ELECTRON MICROSCOPIC STUDIES ON PERIODIC ACID-SCHIFF-POSITIVE NONGLYCOGENIC STRUCTURES IN HUMAN LIVER CELLS

CLAUDE BIAVA, M.D.

From the Departments of Pathology, Mount Sinai Hospital and The Chicago Medical School, Chicago, Ill.

Periodic acid-Schiff(PAS)-positive nonglycogenic structures are well known to occur in normal and abnormal human livers. Popper, Paronetto and Barka[1] have recently contributed a detailed morphologic and histochemical characterization of these structures and discussed the subject thoroughly. Among the various types of diastase-resistant PAS positive (D-PAS) structures described in their study, these authors attached particular importance to the D-PAS granules appearing in liver cells in a variety of liver diseases. They observed that these granules were coarser and more numerous than the granules corresponding to lipofuscin pigment which are usually seen in the peribiliary cytoplasm of normal liver cells and which may also react positively with the PAS technique. Aside from these differences, however, they found that the two types of D-PAS granules exhibited essentially similar staining and histochemical properties as well as autofluorescence and acid phosphatase activity. They concluded, therefore, that D-PAS granules in abnormal livers were at least in part related to lipofuscin pigment. On the basis of the bile-pigmented appearance exhibited by some of the granules in patients with cholestasis, they also suggested that the D-PAS material was partly related to bile pigment.

From these results,[1] it is apparent that if basic differences in composition and morphogenesis do exist between normal D-PAS lipofuscin granules and abnormal lipofuscin-like D-PAS granules in liver cells, such differences are not readily demonstrated with the help of light microscopic techniques currently in use and that the relationship between the granules and bile pigment is likewise difficult to establish. Whereas our light microscopic observations have corroborated in most respects the results of Popper and co-workers,[1] observations with the electron microscope have provided convincing evidence that bile pigment and various types of D-PAS granules in normal and abnormal human livers differ significantly in structure and morphogenesis.

In a previous publication,[2] an account was given of the ultrastructure

and possible origin of the bile-pigmented materials seen in livers with intrahepatic and extrahepatic cholestasis. The purpose of the present report is to describe the fine structure of abnormal D-PAS granules in liver cells and to outline their morphogenesis. Their relationship to bile pigment will also be considered together with the contrasting fine structural appearances of normal lipofuscin pigment.

MATERIAL AND METHODS

Liver biopsy specimens from 46 patients were studied by light and electron microscopy. Five specimens were considered histologically normal. They were obtained from patients who did not present clinical evidence of hepatic disease and were undergoing operations for non-neoplastic intra-abdominal conditions. Fifteen specimens were from patients with acute viral hepatitis; 8 of these showed evidence of intrahepatic cholestasis of variable severity.[3] Three specimens were from patients with extrahepatic biliary obstruction due to carcinoma of the head of the pancreas and 6 were from patients with septal or postnecrotic cirrhosis. Fifteen specimens were from patients with miscellaneous conditions including diabetes, anemia, systemic infections and carcinomas; they showed varying degrees of fatty metamorphosis, granulomas or nonspecific reactive hepatitis.

For light microscopy, specimens were fixed in 10 per cent buffered formalin. Sufficient tissue for frozen sections was available from several cases. Frozen and paraffin sections were submitted to hematoxylin and eosin stain; the PAS reaction before and after diastase digestion; peracetic acid-aldehyde fuchsin stain[3]; Perls' reaction for iron; Hall's stain[4] for bilirubin; and Sudan black and oil red O stains for fat. Deparaffinized sections were also examined with ultraviolet light for autofluorescence.

For electron microscopy, specimens were fixed in 1 per cent osmium tetroxide in Millonig's buffer[5] and embedded in Epon 812.[6] A few blocks from several specimens were also embedded in a 1 to 4 mixture of methyl and butyl methacrylate. Thick sections from the latter blocks were treated with the PAS procedure[7] and examined by light microscopy. Adjacent ultrathin sections from fields rich in PAS positive-granules were then examined directly by electron microscopy. Thick sections from Epon-embedded blocks were stained with 0.4 per cent toluidine blue in 1 per cent borax and examined by light microscopy in order to select appropriate fields for ultrathin sectioning. The PAS procedure could not be successfully applied to these sections. Ultrathin sections were cut with a Porter-Blum ultramicrotome, stained with lead hydroxide[8] and examined with an RCA EMU-3G electron microscope.

RESULTS

Increased numbers of D-PAS granules were found in the cytoplasm of liver cells in all of the abnormal specimens. They were most numerous in cases of acute liver disease, particularly viral hepatitis, where they occurred predominantly in liver cells in centrilobular areas and near foci of parenchymal necrosis. In patients with chronic liver disease, they were contained in more irregularly scattered liver cells. Their number varied considerably and appeared to correlate well with the degree of clinical activity of the disease. They were also present in increased

amounts in patients without primary liver disease, as in cases of anemia, systemic infection and carcinoma, in whom involvement of the liver appeared to be secondary and laboratory tests revealed only moderate degrees of hepatic dysfunction. In livers considered normal, D-PAS granules were fewer and located almost exclusively near bile canaliculi where they appeared to correspond to brown granules of lipofuscin pigment. Larger and coarser granules of lipofuscin pigment, however, gave weak or negative PAS reactions. Some of these granules gave a positive Perls' reaction for iron.

A distinction between D-PAS granules in normal and abnormal livers was not readily achieved with the staining techniques employed. Both types of granules appeared as light brown pigment in conventional hematoxylin and eosin-stained sections; both showed a yellow-brown autofluorescence with ultraviolet light and were stained by oil red O in frozen sections and Sudan black in paraffin sections. The most consistent differences were a more variable size and a more diffuse intracellular distribution of D-PAS granules in abnormal livers. In the course of this investigation it was found that the intensity of the color reaction obtained with the PAS procedure was often insufficient for an optimal demonstration of the finer D-PAS granules, particularly in thin paraffin sections (2μ or less). Since in these sections the size and intracellular distribution of the granules could be studied to advantage and correlated more readily with the electron microscopic findings, a number of staining procedures paralleling the results obtained with the PAS reaction but yielding a stronger color reaction were tried. Among these the peracetic acid-aldehyde fuchsin stain recommended by Spicer[3] gave the best results. It stained both normal and abnormal D-PAS granules intensely dark blue, so that even granules approaching in size the resolving power of the light microscope could be clearly appreciated as fine dots sharply demarcated from the surrounding cytoplasm. The paracetic acid-aldehyde fuchsin stain was therefore extensively used in the present study along with the PAS reaction.

In patients with extrahepatic or intrahepatic cholestasis, D-PAS granules were prominent and did not usually show bile staining when examined either in unstained sections or in sections treated with the Hall's procedure.[4] On the other hand, typical masses of hepatocellular and canalicular bile pigment gave negative PAS reactions and unlike D-PAS granules did not bind fat soluble dyes or show autofluorescence. In many liver cells, however, bile pigment and D-PAS granules appeared intimately associated so that it was difficult or impossible to clearly differentiate between them, even in thin paraffin sections.

ELECTRON MICROSCOPIC OBSERVATIONS

Hepatocellular D-PAS Granules in Abnormal Livers. Alternate thick and thin sections of plastic-embedded blocks were respectively examined by light and electron microscopy in order to facilitate the identification of sub-cellular structures corresponding to the D-PAS granules. Such correlation was best achieved in methacrylate-embedded tissues inasmuch as the PAS procedure could not be successfully applied to Epon-embedded tissues. When examined in thick methacrylate sections prior to staining, the granules appeared as dense refractile dots with a distinct brown color due to their considerable binding of the osmium used as fixative. Following the PAS procedure, they appeared as sharply demarcated red dots scattered throughout the cytoplasm of liver cells but with a higher concentration in the vicinity of bile canaliculi (Fig. 1). In thick Epon sections treated with alkaline toluidine blue, similar granules stained intensely deep-blue.

With the electron microscope D-PAS granules were found to correspond to dense osmiophilic bodies with a characteristic vacuolated appearance (Fig. 2). This appearance, together with the ultrastructural features to be described, were sufficiently uniform and distinctive in all of the abnormal livers examined so that the continued use of methacrylate-embedded blocks for the purpose of correlating light and electron microscopic images of D-PAS granules was no longer considered warranted after the initial phases of this study. Simpler vacuolated dense bodies consisted of cytoplasmic vacuoles 300 to 500 mμ in diameter, containing variable amounts of an exceedingly osmiophilic substance forming clumps and rings disposed at the periphery of each vacuole (Fig. 3). Individual vacuoles were usually well circumscribed and separated from the surrounding cytoplasm by a single membrane. Aggregates of similar vacuoles formed larger and more polymorphic dense bodies (Figs. 3 and 4) attaining dimensions of up to several μ. The aggregates seemed to arise either from a secondary coalescence of several single vacuoles or from the simultaneous formation of such vacuoles in adjacent regions of cytoplasm. The intracellular distribution of the vacuolated dense bodies correlated closely with that of D-PAS granules as seen by light microscopy. Although they did not spare any particular region of cytoplasm, they seemed to occur more commonly in pericanalicular and glycogen areas than in ergastoplasmic areas of liver cells. They were generally more numerous than D-PAS granules, presumably because many of the bodies were too small to be detectable with the light microscope.

In patients with acute liver diseases, particularly viral hepatitis, a

structural variant of vacuolated dense bodies was frequently observed. This consisted of much larger cytoplasmic vacuoles surrounded by an irregular and often discontinuous single membrane containing whorls and ring-shaped clumps of osmiophilic substance (Fig. 5). This type of vacuolated dense body occurred predominantly in glycogen areas of liver cells.

At high magnification, the ring-shaped deposits of osmiophilic substance at the periphery of individual vacuoles revealed a myeloid substructure consisting of concentric dense lamellae about 25Å thick alternating with electron lucent spaces about 20Å wide. The concentric lamellae formed layers of variable thickness, and usually enclosed clumps of granular or amorphous material with variable degrees of osmiophilia. In these clumps there often were some imperfectly formed myeloid lamellae which extended to, and became continuous with, the inner lamellae of the peripheral myeloid layers (Fig. 6).

From an examination of a large number of electron micrographs, images interpretable as possible sequential stages in the development of vacuolated dense bodies were detected. In general, these bodies seemed to arise from the sequestration and breakdown of circumscribed portions of hepatocellular cytoplasm. The modalities of such sequestration varied somewhat, however, in the case of vacuolated bodies of different sizes. Within the limitations inherent in the study of dynamic events from static images, it has seemed possible to delineate three main modalities. The first occurred commonly in patients with chronic or subsiding liver diseases and involved protrusion of small portions of ground cytoplasm into dilated vesicles of endoplasmic reticulum (Figs. 7, 8 and 9). The protruding clumps of ground cytoplasm often contained a few ribosomes or glycogen particles (Fig. 7) and were frequently accompanied by a single altered vesicle of agranular reticulum exhibiting a reticular membrane considerably more osmiophilic than normally seen (Fig. 8). Similar intrareticular cytoplasmic projections were partly or completely separated from the surrounding cytoplasm and became sequestered within the dilated vesicles of endoplasmic reticulum, thus forming early vacuolated bodies (Fig. 10). In several vacuoles, the sequestered cytoplasmic components were found in various stages of degradation as indicated by their increasing density and osmiophilia, while in other vacuoles they appeared completely transformed into solid or ring-shaped clumps of osmiophilic material (Figs. 9 and 11). A few myeloid lamellae could usually be detected in this material (Fig. 9).

