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STUDIES ON VINBLASTINE-INDUCED AUTOPHAGOCYTOSIS IN MOUSE LIVER

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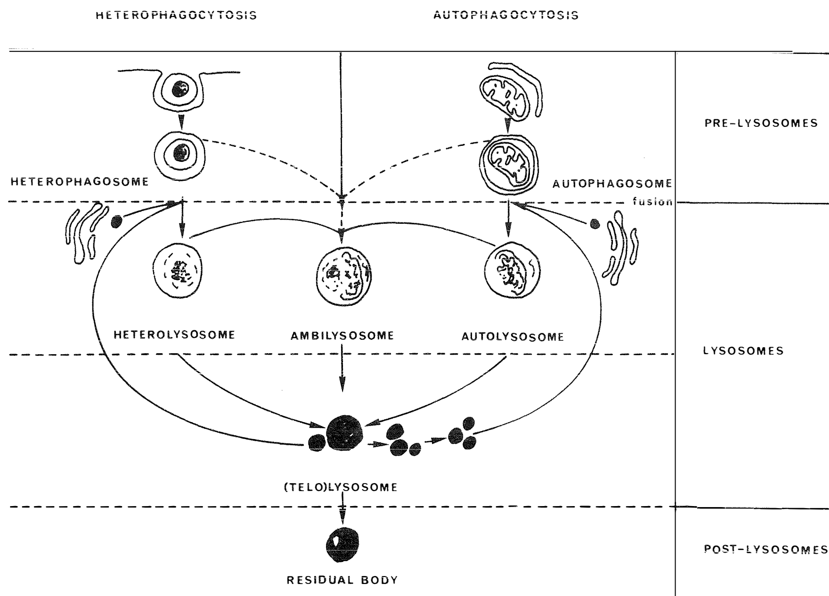
INTRODUCTION

Autophagocytosis

Autophagocytosis is a process in which cells digest their own organelles. Together with heterophagocytosis it forms the lysosomal digestion system of the cell. Novikoff (1959) formulated the concept of autophagocytosis as understood today. He combined de Duve's (1959) idea of lysosomes with the electron microscopic findings of membrane-limited, degenerating cytoplasmic organelles and proposed that these might together accomplish cell autolysis. Hruban *et al.* (1963) conducted the first studies on drug-induced autophagocytosis, using the term "focal cytoplasmic degeneration". Since these findings, autophagocytosis has been observed in various tissues under both physiological and pathological conditions.

Terminology. The most commonly used terminology for the various components of the lysosomal system was proposed by de Duve and Wattiaux (1966): *Pre-lysosomes* are membrane-limited organelles which do not contain lysosomal enzymes. On the other hand they contain material destined to be degraded by lysosomal enzymes. The material can be either of extracellular origin, in which case the structure is termed a *heterophagosome*, or of intracellular origin, in which case the structure is an *autophagosome.* Pre-lysosomes acquire lysosomal enzymes presumably by fusing with *primary* and/or *secondary lysosomes.* Primary lysosomes are thought to be derived from Golgi vesicles containing only lysosomal enzymes. Pre-lysosomes which acquire lysosomal enzymes become secondary lysosomes, either *heterolysosomes* or *autolysosomes.* If hetero- and autolysosomes are fused, they form an *ambilysosome.* During destruction of their contents by hydrolases, hetero-, auto- and ambilysosomes are converted to *(telo)lysosomes.* In *residual bodies* or *post-lysosomes* the degradation of the internal material is incomplete. The term *autophagic vacuole* (AV) refers to both autophagosomes and autolysosomes (de Duve 1963). It is

frequently used when it is not known whether or not lysosomal hydrolases are present. The components of the lysosomal system are presented in Text-fig. 1.



Text-fig. 1. The components of the lysosomal system during pre-lysosomal, lysosomal and post-lysosomal phases.

