours. × 1800.

one, contained unusually prominent endoplasmic reticulum measuring up to 0.5 μ in width (Fig. 16). Some of the reticulum appeared as fine branching dark lines radiating from the nucleus and vacuolated perinuclear droplets. More peripherally, the reticulum was more disorganized and broken up. Over the following 11 minutes, it was directly observed that, as the cell continued to swell, further fragmentation of the radially oriented endoplasmic reticulum seemed to be followed by a rearrangement of some of the segments of the endoplasmic reticulum into complex oval bodies comprising phase-dark elements disposed in various curvilinear arrays (*MF* in Fig. 17). Subsequently these complex oval structures underwent various modifications in configuration and then largely disaggregated to become more or less unconnected reticulum fragments. Both from their general appearance and considerable structural lability these complex oval structures resembled myelin figures.

Perinuclear Sacs

Nuclei were obscured to a variable degree by superimposed cytoplasmic structures, such as perinuclear droplets, fat droplets, or granules, which thus modified the nuclear appearance (Figs. 1, 2, 26 to 31). Nuclear images of severely injured pancreatic acinar cells, however, were modified by superimposed phase-lucent images which are interpreted to be perinuclear expansile sac-like structures here termed "perinuclear sacs." Their development is shown in time-lapse photomicrographs (Figs. 18 and 19). Fig. 18 shows two compressed pancreatic acinar cells whose nuclei appear as complex phase-dark and phase-lucent patterns. Fig. 19, showing the same cells 80 minutes later, indicates the altered shape and expansion of the phase-lucent areas which now obscure much of the nuclei and, in the lower cell, project into the surrounding cytoplasm (see arrows). Fig. 20 provides further evidence on this point: greatly compressed nuclei are seen as a composite of phase-dark and phase-lucent areas, but since the latter appear directly continuous with surrounding extranuclear phase-lucent regions, they are interpreted to represent sac-like structures which loosely envelope the nucleus. The perinuclear structures survive for a time after cell rupture but eventually disappear, leaving only the phase-dark pyknotic nuclei (Fig. 21). These expansile phase-lucent structures are probably derived from the innermost cisternae of the endoplasmic reticulum, dilated by injury, but localized dilatations of the nuclear envelope cannot be excluded. Since positive identification of the structures affected has not been possible, they have been designated simply "perinuclear sacs."

Nuclear Membranes

Pathological fluid collections around compressed nuclei have, by widely separating the two layers of the nuclear envelope, shown them as distinct membranous structures (NM_1 and NM_2, Fig. 22). These membranes were clearly seen in compressed macrophages (Fig. 23) where the fluid appeared to accumulate inside the inner nuclear membrane (NM_1) as well as between the two membranes (NM_1 and NM_2). Phase-dark particles adhering to the inner nuclear membrane (NM_1) indicate that its image is not simply a phase-contrast artifact.

Secretion Granules, Perinuclear Droplets, and Mitochondria

Secretion granules appeared as very dark, approximately spherical bodies (0.5 μ to 1.0 μ in diameter) fairly closely packed within the apical poles of the cells. Perinuclear droplets were larger (up to 2.5 μ in diameter), less dark, readily deformable bodies located about the nucleus and in the apical cytoplasm where they merged with the secretion granules. Although secretion granules and perinuclear droplets were typically distinct, apparent transition forms occurred in the junctional zone where the two types met (Figs. 1 and 2). Some structures observed in the perinuclear area may have been mitochondria, but positive identification was not possible, even when using Janus green B.

As evidenced by their highly phase-lucent surroundings both secretion granules and perinuclear droplets were associated with a widespread vacuolar system extending from cell apex to the perinuclear sacs (Figs. 3 and 4). In addition some cells showed apical vacuoles which appeared to communicate with the perinuclear sacs (Figs. 18 and 19). However, with the present method, no Golgi complex structures were identified in the centrosphere region of these cells.

Although cell compression resulted in minimal displacement of the more apical perinuclear droplets, it caused considerable centrifugal droplet movement into the basal cytoplasm. This was accompanied by pronounced droplet deformation,

and displaced droplets assumed a variety of elongate and even branching forms (Figs. 1 and 18). Whilst these changes in shape suggested the possibility that droplet material had been forced through fine cytoplasmic channels, such structures were never observed in direct association with droplet material.

Eventually both secretion granules and perinuclear droplets became pale, swollen, and vacuolated (Figs. 4, 6, and 7); however this change was more pronounced and occurred earlier in the case of the perinuclear droplets. Vacuoles formed in this way remained relatively small, thick-walled spheres bearing little resemblance to the other cytoplasmic vacuoles described.

Walker Carcinoma Cells

General Observations

The granule-free cytoplasm of some saline-washed, compressed Walker carcinoma cells was noted to contain phase-dark linear images, similar to those seen in pancreatic acinar cells. In Walker carcinoma cells, however, the dark lines lacked any parallel arrangement but instead formed a delicate branching network which is here interpreted as endoplasmic reticulum. Although usually appearing as fine dark lines of uniform width, some reticulum showed one or more small, central, spheroidal phase-lucent areas which thus appeared as the smallest observable pathological vacuoles (Fig. 24).

The granule-free cytoplasm of other compressed Walker carcinoma cells showed, besides phase-lucent vacuoles, networks of phase-lucent curvilinear structures whose disposition suggested a likely origin from endoplasmic reticulum (Figs. 25 and 26). Following continued compression, the phase-lucent linear structures dilated, coalesced, then broke up to form isolated spheroidal vacuoles (Fig. 27). These, in greatly compressed cytoplasm, appeared as phase-lucent structures enclosed by a fine membrane (Fig. 28). Occasionally such vacuoles were extruded through the plasma membrane into the surrounding saline medium where they remained as independent structures (Figs. 27 and 28).