A second modality involved a more direct sequestration of somewhat larger portions of ground cytoplasm containing particulate structures such as ribosomes and glycogen particles. The earliest detectable changes

consisted of focal areas of condensed cytoplasm which were separated from the surrounding cytoplasm, either by an electron-lucent narrow space or by an apparently newly formed single membrane (Figs. 12 and 13). The vacuolated bodies formed in this manner were of the type most commonly found in the present study (Figs. 2, 3 and 4). Characteristically they contained clumps of sequestered cytoplasm in all stages of degradation and transformation into whorls and rings of intensely osmiophilic material (Figs. 12 and 13). Granular material representing residual ribosomes and glycogen particles often remained recognizable in the vacuoles when the associated ground cytoplasm appeared to be in advanced stages of degradation (Figs. 6, 12 and 13). The development of myeloid structures was apparently associated with progressive degradation of the sequestered cytoplasmic components and was attended by a proportional decrease in the amounts of granular and amorphous materials in the vacuoles (Fig. 6). In large multivacuolated dense bodies, individual unit vacuoles were commonly found in different stages of development and contained cytoplasmic components in various stages of degradation (Fig. 14). This asynchronous development suggested that large multivacuolated bodies were formed, at least in part, by successive accretion of newly formed unit vacuoles in adjacent regions of cytoplasm.

A third modality differed from the preceding only in that more conspicuous portions of cytoplasm were at one time sequestered into large cytoplasmic vacuoles. Most commonly these vacuoles occurred in glycogen areas of liver cells and the sequestered portions of cytoplasm characteristically contained numerous vesicular elements of agranular endoplasmic reticulum (Figs. 15 and 16). Degradation of these elements and of the associated ground cytoplasm led to the formation of the multiple whorls and ring-shaped clumps of osmiophilic substance seen in large vacuolated dense bodies (Fig. 5). This modality seemed to reflect a more severe and explosive type of cytoplasmic vacuolation and sequestration and, as mentioned earlier, occurred particularly in patients with acute viral hepatitis. In addition to agranular endoplasmic reticulum, other cytoplasmic organelles such as mitochondria and ergastoplasmic cisternae were on occasion sequestered in these larger vacuoles. In patients with intrahepatic or extrahepatic cholestasis, some of the vacuoles also contained relatively large granular masses of clumped ground cytoplasm similar to those previously shown to correspond to bile pigment[2] (Figs. 16 and 17). Condensation and partial degradation of the various structures contained in these vacuoles led to the formation of mixed types of bodies composed of ring-shaped clumps of osmiophilic substance embedded within compact masses of altered ground cytoplasm (Figs. 17 and 18). These bodies are believed to correspond to mixed types of

granules which by light microscopy appeared either as bile pigmented D-PAS granules or as clumps of bile pigment containing D-PAS material.

Extracellular D-PAS Granules. Whorls and rings of osmiophilic material derived from breakdown of hepatocellular cytoplasmic components were found extracellularly in two locations. A few were frequently present in bile canaliculi, where they arose in part from degradation of microvilli separated from liver cells and shed into canalicular lumens (Fig. 19). They were never sufficiently numerous to obstruct bile canaliculi. In cases of cholestasis, several osmiophilic clumps were at times found clustered and compressed at the periphery of bile thrombi (Fig. 20) with an appearance reminiscent of the mixed types of bodies described above in liver cells (Figs. 17 and 18).

Similar clumps of osmiophilic material were observed also within vacuole-like focal expansions of intercellular spaces occurring frequently in abnormal livers, particularly at the level of the dovetail interdigitations normally seen between adjacent liver cells (Fig. 21). Formation of such vacuole-like intercellular spaces was frequently associated with swelling and disruption of cytoplasmic processes projecting into them (Fig. 22). When tangentially sectioned these spaces appeared as pseudo-cytoplasmic vacuoles distributed in rows along the cellular borders of adjacent liver cells and contained cross-sectioned cytoplasmic processes in their centers (Fig. 23). Separation and degradation of altered cytoplasmic processes led to formation of dense osmiophilic clumps which, because of the variable plane of sectioning, were seen either within dilated intercellular spaces or within pseudocytoplasmic vacuoles at the periphery of liver cells (Fig. 24). Such images were at times deceptively suggestive of an outward movement of cytoplasmic vacuolated dense bodies and of a discharge of their osmiophilic contents into intercellular spaces.

D-PAS Lipofuscin Granules in Normal Livers. In normal human livers, hepatocellular lipofuscin pigment appeared as solid clumps of intensely osmiophilic and homogenous material deposited within membrane-bound pericanalicular bodies (Fig. 25). These bodies were similar to those found in rat livers and are believed to correspond to lysosomes.[9] They were composed of a compact and coarsely granular matrix of moderate electron density which usually contained moderate numbers of electron-dense ferritin particles. Characteristically, their outer single membrane appeared separated from the matrix by a narrow electron-lucent space about 100 Å wide (Fig. 25). The masses of lipofuscin pigment deposited within pericanalicular bodies varied greatly in size and number. As the amount of pigment increased, larger and denser pericanalicular bodies were formed which contained decreasing amounts of

matrix substance. At high magnification, lipofuscin pigment maintained an homogenous appearance with no tendency to form crystalline myeloid or lamellar structures.

Cholesterol Crystals in Abnormal Livers. In addition to the vacuolated dense bodies previously described, some abnormal liver cells showed elongated cigar-shaped cytoplasmic clefts, most commonly found in glycogen areas in patients with cholestasis (Fig. 26). Similar clefts were also seen in the cytoplasm of Kupffer cells. They exhibited unusually sharp borders and were often surrounded by a thin layer of dense material but not by a distinct outer membrane. Their shape was highly reminiscent of the spaces seen by light microscopy in various pathologic tissues and known to be produced by extracted cholesterol crystals. Their electron microscopic appearances were identical to those exhibited by typical cholesterol crystals of larger size found in hemorrhagic tissues (personal observation) and in atheromatous plaques of human aortas.[10] The cigar-shaped cytoplasmic clefts in liver cells were therefore believed to represent artifactually empty spaces originally occupied by small cholesterol crystals removed during dehydration of tissues with ethyl alcohol.

DISCUSSION

The types of vacuolated dense bodies described represented some of the most common ultrastructural hepatocellular changes thus far encountered in our series of liver biopsies. Their occurrence seemed independent of the type of liver disease affecting patients and of complicating factors, such as cholestasis and unusually prolonged clinical course intervening in individual cases. This is in keeping with their correspondence to abnormally increased hepatocellular D-PAS granules which have also been noted by light microscopy in various liver diseases.[1] Since, like D-PAS granules, they correlated well with the severity and extent of hepatic damage, they may be similarly regarded as a general index of such damage. In view of the exceedingly discrete size and number of the bodies detectable with the electron microscope, they may indeed constitute a particularly sensitive index of hepatocellular injury.

The frequency with which similar vacuolated dense bodies have been recorded in electron microscopic studies of human liver diseases undoubtedly reflects the high incidence found in our material. There are, however, widely differing opinions expressed in the literature concerning their nature. Essner and Novikoff [11] first described them in the liver cells of a patient with obstructive jaundice and believed them to represent bile pigment contained in lysosomes. This interpretation was later accepted by several investigators, particularly in electron microscopic studies on human cholestasis.[12-21] Steiner and Carruthers,[22] however,

noted similar structures in liver cells and bile canaliculi of patients with cholestasis and descriptively referred to them as lipid bodies and lipid emboli respectively, rather than as bile pigment. In cases of viral hepatitis, similar bodies were considered by Gueft [23] as a type of viral particle, a view later revised in favor of the possibility that they represented polymorphic products of cytoplasmic breakdown.[24]

In our material, the currently prevalent opinion relating these bodies to bile pigment could not be confirmed. Their occurrence did not correlate with the presence or absence of histologic evidence of cholestasis. Neither could a correspondence be established between them and the bile stained cytoplasmic structures visible by light microscopy.[2] It was found, however, that in patients with cholestasis, they often developed in close association with, and frequently became incorporated within, bile-stained masses of clumped ground cytoplasm corresponding to bile pigment. It is conceivable that the intimate association between these two types of abnormal cytoplasmic structures and their variable predominance in the resulting mixed bodies, accounts for the presence of PAS-positive substances in some clumps of bile-pigment, or for the bile-pigmented appearance of some D-PAS granules noted by light microscopy.[1]

The ultrastructural findings are believed to provide convincing evidence that D-PAS granules in abnormal liver cells originated from a particular type of focal breakdown of cytoplasmic constituents. Early stages of this process involved the sequestration of small portions of cytoplasm within dilated elements of endoplasmic reticulum or within apparently newly formed vacuoles in the ground cytoplasm of liver cells. Degradation of the sequestered cytoplasmic constituents seemed to ensue rapidly and was characteristically accompanied by the development of intensely osmiophilic myeloid structures. The resulting dense bodies were not unlike those observed experimentally to develop in different types of cells under the influence of various physiologic and pathologic stimuli.

Hruban and colleagues [25] have aptly referred to this process as focal cytoplasmic degradation. In some tissues, for instance in hamster seminal vesicles,[26] dense bodies resulting from this process have been shown to correspond to lipofuscin-like D-PAS granules as in the case of the vacuolated dense bodies reported here in human liver cells. Unfortunately there is a perplexing variety of terms currently in use to designate the polymorphic structures representing intermediate and end stages of focal cytoplasmic degradation. Dense bodies,[27–29] lamellar bodies,[30,31] lysosome-like bodies,[32,33] cytolysomes [34–36] and cytosomes [37–39] may be cited among the most common designations. In recognizing the difficulties

likely to arise from a confusing terminology, De Duve,[40] who originally developed the lysosome concept and anticipated the heterogeneous nature of lysosomal structures,[41] has recently proposed that the term autophagic vacuole be used to indicate membrane-bound cytoplasmic vacuoles containing recognizable cellular structures undergoing digestion. In his view, this term would present the combined advantage of being descriptively adequate and of avoiding problems as yet unsolved concerning the relationships between autophagic vacuoles and lysosomes and the source of the lysosomal hydrolytic enzymes often found associated with autophagic vacuoles.

In concurring with the views of Hruban and colleagues,[25] and of De Duve,[40] we consider the vacuolated dense bodies corresponding to D-PAS granules in abnormal human livers as particular forms of focal cytoplasmic degradation and propose to refer to them as autophagic vacuoles when containing recognizable cellular components, and as residual vacuolated bodies when containing only the final products of degradation of the sequestered portions of cytoplasm.

The intense osmiophilia exhibited by the products of degradation in autophagic vacuoles strongly indicates that they consist predominantly of lipids. Their tendency to crystallize into myeloid structures further suggests that they are composed of polar lipids, probably phospholipids.[42] This interpretation is consonant with the lipid character of D-PAS granules deduced by Popper and co-workers [1] from histochemical data. It is presumed that the increasing amounts of lipids detectable during the evolution of autophagic vacuoles into residual vacuolated bodies reflect a progressive liberation or unmasking of the structural lipids of sequestered cytoplasmic components as a consequence of a more readily achieved digestion of their structural proteins. Such a process would therefore represent an example of lipophanerosis at the subcellular level.[43] It is of interest that the liberated lipid moiety appeared to be contributed not only by the sequestered lipoprotein cytoplasmic membranes but by the associated ground cytoplasm as well. A striking example of lipophanerosis with formation of myeloid structures involving predominantly ground cytoplasm has been described by Policard, Bessis and Breton-Gorius [44] in red cells undergoing digestion within bone marrow macrophages.