Pre-lysosomal phase. The origin of the membrane surrounding an AV is still unknown. Two possible sources of AV membrane material have been proposed: 1) Membrane formation by *de novo* synthesis (Ashford & Porter 1962, Pfeifer 1971) or 2) the utilization of pre-existing cytoplasmic membranes. Most previous studies have suggested that the membranes of an AV originate from pre-existing membranes within the cell, most probably from the endoplasmic reticulum (Novikoff *et al.* 1964, Novikoff & Shin 1964, Ericsson *et al.* 1965, Glinsmann & Ericsson 1966, Arstila & Trump

1968, Helminen & Ericsson 1971, Kovács & Réz 1974, McDowell 1974). The utilization of plasma membrane has also been suggested (Quataker 1971).

The transformation from AV without enzyme activity to autolysosomes may occur by fusion with pre-existing lysosomes as has been proposed by de Duve and Wattiaux (1966). Another opinion is that lysosomal hydrolases may be acquired directly from the cavity of the surrounding membranes without a pre-lysosomal phase. According to this theory the membranes of an AV originate from lysosomes (Saito & Ogawa 1974), from a cisterna of endoplasmic reticulum containing lysosomal hydrolases (Novikoff *et al.* 1964, Novikoff & Shin 1964, de Priester 1972) or from Golgi apparatus (Frank & Christensen 1968, Trout & Viles 1979) or Golgi-associated endoplasmic reticulum related to lysosomes (GERL) (Holtzman *et al.* 1967, Paavola 1978b, Trout & Viles 1979).

The existence of a pre-lysosomal phase has, however, been indicated by histochemical studies (Arstila & Trump 1968) and by labelling the pre-existing lysosomes with iron (Deter 1975b).

Beginning of the lysosomal phase. The newly formed AV are limited by smooth, single, double (Arstila & Trump 1968) or multiple (Marzella & Glaumann 1980b) membranes. Some kind of transformation occurs in the membranes of a newly formed, double membrane-limited AV, since its inner membrane appears to thicken shortly after fusion with lysosomes. Later the outer membrane also thickens and then the inner membrane disappears. The disappearance occurs probably as a result of digestion by the lysosomal hydrolases (Arstila & Trump 1968, 1969).

The fusion of AV with lysosomes has been proposed to occur by random collision (Deter 1975a). However, the uneven distribution of lysosomes and AV found in liver parenchymal cells (Ericsson & Glinsmann 1966) and in kidney tubule cells (Pfeifer & Scheller 1975) does not support this hypothesis.

In biochemical studies the free activity of lysosomal hydrolases increases after increased autophagocytosis. The reason is obviously that the newly formed AV are larger than the pre-existing lysosomes, having smaller osmotic resistance and being more easily disrupted during the homogenization (Deter 1971).

Destruction and degradation. The destruction of the engulfed material within AV occurs to a certain extent without lysosomal hydrolases (Arstila *et al.* 1972, Pfeifer 1972, Deter 1975b), but degradation to the molecular level does not occur unless hydrolytic enzymes are present (Tappel 1969, Deter 1975b). The lysosomal hydrolases are capable of degrading proteins, carbohydrates, ribonucleic acids and lipids (Tappel 1969). The proteolysis to the level of amino acids and dipeptides is a rapid process (Coffey & de Duve 1968), whereas lipid degradation occurs more slowly (Glaumann & Trump 1975).

The average half-life of an AV is estimated to 8-9 min in the liver (Mortimore & Schworer 1977, Pfeifer 1978). However, the degradation times of various cytoplasmic components may vary (Glaumann *et al.* 1975a, 1975b, Glaumann & Trump 1975) and probably depend on the physiological or pathological state of the cell.

When the degradation of the engulfed material is not complete, the end product of an AV is called a residual body (de Duve & Wattiaux 1966). In non-dividing cells such as neurons (Ericsson 1969) and cardiac muscle cells (Brandes 1965), these bodies accumulate in the cytoplasm during aging. The aging pigment of human liver is apparently also a result of autophagocytosis (Essner & Novikoff 1960). By contrast, rat hepatocytes are capable of excreting their residual bodies by exocytosis of lysosomal material into the bile canaliculi and the space of Disse (Kerr 1970, Jacques 1975, Marzella & Glaumann 1980b).