As in pancreatic acinar cells, Walker carcinoma cells contained phase-lucent perinuclear sacs which, together with phase-lucent vacuoles and dark granules, formed a pattern over the nuclear surface (Fig. 29). In contrast to pancreatic acinar

FIGURE 12

Margin of ruptured pancreatic acinar cell showing, besides secretion granules (*S*), a disorganized tangle of endoplasmic reticulum (*ER*). To the left of the cell remnants, floating amongst fragments of the endoplasmic reticulum, are two complex myelin figure-like bodies (*MF*) whose unevenly thickened phase-dense lines, disposed in curious configurations, suggest that they are composed of endoplasmic reticulum. T, 1 hour 20 minutes; t, 30 minutes. × 2400.

FIGURE 13

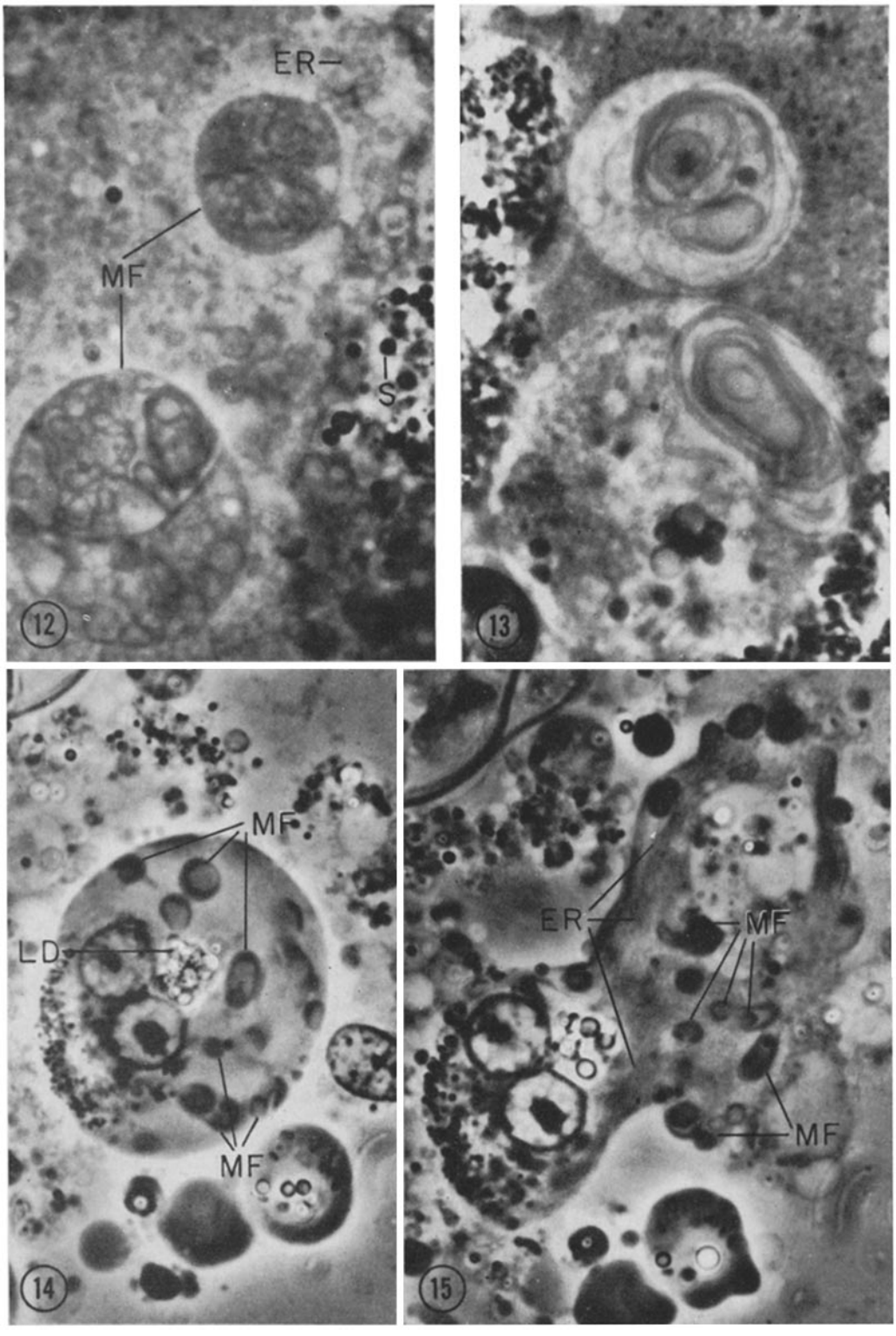
Floating among the fragments of the endoplasmic reticulum and other cell debris are two vesicles containing concentrically laminated membranous structures whose appearance is characteristic of the "artificial" myelin figures derived from extracted phosphatides. T, 3¼ hours; t, 5 minutes. × 2250.