The clumps of osmiophilic substance found in bile canaliculi and intercellular spaces, and apparently derived from microvilli and cytoplasmic processes of liver cells, are presumed to consist of lipid moieties similar in composition to those developing in autophagic cytoplasmic vacuoles. The suggestion that they may represent cholesterol [22] would not seem to be supported by the findings presented here relative to their origin

and to the differing appearances of cholesterol crystals in liver cells.

The ultrastructural features characterizing lipofuscin granules in normal human livers conformed to the description of Essner and Novikoff[11] and differed considerably from the appearances of autophagic vacuoles in abnormal livers. The lipid moiety composing the pigment lacked a crystalline structure and was deposited in the matrix of pre-existing cytoplasmic bodies, presumably lysosomes,[9] in the peribiliary cytoplasm of normal liver cells. The nature and origins of this material remain obscure at present.

The nature of the inducing stimuli and the cellular mechanisms at play in the formation and evolution of autophagic vacuoles in abnormal human livers are not known. The process of sequestration described most often involved unusually discrete portions of hepatocellular cytoplasm. This seemed to account for the small and relatively uniform size of the unit vacuoles composing residual vacuolated bodies and conferring on them their characteristic electron microscopic appearances.

The recurrence of their main structural features in all of the abnormal livers examined was striking and can be best interpreted as indicating that the mechanisms operative in the morphogenesis of autophagic vacuoles constitute a precisely patterned cellular reaction to injury rather than a disorganized lytic process. This cellular response would seem to reflect undefined properties of human liver cells more than the influence of particular types of pathologic stimuli. Consistent with this impression is some indirect evidence indicating that the activity of living cells is necessary for the occurrence of this type of autophagic vacuoles, and of focal cytoplasmic degradation in general.

In the present study, early phases of the autophagic process, namely separation of portions of cytoplasm within liver cells and the formation of limiting membranes around the resulting vacuoles, were generally observed to take place in cells otherwise structurally well preserved and apparently viable. Poux[45] described autophagic vacuoles in vegetable cells and likewise remarked that they occurred in cells which, outside the vacuoles, showed an essentially normal cytoplasm. On the other hand, Hruban and colleagues[25] were unable to detect areas of focal cytoplasmic degradation in animal livers undergoing post-mortem autolysis. In a similar investigation, we examined human livers sampled at different intervals up to 48 hours after death and failed to find any evidence that post-mortem autolysis was attended by formation of autophagic vacuoles similar to those seen in liver disease. In a study of dense bodies induced by acridine orange in HeLa cells, Robbins, Marcus and Gonatas[46] found that the formation of such bodies as well as the development of myeloid structures within them were energy-dependent processes sensitive to in-

hibitors of the glycolytic and Krebs cycles. These results are of considerable interest inasmuch as the ultrastructural features exhibited by the acridine orange-induced dense bodies illustrated by Robbins and co-workers were strikingly similar to those described here in autophagic vacuoles and residual vacuolated bodies in human liver tissue.

From the foregoing considerations it can be tentatively concluded that the D-PAS granules in abnormal human livers, although derived from focal breakdown of cytoplasmic structures, may not represent simply a degenerative process but also an active cellular response to injury which in several structural details is fairly characteristic of human liver cells. It is possible that the development of this type of cellular reaction requires the expenditure of metabolic energy, and that it helps the survival of liver cells under adverse conditions. One of its useful functions could reside in the elimination of toxic products bound to altered cytoplasmic structures and not readily metabolized by liver cells. By contrast, it may be noted that the development of other types of hepatocellular lesions, such as Mallory's alcoholic hyalin [47] and Councilman-like acidophilic bodies [21, 48] is not accompanied by sequestration and degradation of the corresponding altered cytoplasmic structures. In the case of bile pigment,[2] only a comparatively late and sluggish autophagic process occurs.

SUMMARY

Diastase-resistant periodic acid-Schiff-positive (D-PAS) granules in human liver cells were studied by light and electron microscopy. By light microscopy, D-PAS granules in abnormal liver cells closely resembled the appearance and staining reactions of the D-PAS granules of lipofuscin pigment seen in normal liver cells, except that they were more numerous and variable in size and were more diffusely distributed in the cytoplasm. In patients with cholestasis, they could not always be differentiated from bile pigment. By electron microscopy, they showed characteristic ultrastructural features which permitted their differentiation from both lipofuscin and bile pigment.

Abnormal D-PAS granules appeared to arise from the sequestration and degradation of small portions of hepatocellular cytoplasm within elements of endoplasmic reticulum or in newly formed cytoplasmic vacuoles. They were, therefore, considered as autophagic vacuoles concerned with the prompt removal and disposal of altered cytoplasmic components. Their development in association with various liver diseases seemed to reflect a nonspecific and widely occurring type of hepatocellular reaction to injury.

REFERENCES

1. POPPER, H.; PARONETTO, F., and BARKA, T.   PAS-positive structures of non-glycogenic character in normal and abnormal liver. *Arch. Path.*, 1960, **70**, 300–313.

2. BIAVA, C.   Studies on cholestasis. The fine structure and morphogenesis of hepatocellular and canalicular bile pigment. *Lab. Invest.*, 1964, **13**, 1099–1123.

3. SPICER, S. S.   Siderosis associated with increased lipofuscins and mast cells in aging mice. *Am. J. Path.*, 1960, **37**, 457–475.

4. HALL, M. J.   A staining reaction for bilirubin in sections of tissue. *Am. J. Clin. Path.*, 1960, **34**, 313–316.

5. MILLONIG, G.   Advantages of a phosphate buffer for OsO_4 Solutions in fixation. (Abstract) *J. Appl. Physics*, 1961, **32**, 1637.

6. LUFT, J. H.   Improvements in epoxy resin embedding methods. *J. Biophys. & Biochem. Cytol.*, 1961, **9**, 409–414.

7. JENNINGS, B. M.; FARQUHAR, M. G., and MOON, H. D.   Staining methods for osmium-methacrylate sections. *Am. J. Path.*, 1959, **35**, 991–997.

8. MILLONIG, G.   A modified procedure for lead staining of thin sections. *J. Biophys. & Biochem. Cytol.*, 1961, **11**, 736–739.

9. NOVIKOFF, A. B.; BEUFAY, H., and DE DUVE, C.   Electron microscopy of lysosome-rich fractions from rat liver. *J. Biophys. & Biochem. Cytol.*, 1956, **2**, (suppl.), 179–184.

10. BALIS, J. U.; HAUST, D. M., and MORE, R. H.   Electron-microscopic studies in human atherosclerosis. Cellular elements in aortic fatty streaks. *Exper. Molec. Path.*, 1964, **3**, 511–525.

11. ESSNER, E., and NOVIKOFF, A. B.   Human hepatocellular pigments and lysosomes. *J. Ultrastruct. Res.*, 1960, **3**, 374–391.

12. TANIKAWA, K.   Electron microscopic study of viral hepatitis with special attention to cholestatic form. *J. Jap. Soc. Intern. Med.*, 1961, **50**, 842–854.

13. ICHIDA, F.   Morphological observations on cases of nonfatal viral hepatitis with protracted jaundice. *Ann. Rep. Inst. Virus Res., Kyoto Univ.*, 1961, **4**, 153–171.

14. SASAKI, H., and ICHIDA, F.   Electron Microscopic Studies of Cholestasis. *Ann. Rep. Inst. Virus Res., Kyoto Univ.*, 1961, **4**, 172–191.

15. ALBOT, G.; JÉZÉQUEL, A. M., and LUNEL, J.   Les Épisodes cholostatiques au cours des ictères par hepatites virales. *Acta Hepatosplen. (Stuttgart)*, 1962, **9**, 140–154.

16. ORLANDI, F.   Electron-microscopic observations on human liver during cholestasis. *Acta Hepatosplen. (Stuttgart)*, 1962, **9**, 156–164.

17. JÉZÉQUEL, A. M., and ALBOT, G.   Les modifications de l'ultrastructure du foie au cours des ictères par hépatite virale et la pathogénie de l'ictère par hépatite. I. Formes de courte duree. *Sem. Hôp. Paris*, 1962, **38**, 523–539.

18. JÉZÉQUEL, A. M., and ALBOT, G.   Les modifications de l'ultrastructure du foie au cours des ictères par hépatite virale et la pathogénie de l'ictère par hépatite. II. Formes prolongées simples ou compliquées d'épisodes cholostatiques. *Sem. Hôp. Paris*, 1962, **38**, 550–567.

19. COSSEL, L.   Elektronenmikroskopische Befunde bei chronischer Virushepatitis und Lebercirrhose. *Virchows Arch. path. Anat.*, 1963, **336**, 354–367.

20. SCHAFFNER, F.; BARKA, T., and POPPER, H.   Hepatic mesenchymal cell reaction in liver disease. *Exp. Molec. Path.*, 1963, **2**, 419–441.

21. RUEBNER, B. H., and MIYAI, K. The Pathology of Neonatal hepatitis and biliary atresia with particular reference to hemopoiesis and hemosiderin deposition. *Ann. New York Acad. Sc.,* 1963, 111, 375–391.

22. STEINER, J. W., and CARRUTHERS, J. S. Studies on the fine structure of the terminal branches of the biliary tree. II. Observations of pathologically altered bile canaliculi. *Am. J. Path.,* 1961, 39, 41–63.

23. GUEFT, B. Viral hepatitis under the electron microscope. *Arch. Path.,* 1961, 72, 61–69.

24. KIKKAWA, Y., and GUEFT, B. Virus-like hepatic cell particles in patients with chronic alcoholism. *Gastroenterology,* 1963, 44, 243–250.

25. HRUBAN, Z.; SPARGO, B.; SWIFT, H.; WISSLER, R. W., and KLEINFELD, R. G. Focal cytoplasmic degradation. *Am. J. Path.,* 1963, 42, 657–683.

26. CAVAZOS, L. F.; BELT, W. D.; SHERIDAN, M. N., and FEAGANS, W. M. The fine structure of the hamster seminal vesicle with special reference to pigment formation. *Z. Zellforsch.,* 1964, 63, 179–193.

27. DAEMS, W. TH., and VAN RIJSSEL, TH. G. The fine structure of the peribiliary dense bodies in mouse liver tissue. *J. Ultrastruct. Res.,* 1961, 5, 263–290.

28. HERMAN, L., and FITZGERALD, P. J. The degenerative changes in pancreatic acinar cells caused by DL-ethionine. *J. Cell Biol.,* 1962, 12, 277–296.

29. HRUBAN, A.; SWIFT, H., and WISSLER, W. Analog-induced inclusions in pancreatic acinar cells. *J. Ultrastruct. Res.,* 1962, 7, 273–285.

30. MENEFEE, M. G., and EVANS, V. J. Structural differences produced in mammalian cells by changes in their environment. *J. Nat. Cancer Inst.,* 1960, 25, 1303–1323.

31. CECIO, A. Electron microscopic observations of young rat livers. I. Distribution and structure of myelin figures (lamellar bodies). *Z. Zellforsch.,* 1964, 62, 717–742.

32. ASHFORD, T. P., and PORTER, K. R. Cytoplasmic components in hepatic cell lysosomes. *J. Cell Biol.,* 1962, 12, 198–202.