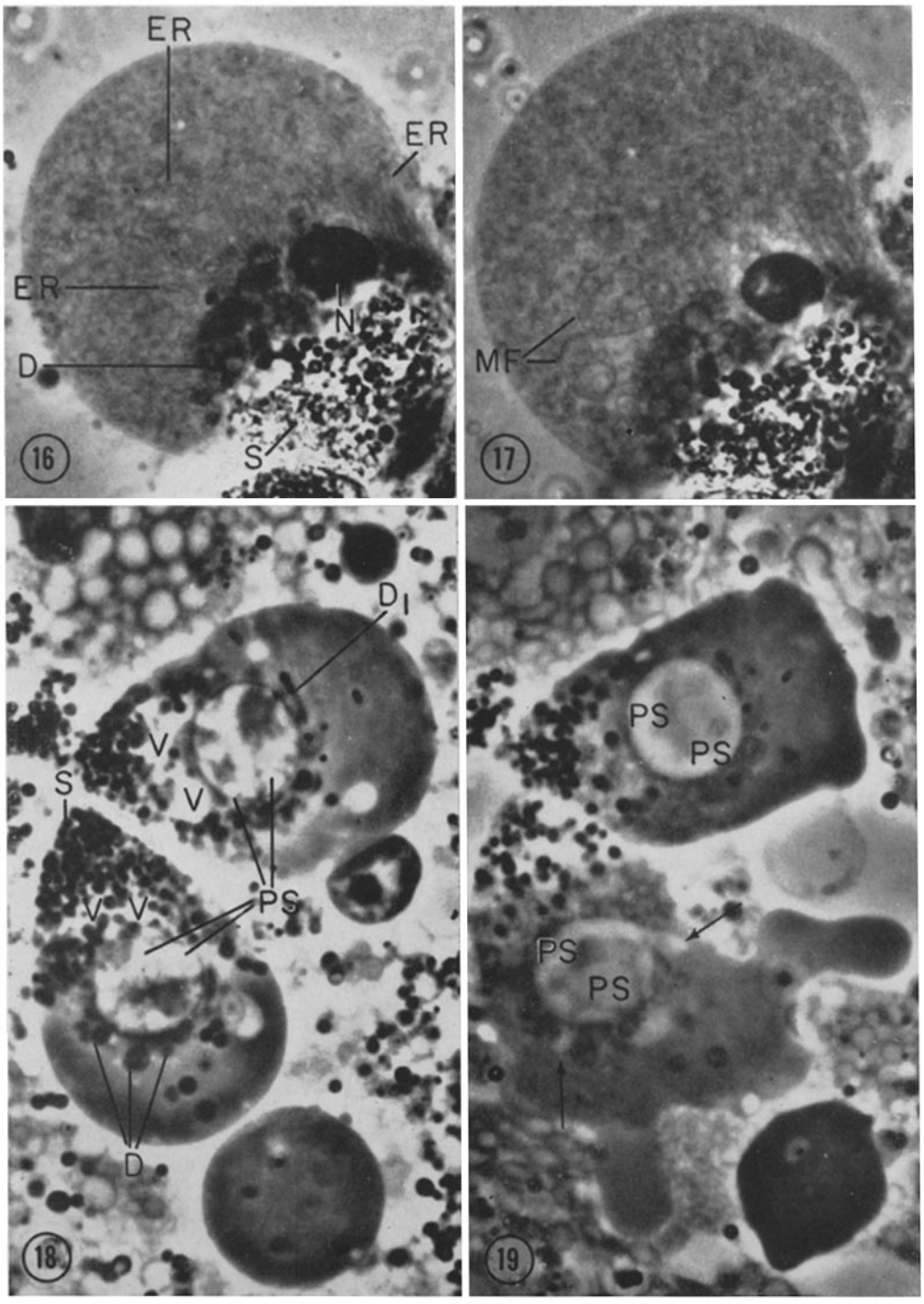
FIGURE 14

Rat pancreatic acinar cell containing, besides nuclei, perinuclear droplets and secretion granules, more refractile lipid droplets (*LD*) and numerous compact globular and ovoid myelin figures (*MF*). T, 2 hours; t, 18 minutes. × 1650.

FIGURE 15

The same cell (see Fig. 14) 39 minutes later showing nuclear compression, some expansion of the myelin figures (*MF*) and spreading of the cytoplasm. Now, in the thinly spread cytoplasm, endoplasmic reticulum (*ER*) can be seen surrounding the myelin figures. T, 2 hours 39 minutes; t, 57 minutes. × 1650.


ER
MF
S
12
13
MF
LD
MF
14
ER
MF
MF
15


ER
ER
ER
N
D
S
16
MF
17
DI
V
S
V
V
V
PS
D
18
PS
PS
PS
PS
19

cells, injured Walker carcinoma cells were not seen to develop concentrically laminated myelin figures.

Enzyme and Enzyme-Inhibitor Studies

PHOSPHATIDASE A: Vacuolated Walker carcinoma cells treated with phosphatidase A solution at first became more phase-lucent, revealing somewhat greater cytoplasmic detail (Figs 29 and 30). Then, over the following 10 minutes, the cell margins retracted and developed a ragged "moth-eaten" appearance and the cytoplasmic vacuoles and perinuclear sacs disappeared. The cell nucleus and dark cytoplasmic granules appeared unaffected (Fig. 31).

FLUORIDE AND CYANIDE: The medium containing fluoride and cyanide neither inhibited the formation of new cytoplasmic vacuoles nor prevented the further development of existing ones.

OXALATE AND EDTA: Pathological vacuole formation was not prevented in cells bathed in either the oxalate solution or that containing ethylenediaminetetraacetic acid.

DISCUSSION

The principal findings here are appearances that may reasonably be interpreted as: endoplasmic reticulum in unfixed pancreatic acinar and Walker carcinoma cells; pathological vacuole formation by dilatation of endoplasmic reticulum; direct participation of endoplasmic reticulum in forming simple myelin figures; and the disruption of vacuoles and other membrane-limited structures by phosphatidase A. Other topics for discussion relate to the perinuclear sacs, the nuclear membranes and the secretion granules, perinuclear droplets and mitochondria.

Identification of Endoplasmic Reticulum in Unfixed Cells

Previous light microscopic findings of the endoplasmic reticulum in unfixed cells were made by Porter (21), Palay and Wissig (22), Shelton (23),

FIGURE 16

Swollen compressed pancreatic acinar cell containing, besides nucleus (*N*), perinuclear droplets (*D*) and secretion granules (*S*), fragmented and elongate elements of the endoplasmic reticulum (*ER*). T, 3¼ hours; t, 6 minutes. × 1650.

FIGURE 17

The same cell (see Fig. 16) 11 minutes later, showing that numbers of elongate elements of the endoplasmic reticulum have by now become disposed in the form of two complex oval bodies (*MF*) comprising curvilinear arrays of endoplasmic reticulum. In their curious curvilinear structure and pronounced lability of form these oval bodies resemble myelin figures. T, 3 hours 26 minutes; t, 17 minutes. × 1650.

FIGURE 18

Two compressed pancreatic acinar cells containing secretion granules (*S*) and perinuclear droplets (*D*) some of which have undergone compression-induced displacements and distortions of shape (D_1). The nucleus of each cell shows a light and dark pattern which is in part due to the presence of superimposed highly phase-lucent structures, the perinuclear sacs (*PS*). These appear to connect with a phase-lucent vacuolar system (*V*) associated with the apical perinuclear droplets and secretion granules. T, 3 hours; t, 5 minutes. × 2100.

FIGURE 19

The same cell 80 minutes later showing, in addition to swelling and fading of the perinuclear droplets, dilatation of perinuclear sacs (*PS*) which, in the lower cell, now appear as a series of irregularly bounded phase-lucent zones. In both cells the process has given rise to veiling of the nuclei and, at some points, overlapping of the nuclear boundaries by localized extensions of the sacs (see arrows). Two cytoplasmic vacuoles seen in Fig. 18 have now disappeared. T, 4 hours 20 minutes; t, 1 hour 25 minutes. × 2100.

Thiéry (24), Fawcett and Ito (25) and Rose and Pomerat (26). The present work on unfixed pancreatic acinar cells shows parallel arrays of phase-dark lines whose distribution corresponds to that of the endoplasmic reticulum in electron micrographs of similar cells (27, 28). This indicates that the phase-dark lines represent cisternae of the endoplasmic reticulum of the cell. Likewise, the observation, in unfixed Walker carcinoma cells, of a net-like arrangement of similar phase-dark structures identifies these as elements of the endoplasmic reticulum. Although the finding of endoplasmic reticulum in unfixed cells appears of limited value in the study of its detailed structure, the ability to directly examine this organelle opens up greater possibilities for the study of its behaviour under various circumstances. The present observations indicate some aspects of the behaviour of endoplasmic reticulum under injurious conditions.

Endoplasmic Reticulum and Vacuole Formation

The appearance of tiny phase-lucent spheroidal vacuoles within individual elements of endoplasmic reticulum in injured Walker carcinoma cells indicates that vacuoles may arise by localized dilatation of reticulum. Whole networks of phase-

FIGURE 20

Greatly compressed pancreatic acinar cells whose phase-dark nuclei (*N*) are largely camouflaged by enveloping phase-lucent structures, perinuclear sacs (*PS*), which can be seen extending beyond the nuclear borders (arrows). T, 4 hours 36 minutes; t, 1 hour 36 minutes. × 2700.

FIGURE 21

Nuclei, separated from pancreatic acinar cells following cell rupture, showing reduction of the area covered by the phase-lucent perinuclear sacs (*PS*). In some nuclei this change has proceeded so far that only the dark nuclear image (*N*) remains. T, 2 hours 39 minutes; t, 1 hour 22 minutes. × 2700.

FIGURE 22

Isolated pancreatic acinar cell nuclei showing both inner (NM_1) and outer (NM_2) nuclear membranes separated from one another by fluid which thus forms paranuclear vacuoles (*PV*). T, 21 hours; t, 2 minutes. × 1920.

FIGURE 23

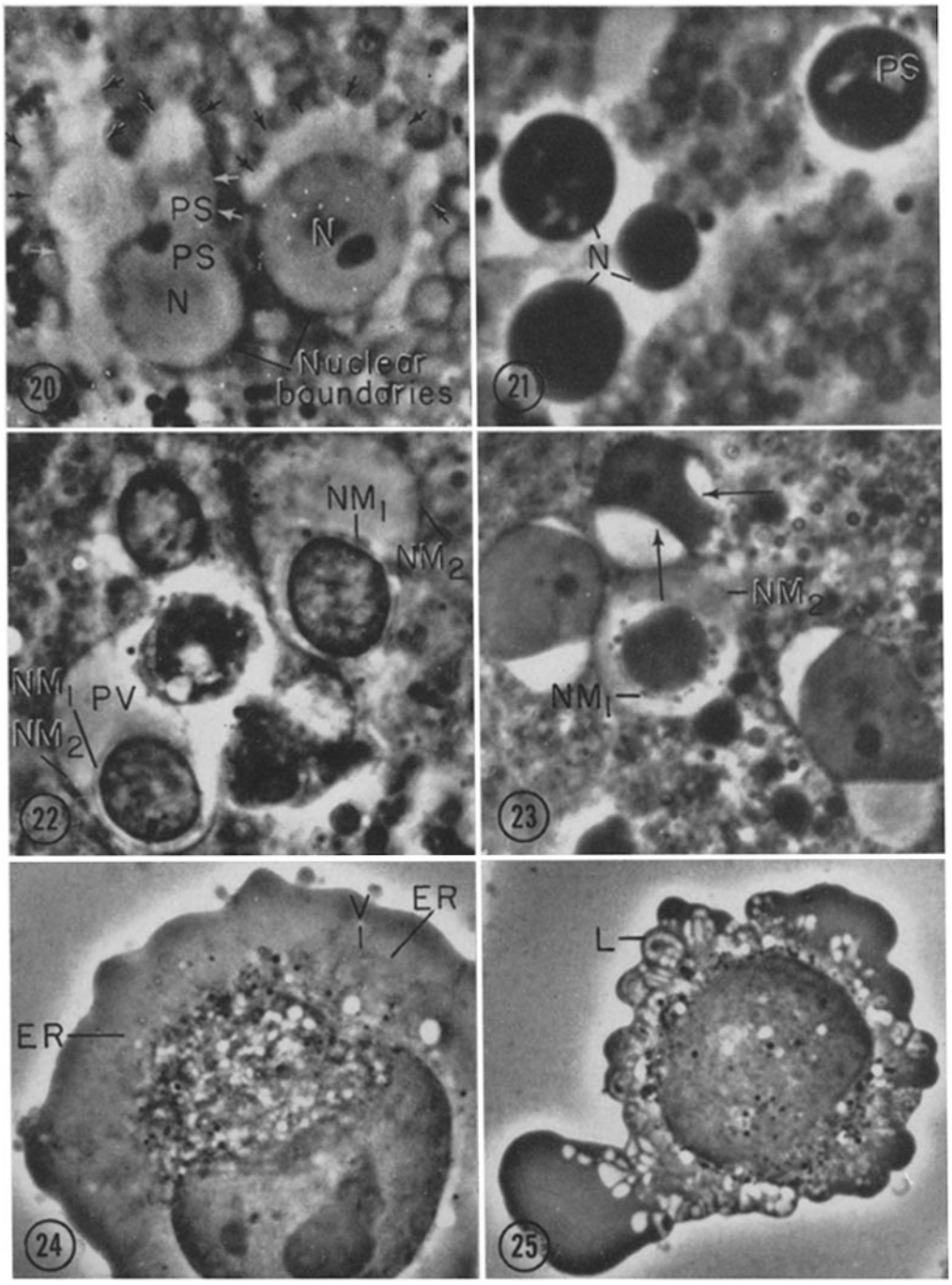
Nuclei isolated from human macrophages, obtained from sputum, illustrating inner (NM_1) and outer (NM_2) nuclear membranes. In the central cell, separation of the nuclear membranes has isolated the pyknotic nucleus within an all encompassing perinuclear vacuole. The curious indentations (arrows) shown by all the nuclei are suggestive of inward displacement of nuclear material by localized fluid accumulations inside their inner nuclear membranes. Note the absence of any phase-lucent structures resembling perinuclear sacs. t, 14 minutes. × 1800.