33. TAKAHASHI, N., and MOTTET, N. K. Further observations on the cytoplasmic spherical bodies in HEp-3 carcinoma cells. *Lab. Invest.,* 1962, 11, 743–752.

34. NOVIKOFF, A. B. Biochemical and Staining Reactions of Cytoplasmic Constituents. In: Developing Cell Systems and Their Control. RUDNICK, D. (ed.). Ronald Press Co., New York, 1960, pp. 167–203.

35. NOVIKOFF, A. B., and ESSNER, E. Cytolysomes and mitochondrial degeneration. *J. Cell Biol.,* 1962, 15, 140–146.

36. NAPOLITANO, L. Cytolysomes in metabolically active cells. *J. Cell Biol.,* 1963, 18, 478–481.

37. TRUMP, B. F., and JANIGAN, D. T. The pathogenesis of cytologic vacuolization in sucrose nephrosis. An electron microscopic and histochemical study. *Lab. Invest.,* 1962, 11, 395–411.

38. TRUMP, B. F.; GOLDBLATT, P. J., and STOWELL, R. E. An electron microscopic study of early cytoplasmic alterations in hepatic parenchymal cells of mouse liver during necrosis *in vitro* (autolysis). *Lab. Invest.,* 1962, 11, 986–1015.

39. JORDAN, S. W. Electron Microscopy of Hepatic Regeneration. *Exp. Molec. Path.,* 1964, 3, 183–200.

40. DE DUVE, C. The Lysosome Concept. In: Lysosomes. Ciba Foundation Symposium. DE REUCK, A. V. S., and CAMERON, M. P. (eds.). Little, Brown & Co., Boston, 1963, pp. 1–35.

41. DE DUVE, C. Lysosomes, a New Group of Cytoplasmic Particles. In: Sub-

cellular Particles. HAYASHI, T. (ed.). Ronald Press Co., New York, 1959, pp. 128–159.

42. STOECKENIUS, W.   Some electron microscopical observations on lipid-crystalline phases in lipid-water systems. *J. Cell Biol.*, 1962, **12**, 221–229.

43. POLICARD, A.; COLLET, A., and PREGERMAIN, S.   Étude au microscope électronique de la lipophanerose cytoplasmique. Fourth International Conference on Electron Microscopy, Berlin, Sept. 10–17, 1958, Springer-Verlag, Vol. 2, 1960, pp. 258–261.

44. POLICARD, A.; BESSIS, M., and BRETON-GORIUS, J.   Structures myéliniques observées au microscope électronique sur des coupes de globules rouges en voie de lyse. *Exp. Cell Res.*, 1957, **13**, 184–186.

45. POUX, N.   Sur la présence d'enclaves cytoplasmiques en voie de dégénérescence dans les vacuoles des cellules végétales. *C.R. Acad. Sci. (Paris)*, 1963, **257**, 736–738.

46. ROBBINS, E.; MARCUS, P. I., and GONATAS, N. K.   Dynamics of acridine orange-cell interaction. II. Dye-induced ultrastructural changes in multivesicular bodies (acridine orange particles). *J. Cell Biol.*, 1964, **21**, 49–62.

47. BIAVA, C.   Mallory alcoholic hyalin: a heretofore unique lesion of hepatocellular ergastoplasm. *Lab. Invest.*, 1964, **13**, 301–320.

48. BIAVA, C.   Electron microscopic observations on Councilman-like acidophilic bodies and other forms of acidophilic changes in human liver cells. *Am. J. Path.* (In press)

———————————

[Illustrations follow]

Legends for Figures

All of the electron micrographs except figures 1 and 2 are from Epon-embedded tissue and lead hydroxide stained sections. Figure 2 is unstained.

FIG. 1.   Liver cells contain numerous D-PAS granules in a case of acute viral hepatitis. Methacrylate embedding, PAS stain. $\times$ 1,800.

FIG. 2.   Portions of two liver cells in an adjacent section of the same field shown in Fig. 1. Numerous vacuolated dense bodies are scattered throughout the cytoplasm. Nucleus, N; Golgi apparatus, G. $\times$ 9,500.

FIG. 3.   Detail of vacuolated dense bodies in glycogen areas of a liver cell. Vacuoles are arranged singly and in clusters and are well demarcated by outer single membranes. They contain characteristic ring-shaped clumps of a dense osmiophilic substance. $\times$ 44,000.
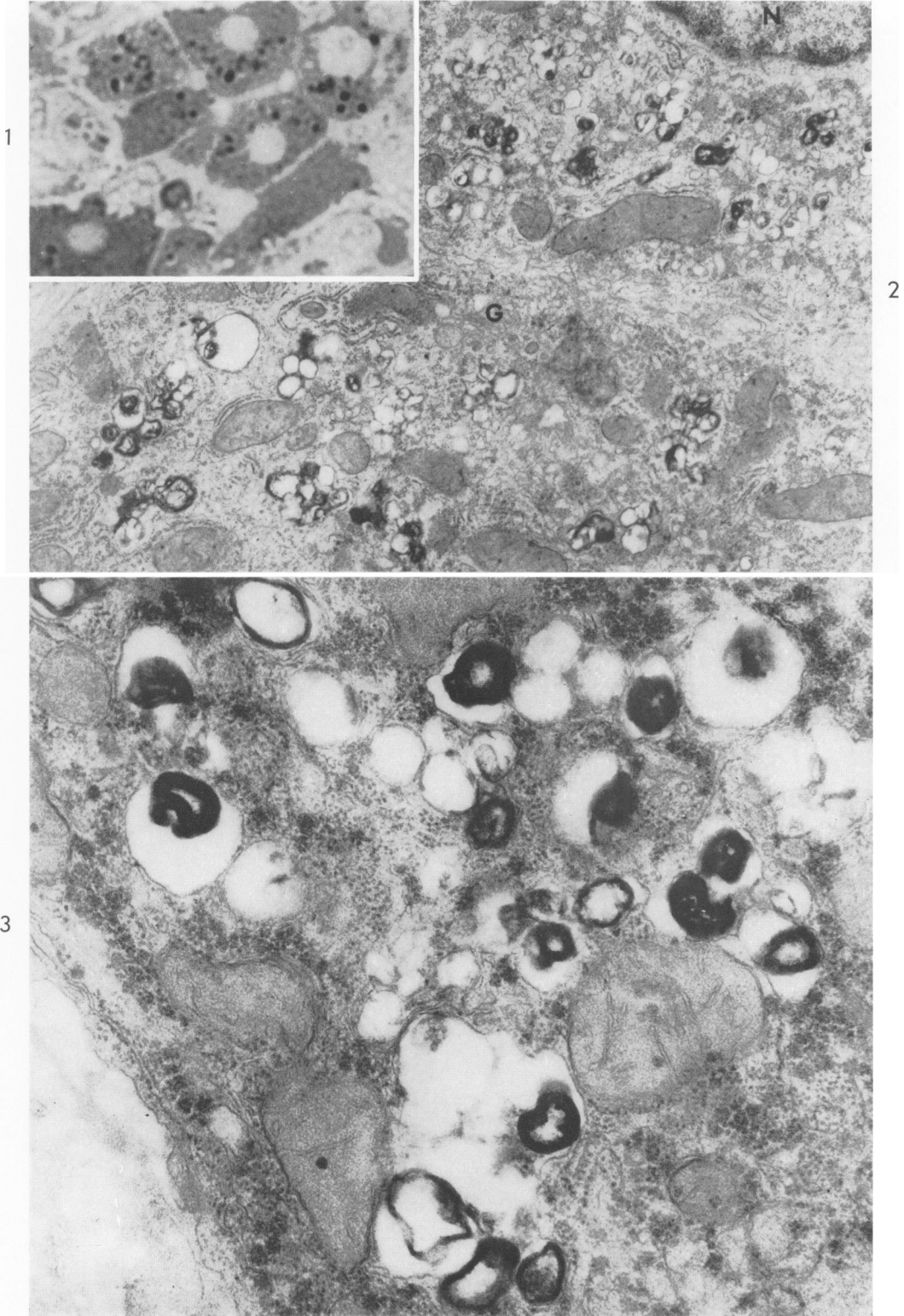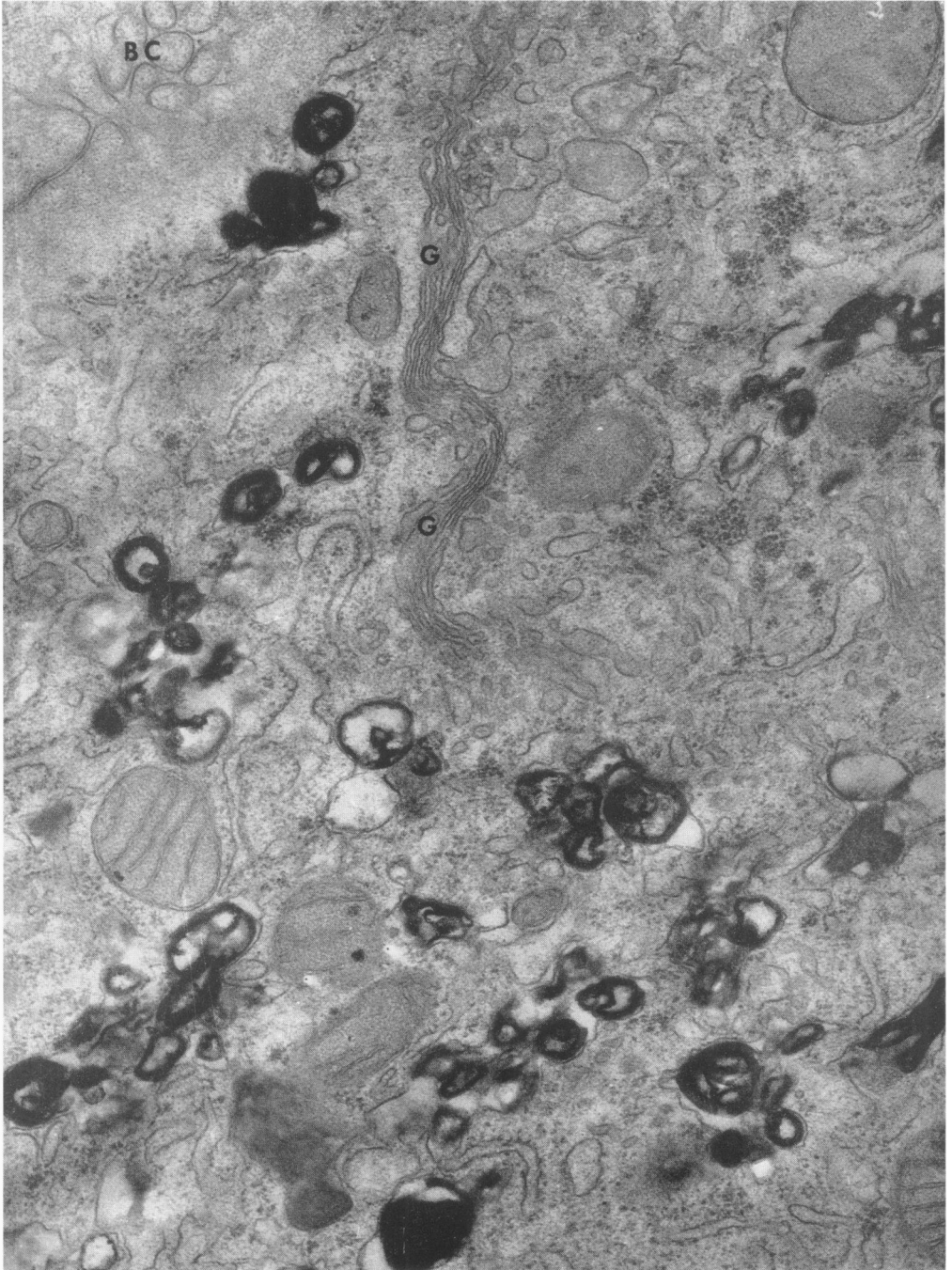
FIG. 4.   Vacuolated dense bodies of variable size in the peribiliary cytoplasm of a
liver cell. Larger bodies contain several ring-shaped clumps of dense substance.
The adjacent cytoplasm is well preserved. The Golgi apparatus (G) is
moderately hypertrophic. Bile canaliculus, BC. × 40,000.
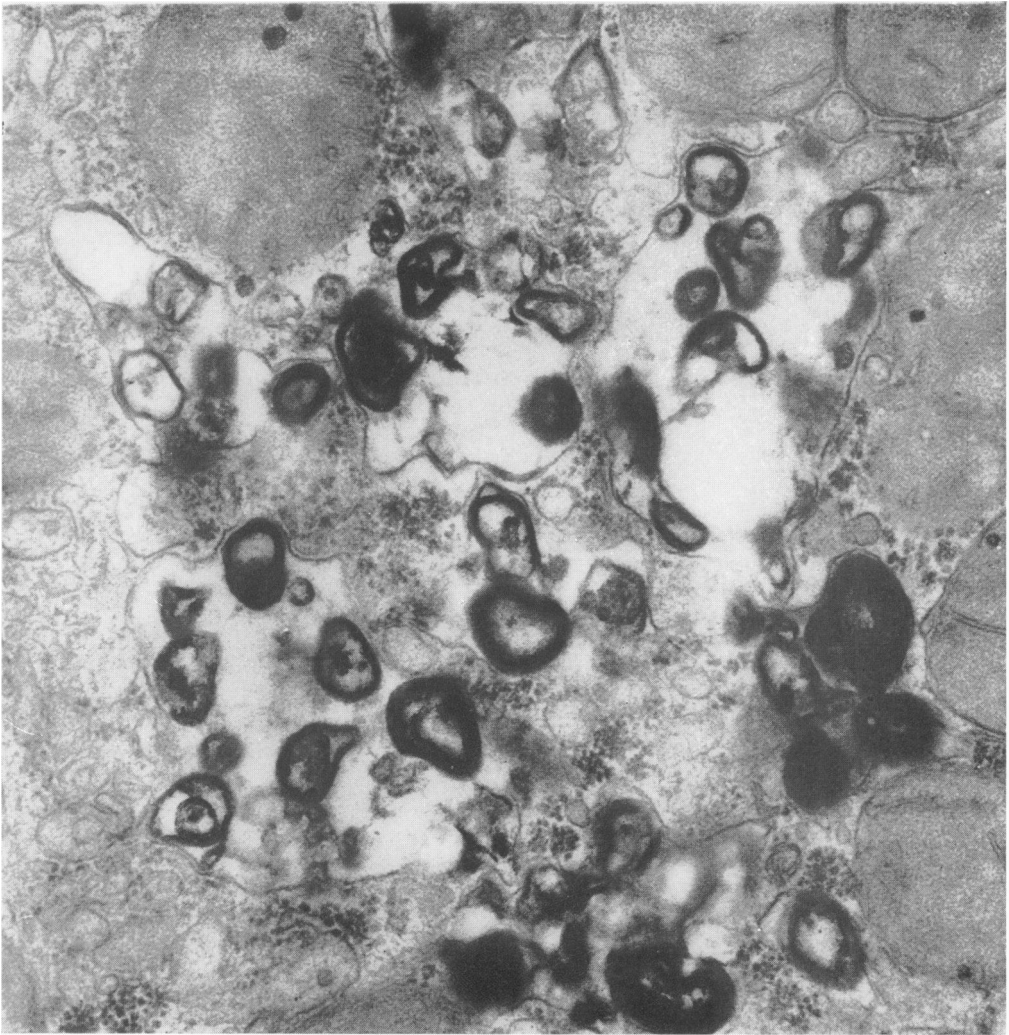
FIG. 5.   Large vacuolated dense bodies in a glycogen area of a liver cell in a case of acute viral hepatitis. The vacuoles are outlined by poorly defined outer membranes and contain several clumps and whorls of dense substance. $\times$ 52,000.