FIGURE 24

Saline-washed compressed Walker carcinoma cell of the rat containing, within the agranular cytoplasm, a delicate network of fine phase-dense lines, the endoplasmic reticulum (*ER*). Commencing dilatation of these membranes has produced several tiny vacuoles (*V*). T, 36 minutes; t, 1 minute × 1575.

FIGURE 25

Walker carcinoma cell containing vacuoles and elongate phase-lucent structures (*L*) which may represent dilated endoplasmic reticulum. T, 4 hours 20 minutes; t, 6 minutes. × 1500.


PS
PS
N
N
Nuclear boundaries
20
PS
N
21
NM1
NM2
NM1
PV
NM2
22
NM2
NM1
23
V
ER
ER
24
L
25

lucent curvilinear structures surrounded by apparently homogeneous cytoplasm suggest a more pronounced dilatation of extensive segments of endoplasmic reticulum. This interpretation is in accord with the electron microscopic observation that in injured cells the endoplasmic reticulum is dilated about spaces of low electron opacity (27, 29–31). It is also in agreement with Thiéry's (24) phase microscopic finding in plasma cells that, in the course of cellular compression, the ergastoplasm underwent progressive vacuolar dilatation. These and the present observations on Walker carcinoma cells indicate that injured endoplasmic reticulum may, by dilatation, give rise to pathological vacuoles.

The finding, in pancreatic acinar cells, of parallel arrays of phase-lucent linear structures resembling those seen in Walker carcinoma cells suggests the likelihood that these represent dilated cisternae of the endoplasmic reticulum. In this case the phase-dark areas between the phase-lucent zones would represent stacks of non-dilated cisternae. Alternatively, it may be that, while the phase-dark zones correspond to stacks of flattened cisternae, the intervening elongate phase-lucent areas represent reticulum-free, overhydrated cytoplasmic matrix. However the former interpretation is favoured because it accords with the findings in Walker carcinoma cells; here, because of the sparseness and branching of endoplasmic reticu-

FIGURE 26

Compressed Walker carcinoma cell showing extensive curvilinear networks of elongate phase-lucent structures interpreted as dilated endoplasmic reticulum. T, 4 hours 38 minutes; t, 6 minutes. × 1050.

FIGURE 27

The same cell (see Fig. 26) 3 minutes later. Following compression the elongate phase-lucent structures have dilated, partially coalesced, then separated to form numerous spheroidal cytoplasmic vacuoles (V_1). Some of these have been extruded through the plasma membrane into the extracellular fluid where they remain as membrane-limited vesicles (V_2) whose refractive index is approximately the same as that of surrounding fluid. T, 4 hours 41 minutes; t, 9 minutes. × 1050.

FIGURE 28

Three minutes later the cytoplasm has spread further, some vacuoles have become smaller with phase dense contents while others have remained about the same size and appear as non-phase-lucent membrane-bounded spheres (arrow). Also shown are dense droplets superimposed on the cell nucleus. T, 4 hours 44 minutes; t, 12 minutes. × 1050.

FIGURE 29

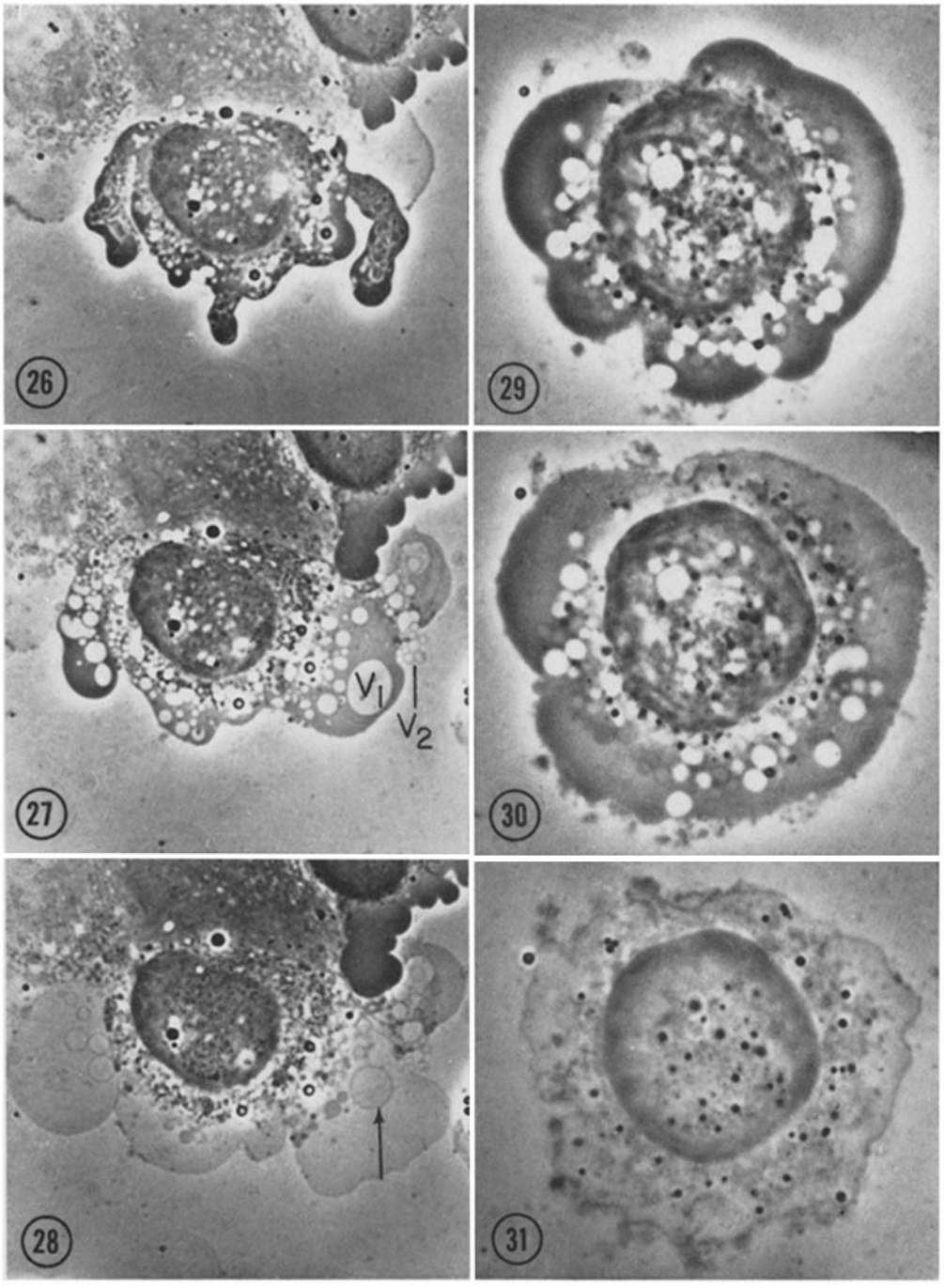
Vacuolated Walker carcinoma cell soon after addition of phosphatidase A solution. Note the perinuclear sac pattern and vacuoles superimposed on the nucleus and the fairly smooth cell margin. T, 1 hour 25 minutes; t, 12 minutes. × 1650.

FIGURE 30

Half a minute later the cell surface has expanded and in many places becomes more phase lucent so that the cell contents are more clearly defined. T, 1 hour 25½ minutes; t, 12½ minutes. × 1650.

FIGURE 31

The same cell 9½ minutes later showing disappearance of phase-lucent vacuoles and perinuclear sacs together with over-all cell shrinkage and an irregular "moth-eaten" appearance of the cell surface. The nucleus and dense cell droplets appear unaffected. T, 1 hour 35 minutes; t, 22 minutes. × 1650.


26
29
27
V_1
V_2
30
28
31

lum, the phase-lucent curvilinear networks cannot be accounted for by fluid accumulation *between* elements of endoplasmic reticulum but only by fluid accumulation *within* the reticulum.

Endoplasmic Reticulum and Myelin Figure Formation

Myelin figures (32–34) in injured cells have been noted frequently with both the light (35–39, 8) and electron microscopes (40–42). Such findings have been accounted for (8, 14, 39) on the basis of injury-induced liberation of phosphatides from various lipoprotein structures within the cell; once freed, phosphatide molecules associate together, as myelin figures, in the nearest available fluid space. The progressively developing, concentrically laminated, intracellular myelin figures seen in the present work may belong in this category.

By contrast, the curious intracellular myelin figure-like structures formed by apparently intact elements of the endoplasmic reticulum must be accounted for somewhat differently. Since the ultrastructural basis of myelin figures is the continuous bimolecular lipid layer (32–34, 43–46), and since the endoplasmic reticulum is known to contain significant amounts of phosphatides (47, 48), the observation that elements of injured endoplasmic reticulum themselves became disposed in the form of simple myelin figures supports the view (45) that the endoplasmic reticulum is limited by membranes whose phosphatides are arranged in continuous bimolecular layers. This interpretation of the finding suggests a possible mechanism by which injured endoplasmic reticulum dilates. *In vitro*, phosphatide molecular layers of extracted phosphatides have a strong inherent capacity for water transfer which results in progressive fluid dilatation of the various tubular and vesicular structures known as myelin figures (32–34). Accordingly, if a bimolecular layer of phosphatide molecules forms the basis of the membrane structure of the endoplasmic reticulum, then injury (by disturbing associated protein and other molecules or ions known to inhibit water transfer across phosphatide membranes *in vitro* (14, 32)) could trigger off water uptake and hence promote fluid dilatation of the endoplasmic reticulum. The same mechanism might contribute to the abnormal accumulations of fluid observed within other membranous cell structures such as the nuclear membranes. Thus this interpretation of the fluid-imbibing behavior of injured cytoplasmic membranes is in accord with Holtfreter's view of the mechanism of fluid imbibition by the damaged plasma membrane (14); the observation that phosphatidase A grossly disturbed the integrity of perinuclear sacs, vacuoles and the plasma membrane of Walker carcinoma cells provides some support for this view.