F<small>IG</small>. 6.   Vacuolated dense bodies at high magnification. Rings of dense substance
exhibit a characteristic myeloid structure consisting of dense lamellae alternating
with electron-lucent bands. Myeloid layers surround variable amounts of
granular or amorphous cytoplasmic debris. Vacuolated bodies (V_1) to (V_4)
show progressively more developed myeloid layers and decreasing amounts of
cytoplasmic debris. The origin of myeloid lamellae within the latter (arrows)
is seen in dense bodies (V_2) and is demonstrated to advantage in dense body
(V_3) illustrated in the *insert*. $\times$ 180,000. *Insert* $\times$ 220,000.
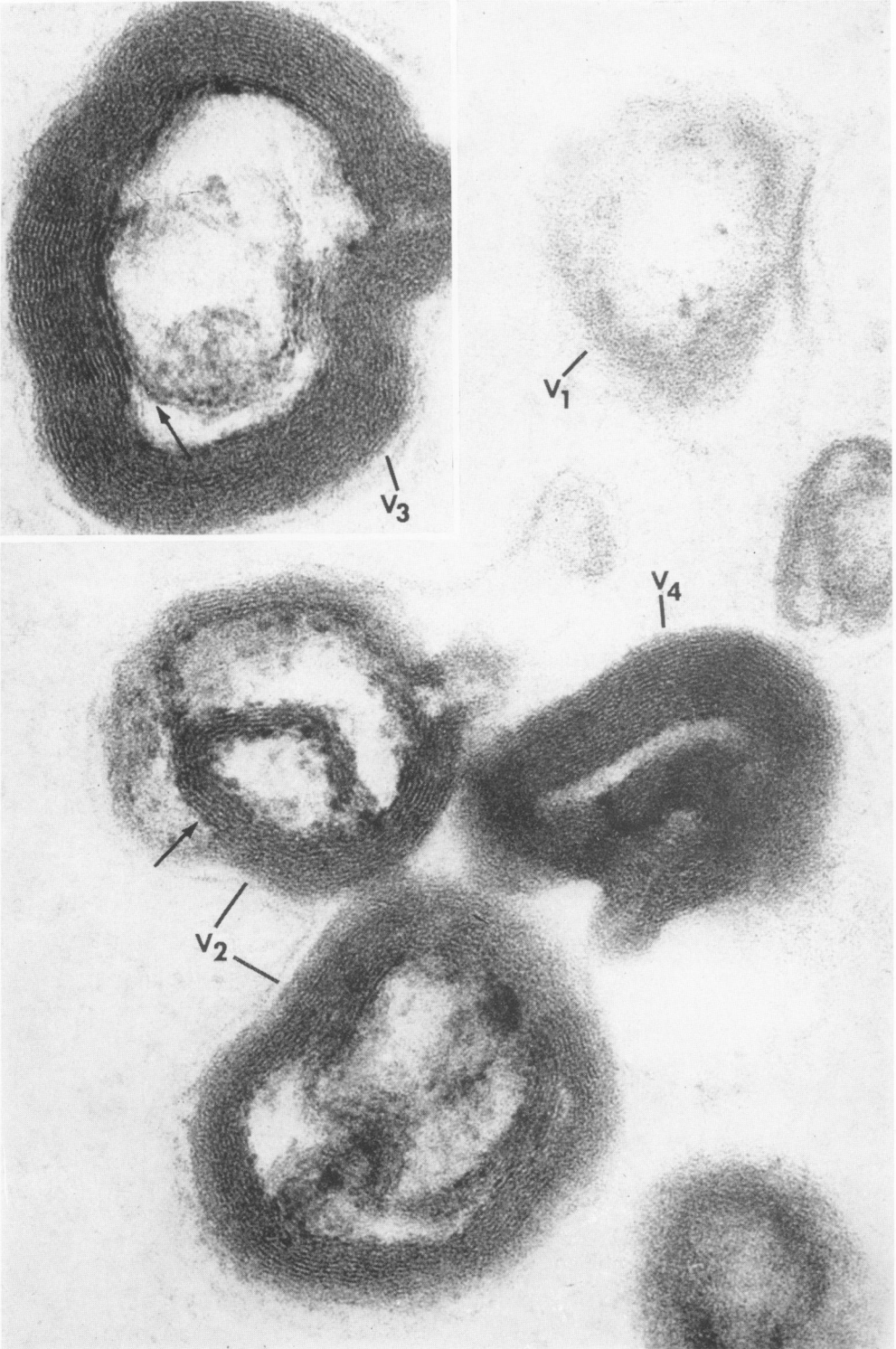
FIG. 7. Small portions of ground cytoplasm project into the dilated vesicles of agranular endoplasmic reticulum. The protruding clumps are cut in different planes. One of them (arrow) contains a glycogen particle and a few ribosomes. × 46,000.

FIG. 8. Two intrareticular cytoplasmic protrusions. Each contains an abnormally dense vesicle of endoplasmic reticulum (arrows) sectioned in different planes. × 60,000.

FIG. 9. A clump of osmiophilic substance with a few indistinct myeloid lamellae (arrow) is contained in a dilated vesicle of agranular endoplasmic reticulum. A partly detached intrareticular cytoplasmic protrusion of similar size is seen in the upper left field. × 60,000.

FIG. 10. A dilated endoplasmic reticular vesicle contains a sequestered cytoplasmic clump. × 60,000.

FIG. 11. An intrareticular cytoplasmic sequester in advanced stage of degradation exhibits a characteristic ring shape and intense osmiophilia. × 60,000.