Myelin Figure Formation and Vacuoles

The question arises as to the formative mechanism of those large vacuoles which are apparently not derived directly, by fluid dilatation, from membrane-limited cell structures. Since phosphatides are major constituents of all animal cells (49) and since, as judged by myelin figure formation, phosphatide liberation from cells and tissues follows many different types of injury (35–42, 8, 20), it is suggested that these cytoplasmic vacuoles may be vesicular myelin figures formed by intracellularly liberated phosphatides. The mechanism proposed is that, in dispersing, liberated phosphatides absorb relatively large quantities of fluid, thereby swelling into large, thin walled, vesicular myelin figures. Thus the process would differ from the formation of concentrically laminated myelin figures only in that whereas these last are caused by *aggregation* of liberated phosphatides, vesicular myelin figures result from fluid uptake by, and *dispersal* of, phosphatides. That vacuoles and laminated myelin figures were not seen together in the same cell may suggest only that intracellular conditions favouring the one process are incompatible with the other.

In support of this phosphatide vesicle concept of pathological vacuole formation, vesicular myelin figures have been noted forming at the periphery of injured cells (36, 38, 12) and tissues (50, 20), indicating that, given appropriate conditions for phosphatide hydration, vesicular myelin figures originate from injured cells. At the same time there is a close morphological resemblance between vesicular myelin figures (33, 34, 20) and cytoplasmic vacuoles, particularly those surviving cell rupture: both appear as elastically deformable membrane-limited translucent spheres. Both cytoplasmic vacuoles (4, 51) and phosphatide vesicles (14, 39) take up and concentrate certain basic dyes such as neutral red; and the fluid-imbibing properties of phosphatide vesicles could explain the ability of vacuoles to deform or displace other cytoplasmic constituents (52, 53, 9) and to con-

tribute to the over-all swelling of injured cells (3, 5, 7, 8, 50).

Perinuclear Sacs

Photographs of the perinuclear sacs indicate that, although adjacent to the nuclei, they are distinct structures. This observation considered together with their expansile sac-like nature suggests that they arose from some membrane-limited cell organelles such as the innermost cisternae of the endoplasmic reticulum (27, 28). However, in the absence of certain identification it has seemed preferable to call them simply "perinuclear sacs."

Nuclear Membranes

These structures, shown by electron micrographs to be a two-layered envelope completely surrounding the nucleus (54), are normally not visible by light microscopy. Even after injury they remained close to one another and to the nuclear material until disorganization of the cell was far advanced. Only then, presumably because of their capacity for fluid uptake, did they separate far enough to become visible; as previously shown by Dustin (55) and Bessis (8), localized fluid accumulation inside a perinuclear membrane formed a paranuclear vacuole, and further extension of this process isolated the nucleus within an all-encompassing vacuole. The mechanism of the fluid collection inside the inner nuclear membrane is not immediately apparent but was associated with nuclear pyknosis and may be related to nucleoprotein coacervation (16).

Secretion Granules, Perinuclear Droplets, and Mitochondria

Although there appears little doubt that the dense granules in the apices of the acinar cells represent the zymogen granules or secretion product of the cell, the identity of the less dense, semiliquid perinuclear droplets is uncertain. However, the occurrence of apparent transition forms between perinuclear droplets and secretion granules, and their like behaviour in eventually swelling and vesiculating, suggests that the perinuclear droplets may represent immature secretion granule material of the kind described by Palade (54). It seems possible that perinuclear droplets, by masking mitochondria and mimicking their forms, may have made the positive identification of mitochondria impossible with the present method.

Appreciation is expressed to Dr. E. Storey for criticism and painstaking help in the preparation of the manuscript, to Mr. K. Johns of the Physics Department for assistance in development of the microcompressor, and to Miss H. M. Doery of the Commonwealth Serum Laboratories for supplies of phosphatidase A.

Received for publication, June 6, 1961.

BIBLIOGRAPHY

1. LEEUWENHOEK, A. VAN, *in* The Collected Letters of Antoni van Leeuwenhoek, (Committee of Dutch Scientists, editors), Amsterdam, Swets and Zeitlinger Ltd., 1948, **3**, 385.
2. DUJARDIN, F., Histoire naturelle des zoophytes. Infusoires, comprenant la physiologie et la classification de ces animaux, et la manière de les étudier à l'aide du microscope, Paris, Libraire Encyclopédique de Roret, 1841.
3. CALKINS, G. N., Protozoology, London, Bailliere, Tindall and Cox, 1910, 127.
4. LEWIS, W. H., and LEWIS, M. R., *in* General Cytology, (E. V. Cowdry, editor), Chicago, The University of Chicago Press, 1924, 385.
5. HEILBRUNN, L. V., The Colloid Chemistry of Protoplasm, Berlin, Verlag von Gebrüder Borntraeger, 1928.
6. HEILBRUNN, L. V., The Dynamics of Living Protoplasm, New York, Academic Press, Inc., 1956.
7. CAMERON, G. R., Pathology of the Cell, London, Oliver and Boyd, 1952.
8. BESSIS, M., Cytology of the Blood and Blood-Forming Organs, (translated by E. Ponder), New York, Grune and Stratton, 1956.
9. MANUELIDIS, E. E., *in* Frontiers in Cytology, (S. L. Palay, editor), New Haven, Yale University Press, 1958, 417.
10. POLICARD, A., and BAUD, C. A., Les structures inframicroscopiques normales et pathologiques des cellules et des tissus, Paris, Masson et Cie, 1958, 59.
11. BUCHSBAUM, R., and KUNTZ, J. A., *Ann. New York Acad. Sc.*, 1954, **58**, 1303.
12. BUCKLEY, I. K., *Australian J. Exp. Biol. and Med. Sc.*, 1960, **38**, 211.
13. FAURÉ-FREMIET, E., *in* Swelling and Shrinking—A General Discussion held by the Faraday Society, London, Gurney and Jackson, 1946, 253.