FIG. 12 AND 13. Vacuolated dense bodies in various stages of development. A clump of condensed ground cytoplasm (V_1) containing ribosomal particles appears separated from the surrounding cytoplasm. A similar clump (V_2) is surrounded by a poorly defined membrane (arrow). Similar clumps containing residual ribosomal particles are seen in various stages of degradation in adjacent vacuoles (V_3) together with developing rings of dense substance. × 52,000.
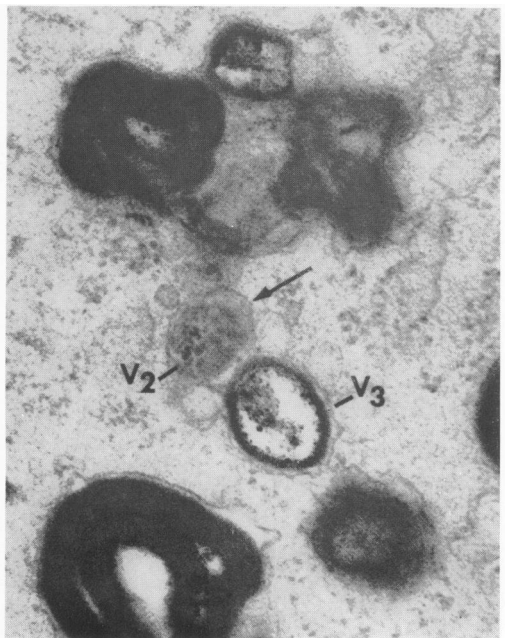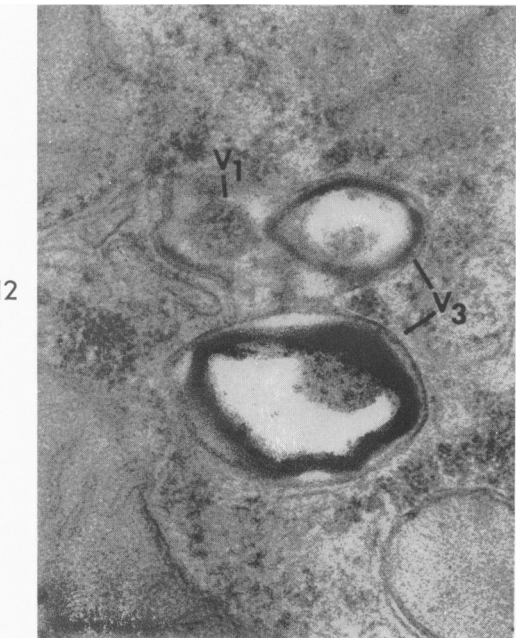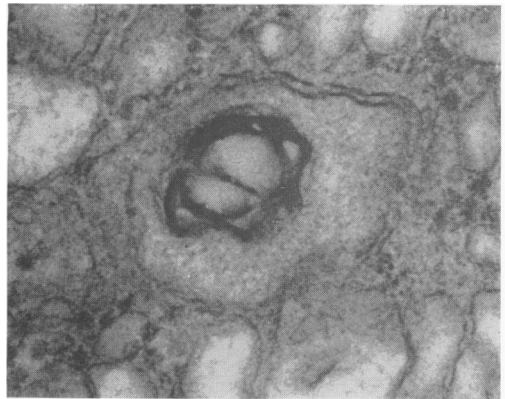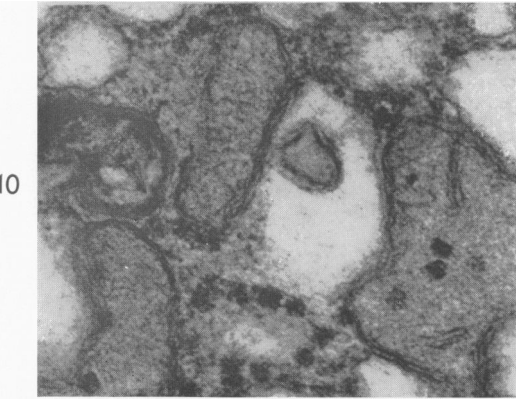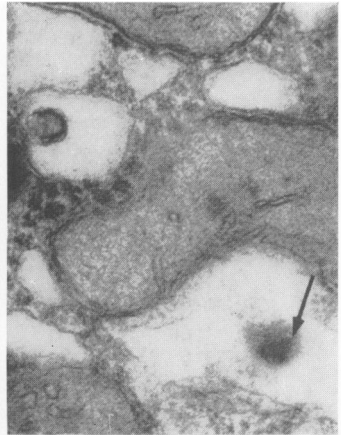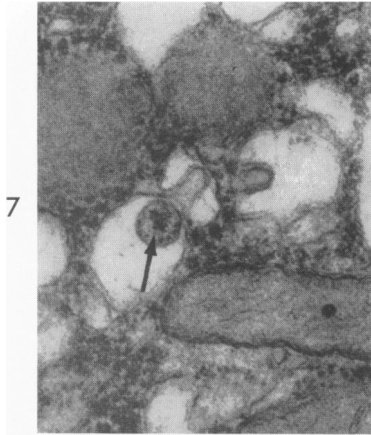
FIG. 14.   A large multivacuolated dense body composed of several unit vacuoles shows early (V1), intermediate (V2) and advanced (V3) stages of cytoplasmic sequestration and degradation with the development of dense myeloid lamellae (arrows). $\times$ 78,000.

FIG. 15.   Glycogen areas of a liver cell from a patient with acute viral hepatitis. There is a circumscribed area of moderately swollen cytoplasm containing disorganized agranular vesicles of endoplasmic reticulum and disrupted glycogen particles. There are suggestions of an incompletely formed limiting membrane (arrows) at the periphery. These appearances are believed to represent one of the earliest recognizable stages in the sequestration of relatively large portions of cytoplasm. A vacuolated dense body containing similar granular debris, probably glycogen, is also present in this field. $\times$ 38,000.
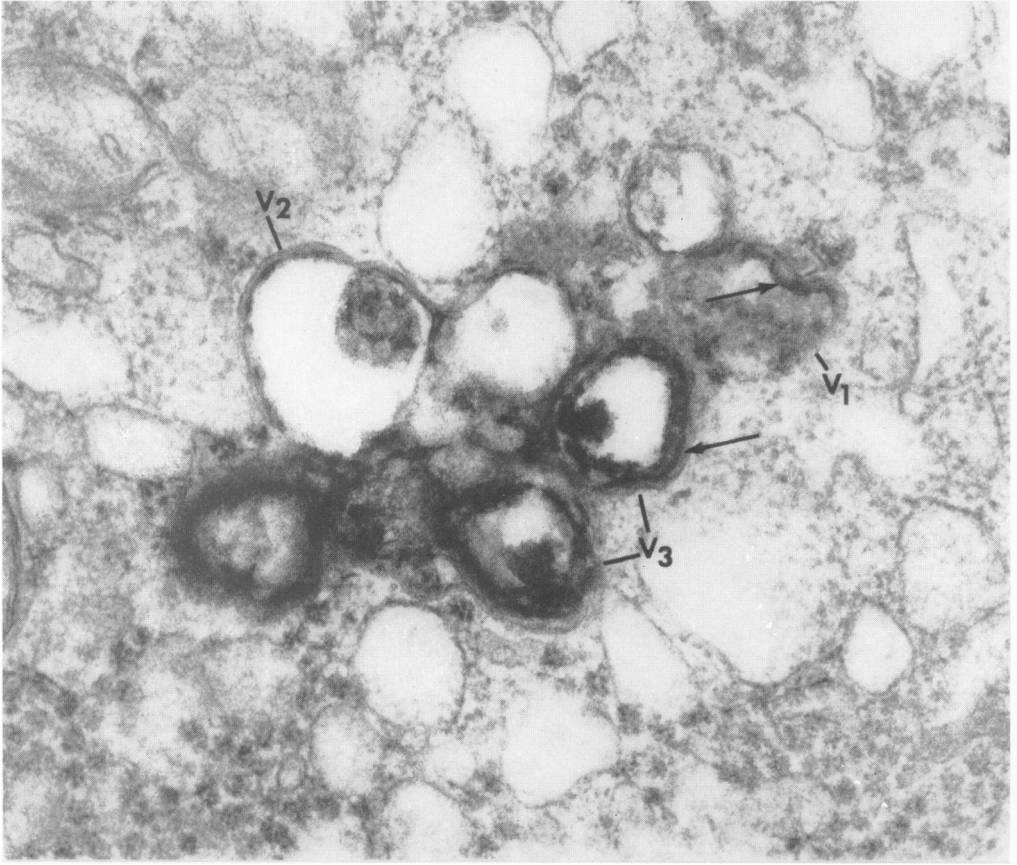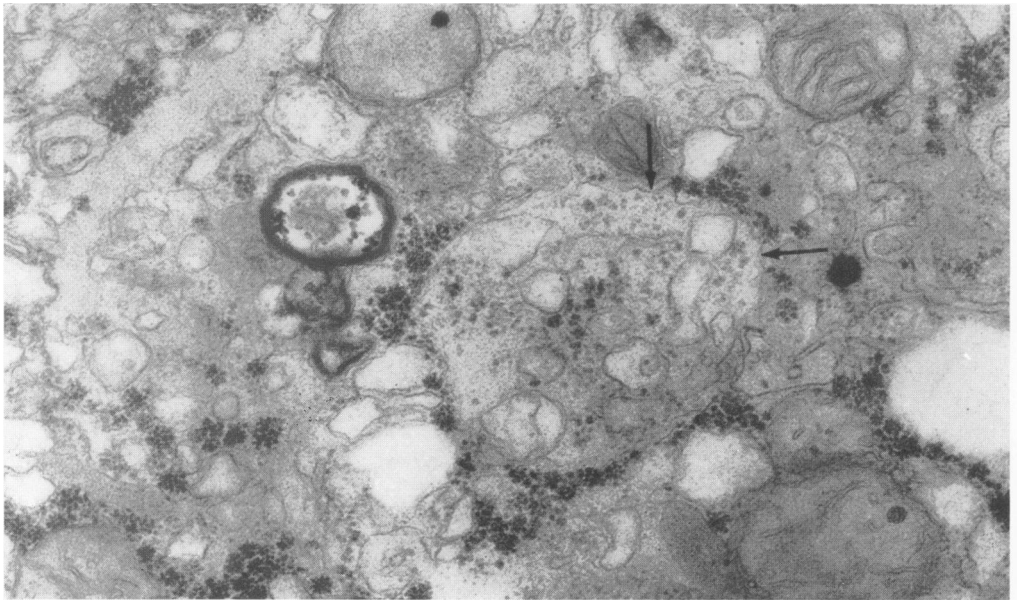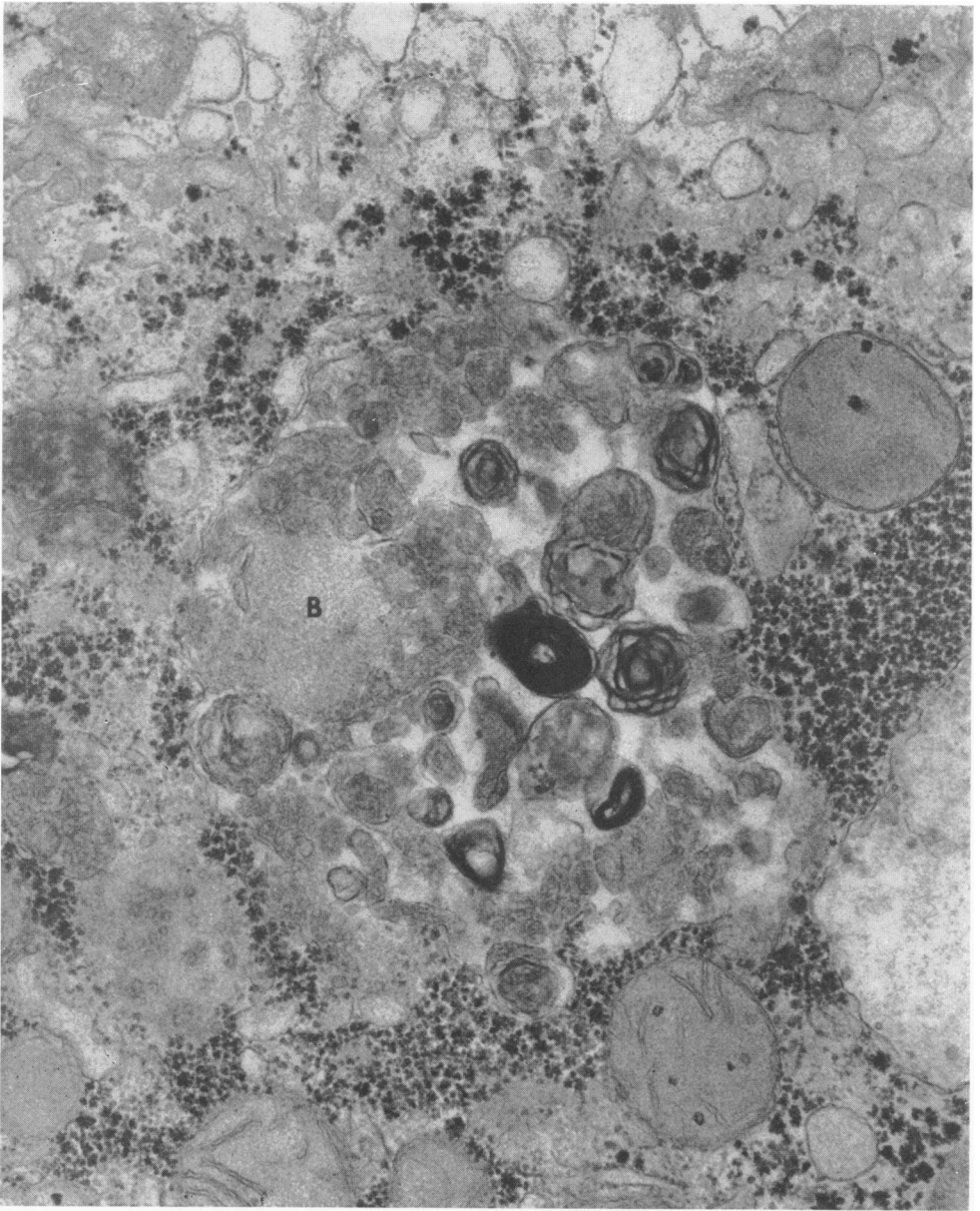
Fig. 16.   Glycogen area of a liver cell from a patient with acute viral hepatitis and intrahepatic cholestasis. A large vacuole is seen containing clumps and whorls of altered cytoplasmic components in various stages of degradation. A conspicuous granular mass of clumped ground cytoplasm corresponding to bile pigment (B) is also present. The vacuole is surrounded by an imperfectly formed limiting membrane. × 42,000.