14. HOLTFRETER, J., *Ann. New York Acad. Sc.*, 1948, **49**, 709.
15. FREY-WYSSLING, A., Submicroscopic Morphology of Protoplasm, Amsterdam, Elsevier Publishing Company, 2nd (English) edition, 1953.
16. BOOIJ, H. L., and BUNGENBERG DE JONG, H. G., *in* Protoplasmatologia Handbuch, (L. V. Heilbrunn and F. Weber, editors), Wien, Springer-Verlag, 1, 1956.
17. ENGSTRÖM, A., and FINEAN, J. B., Biological Ultrastructure, New York, Academic Press, Inc., 1958, 93.
18. BUCKLEY, I. K., *Proc. Roy. Soc. Victoria*, 1961, **74**, 13.
19. DOERY, H. M., and PEARSON, J. E., *Biochem. J.*, 1961, **78**, 820.
20. BUCKLEY, I. K., *Quart. J. Exp. Physiol.*, 1961, **46**, 229.
21. PORTER, K. R., *J. Exp. Med.*, 1953, **97**, 727.
22. PALAY, S. L., and WISSIG, S. L., *Anat. Rec.*, 1953, **116**, 301.
23. SHELTON, E., quoted by Haguenau, F., *Internat. Rev. Cytol.*, 1958, **7**, 477.
24. THIÉRY, J. P., *Rev. Hémat.*, 1955, **10**, 745.
25. FAWCETT, D. W., and ITO, S., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 135.
26. ROSE, G. G., and POMERAT, C. M., *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 423.
27. WEISS, J. M., *J. Exp. Med.*, 1953, **98**, 607.
28. PALADE, G. E., and SIEKEVITZ, P., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 671.
29. LACY, D., and ROTBLAT, J., *in* Fourth International Conference on Electron Microscopy, Berlin, 10–17 September 1958, (W. Bargmann, D. Peters, and C. Wolpers, editors), Berlin, Springer-Verlag, 1960, **2**, 484.
30. EMMELOT, P., and BENEDITTI, E. L., *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 393.
31. MORGAN, C., HOWE, C., and ROSE, H. M., *J. Exp. Med.*, 1961, **113**, 219.
32. LEATHES, J. B., *Lancet*, 1925, **208**, 957.
33. NAGEOTTE, J., *Compt. rend. Acad. sc.*, 1927, **185**, 44.
34. NAGEOTTE, J., *in* Actualités Scientifiques et Industrielles, Paris, Herman et Cie, 1936, parts 431–434.
35. KÖLSCH, K., *in* Zoologische Jahrbücher, (J. W. Spengel, editor), Jena, Gustav Fischer, 1902, **16**, 273.
36. RUNNSTRÖM, J., *Protoplasma*, 1928, **4**, 388.
37. MONNÉ, L., *Protoplasma*, 1939, **32**, 184.
38. PALADE, G. E., and CLAUDE, A., *J. Morphol.*, 1949, **85**, 35.
39. PALADE, G. E., and CLAUDE, A., *J. Morphol.*, 1949, **85**, 71.
40. STOECKENIUS, W., *Exp. Cell Research*, 1957, **13**, 410.
41. POLICARD, A., COLLET, A., and PREGERMAIN, S., *in* Fourth International Conference on Electron Microscopy, Berlin, 10–17 September 1958, (W. Bargmann, D. Peters, and C. Wolpers, editors), Berlin, Springer-Verlag, 1960, **2**, 258.
42. FRIEDMANN, I., and BIRD, E. S., *in* Fourth International Conference on Electron Microscopy, Berlin, 10–17 September, 1958, (W. Bargmann, D. Peters and C. Wolpers, editors), Berlin, Springer-Verlag, 1960, **2**, 266.
43. SCHMITT, F. O., and PALMER, K. J., *Cold Spring Harbor Symp. Quant. Biol.*, 1940, **8**, 94.
44. BEAR, R. S., PALMER, K. J., and SCHMITT, F. O., *J. Cell. and Comp. Physiol.*, 1941, **17**, 355.
45. REVEL, J. P., ITO, S., and FAWCETT, D. W., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 495.
46. STOECKENIUS, W., *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 491.
47. CLAUDE, A., *J. Exp. Med.*, 1946, **84**, 61.
48. PALADE, G. E., and SIEKEVITZ, P., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 171.
49. DAWSON, R. M. C., *Biol. Rev.*, 1957, **32**, 188.
50. HOGUE, M. J., *J. Exp. Med.*, 1919, **30**, 617.
51. LEWIS, M. R., and LEWIS, W. H., *Amer. J. Anat.*, 1915, **17**, 339.
52. LEWIS, M. R., *J. Exp. Med.*, 1920, **31**, 293.
53. LATTA, H., and KUTSAKIS, A., *Lab. Inv.*, 1957, **6**, 12.
54. PALADE, G. E., *in* Electron Microscopy in Anatomy, (J. D. Boyd, F. R. Johnson, J. D. Lever, editors), London, Edward Arnold Ltd., 1961, 176.
55. DUSTIN, P., JR., *Acta clin. belg.*, 1949, **4**, 70.