Fig. 17.   Portion of liver cell from a patient with intrahepatic cholestasis. There are several granular masses of bile pigment (B) some of which contain typical vacuolated dense bodies and thus form bodies of mixed type. Nucleus, N; microbody, mb. × 30,000.

Fig. 18.   Detail of mixed bodies in a liver cell from a patient with extrahepatic cholestasis. Vacuolated dense bodies are incorporated within condensed masses of granular bile pigment (B) in late stages of development as shown by the presence of limiting membranes (arrows). × 54,000.
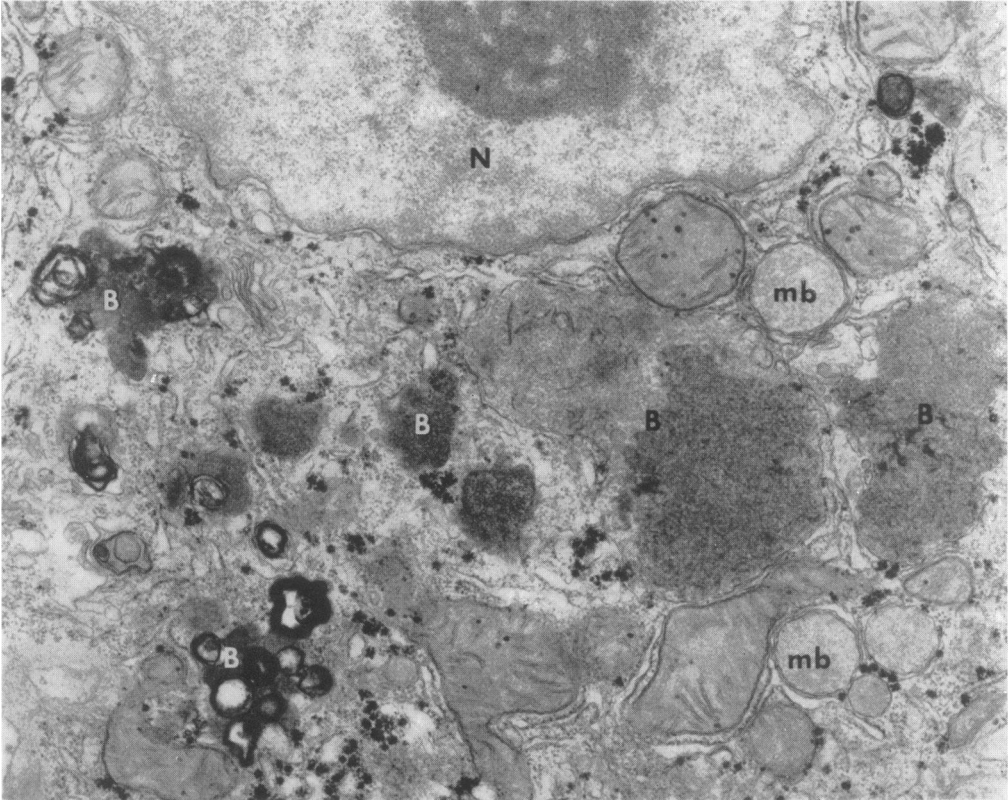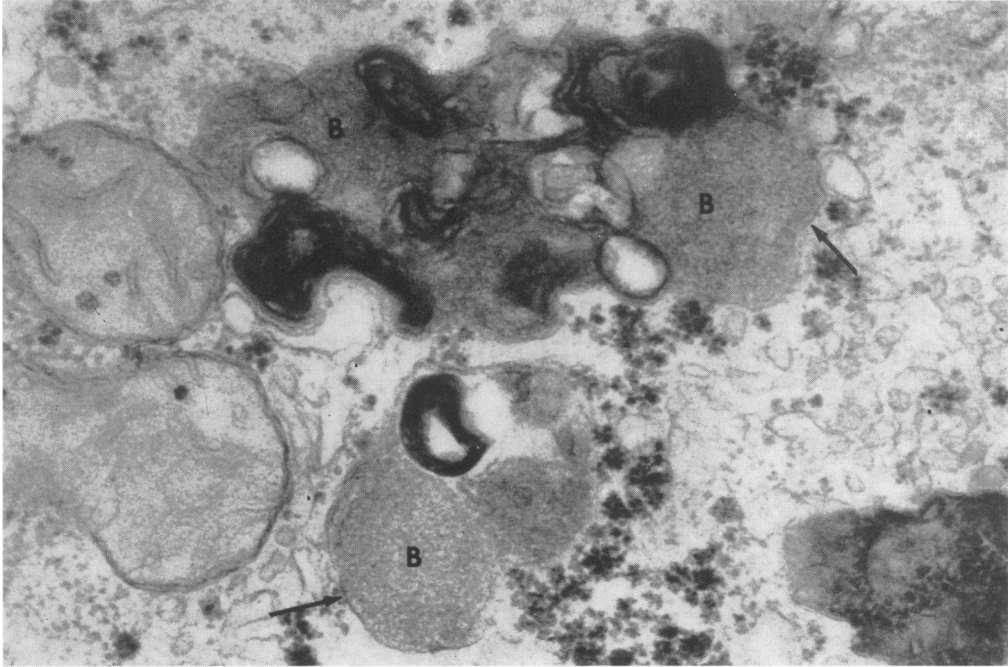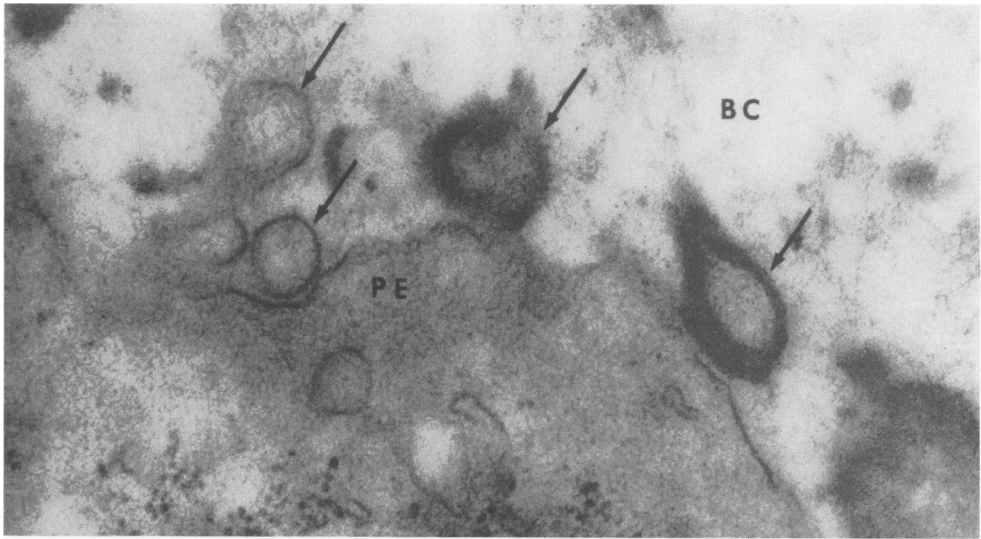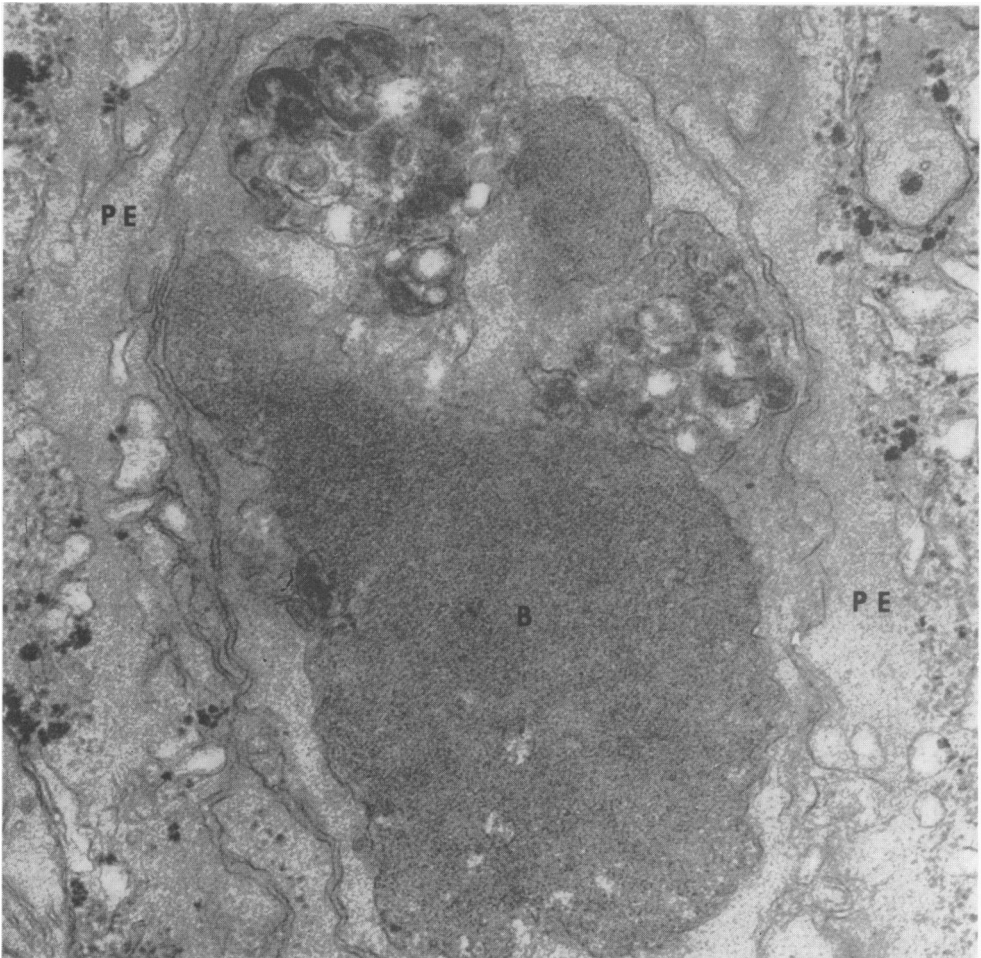
FIG. 19.   Canalicular border of a liver cell. Cross-sectioned microvilli (arrows) in the
canalicular lumen (BC) are undergoing degradation and transformation into
osmiophilic ring-shaped clumps. Pericanalicular ectoplasm, PE. $\times$ 60,000.

FIG. 20.   A canalicular bile thrombus (B) is composed chiefly of granular material
in a patient with intrahepatic cholestasis. Clusters of rings and whorls of dense
substance together with membranous debris are present at the periphery of the
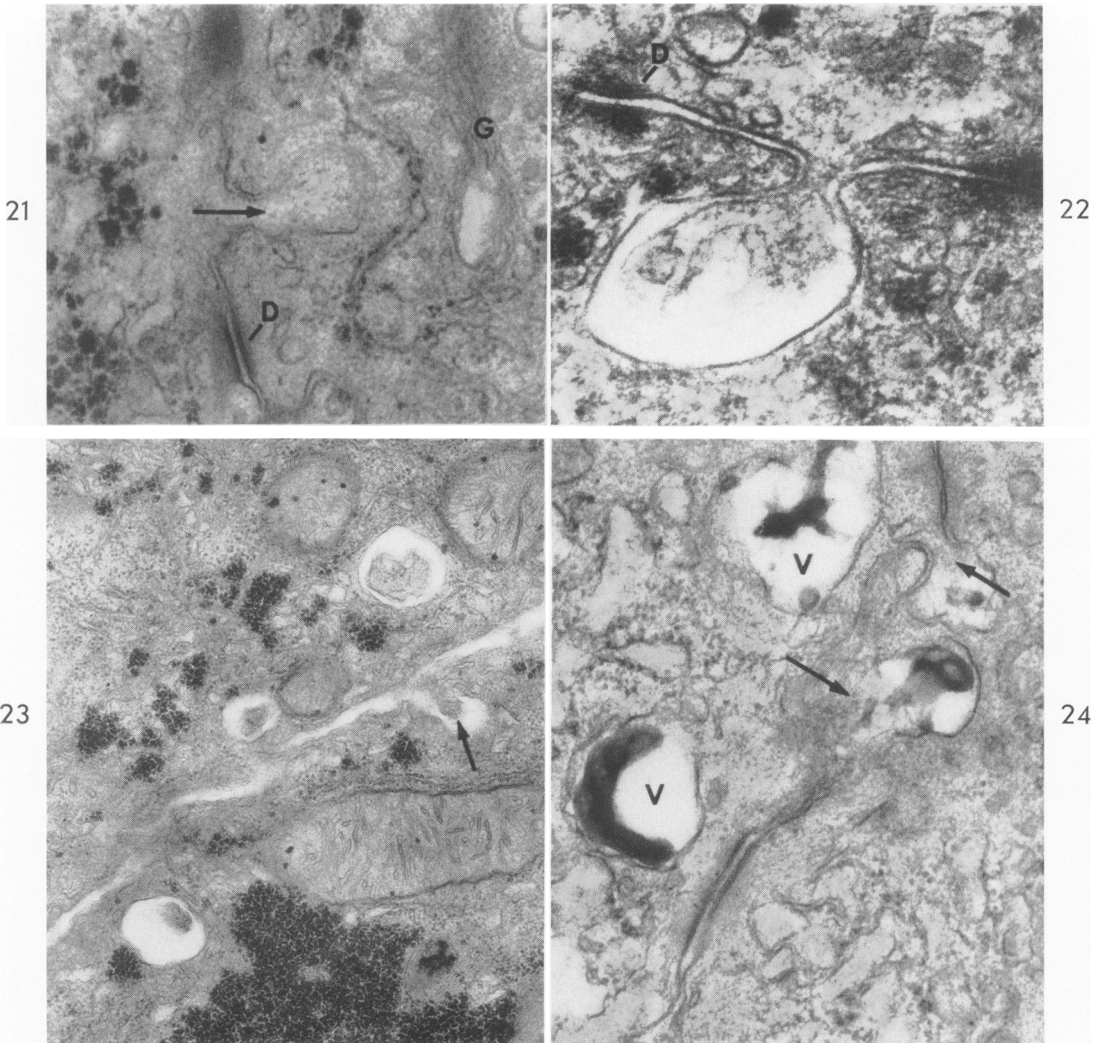bile thrombus. Pericanalicular ectoplasm, PE. $\times$ 48,000.

FIG. 21.   Normal dovetail junction between adjacent liver cells. The cytoplasmic process of one cell (arrow) interdigitates with, and is closely applied to, the cytoplasm of the adjacent cell. Desmosome, D; Golgi apparatus, G. × 40,000.

FIG. 22.   Focal dilatation of the intercellular space at the level of a dovetail junction. There is swelling and disruption of the cytoplasmic process forming part of the junction. Desmosome, D. × 60,000.

FIG. 23.   Pseudocytoplasmic vacuoles near the borders of two adjacent liver cells. The vacuoles are believed to represent tangential sections of focally dilated intercellular spaces such as that pointed out by the arrow and that illustrated in Figure 22. Portions of cytoplasm within each vacuole are believed to represent cross-sectioned cytoplasmic processes projecting into the dilated intercellular spaces. × 30,000.

FIG. 24.   Peripheral portions of two liver cells. Dilated intercellular spaces at the level of altered dovetail junctions (arrows) and pseudocytoplasmic vacuoles (V) are seen containing osmiophilic debris believed to represent portions of cytoplasmic processes in advanced stages of degradation. × 48,000.
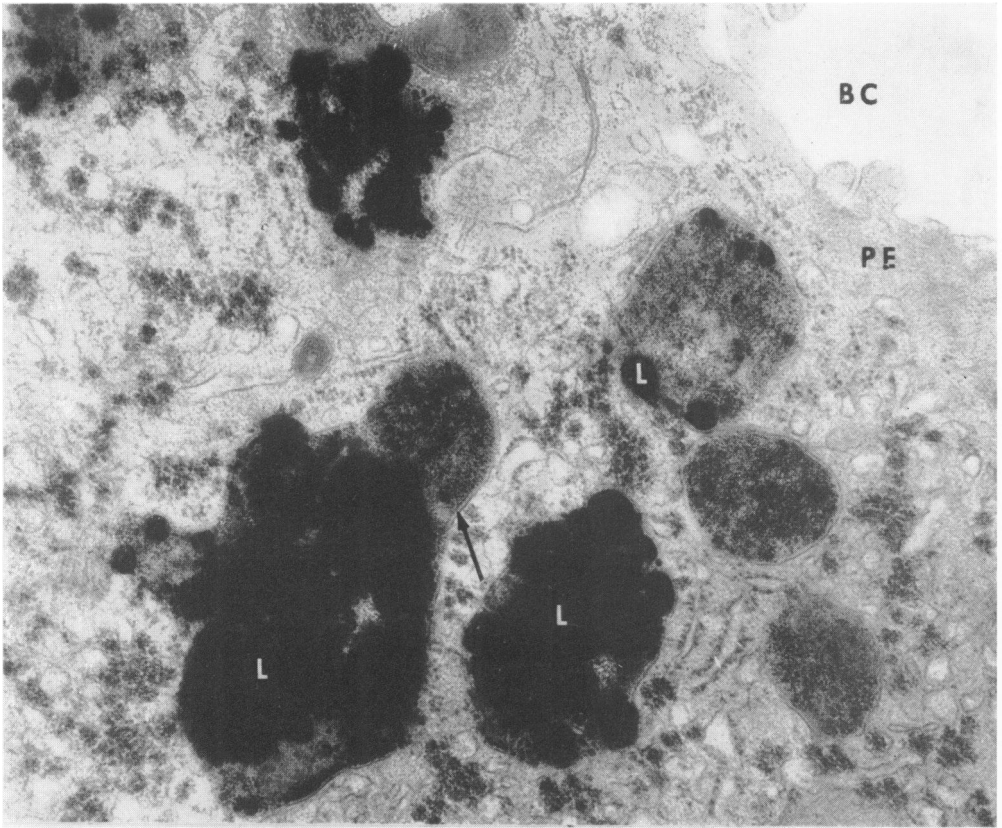
25
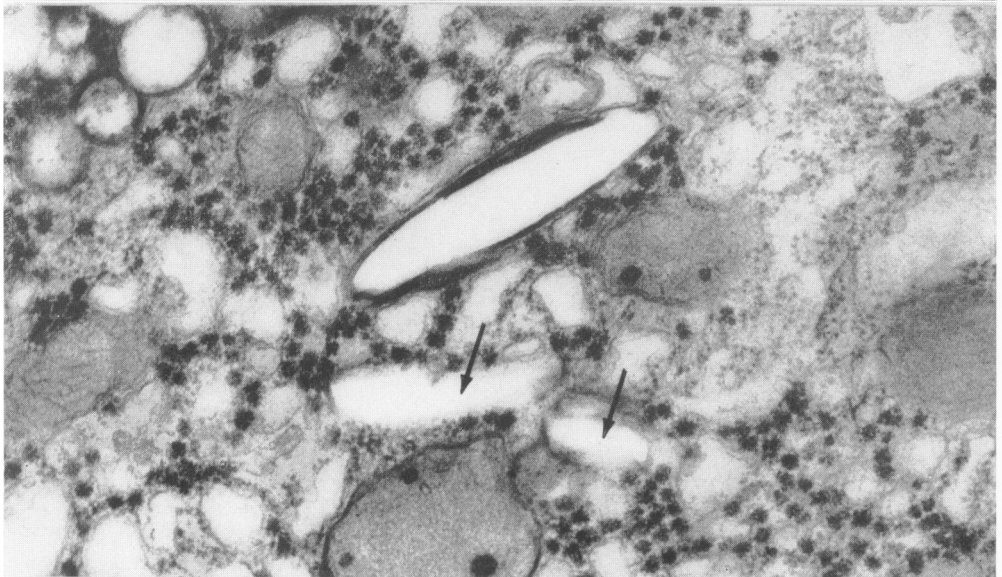
26

FIG. 25.   Peribiliary cytoplasm of normal liver cells. Homogeneous dense masses of
lipofuscin pigment (L) are deposited within membrane-bound pericanalicular
bodies. The matrix of these bodies contains electron dense ferritin particles and
is separated from the outer membranes by a narrow electron-lucent space (ar-
row). Bile canaliculus, BC; pericanalicular ectoplasm, PE. × 38,000.

FIG. 26.   Glycogen area in a liver cell from a patient with extrahepatic cholestasis.
An elongated cigar-shaped cytoplasmic cleft is seen. This is believed to have
been produced by extraction of a cholesterol crystal. The cleft is surrounded by
a thin layer of dense substance. Two similar clefts are seen below (arrows) but
are less typical due to the tangential plane of sectioning. × 54,000.