Significance in normal and pathologically altered cells. Ericsson (1969) has categorized the occurrence of autophagocytosis in different

physiological and pathological conditions as follows: Autophagocytosis occurs 1) in normal cells; 2) during physiological remodelling of cells as in, e.g., metamorphosis; 3) as a physiological survival mechanism for example during starvation and 4) in response to pathological stimuli such as the administration of high doses of various inhibitors, hormones and other physiologically potent chemicals.

Autophagocytosis is a common phenomenon in most normal cells. However, the number of AV in normal physiological conditions is small. It has been proposed that autophagocytosis represents a mechanism whereby old organelles and portions of cytoplasm are degraded for reutilization within the cell (Ericsson 1969). This hypothesis seems improbable because the newly engulfed mitochondria within AV do not show any morphological or histochemical alterations or other signs of "aging" (Arstila *et al.* 1972).

At present, the significance of autophagocytosis in normal cells is not quite clear. It probably represents an important mechanism for intracellular breakdown of cytoplasmic constituents (Mortimore & Schworer 1977, Pfeifer 1978), possibly contributing about 50 % of the intracellular protein degradation in liver (Scornik & Botbol 1976, Pfeifer 1978). Extra-lysosomal hydrolases are also capable of degrading soluble cytoplasmic material or membrane-bound organelles (Ballard 1977).

Autophagy follows a circadian rhythm for example in rat liver, kidney and pancreas. The volume fraction of AV is lowest during the time of activity and feeding in the night, and appears to be highest during the day at the time of lowest activity (Pfeifer 1976).

De Duve and Wattiaux (1966) have suggested that the segregation of cellular material is a random process and that its control is dependent on "statistical factors". However, it has been shown that segregation of cytoplasmic components is to a certain

extent selective in both physiological (Locke & McMahon 1971, Pfeifer 1978) and pathological conditions (Bolender & Weibel 1973). More quantitative research is needed for the elucidation of this problem.

Increased autophagocytosis represents a common sublethal reaction to a variety of injurious stimuli in the cell. The classification of these stimuli is difficult because very different kinds of pathological stimuli can increase cellular autophagocytosis. For example x-ray irradiation (Hugon & Borgers 1966, Volk *et al.* 1966), hypoxia (Abraham *et al.* 1967), excess of glucagon in the liver (Ashford & Porter 1962, Arstila & Trump 1968), amino-acid deprivation in perfused liver (Mortimore & Schworer 1977) and various toxic chemicals (for a review see Ericsson 1969) all increase cellular autophagocytosis. Most older observations on increased autophagocytosis after injurious stimuli are rather subjective because they are based on qualitative electron microscopical studies. Recently, some quantitative studies on increased autophagocytosis have also been published (Deter 1971, Bolender & Weibel 1973, Mortimore & Schworer 1977, Kovács & Kovács 1980). Stereological methods are needed in order to obtain more knowledge on the mechanism of autophagocytosis.

The mechanism of pathological induction of autophagocytosis is not clear. The best studied is the glucagon model in the liver, in which the inducer of autophagocytosis may be the elevated level of intracellular cyclic adenosine monophosphate (cAMP), which mediates the effect of glucagon in the hepatocytes. Cyclic AMP alone is also capable of inducing autophagocytosis in rat liver (Shelburne *et al.* 1973) and kidney (Pfeifer & Guder 1975). The question to be answered is whether the intracellular mechanism of induction of autophagocytosis is always the same regardless of the nature of the stimulus, or whether there exist different pathways by which different kinds of stimuli induce increased autophagocytosis.

Vinblastine

Vinblastine-induced autophagocytosis. The most widely used model in studies on autophagocytosis is the glucagon-induced autophagocytosis in rat liver (Ashford & Porter 1962, Deter & de Duve 1967, Arstila & Trump 1968, Deter 1971). However, the number of AV formed is relatively small in this model. The microtubule inhibitor vinblastine (VBL) is a more potent inducer of autophagocytosis in the liver than glucagon (Arstila *et al.* 1974, Hirsimäki & Pilström 1979). VBL also induces autophagocytosis in neurons (Terry *et al.* 1970), in pancreatic acinar cells (Kovács *et al.* 1975, Nevalainen 1975), in Ehrlich ascites tumor cells (Hirsimäki *et al.* 1975), and in seminal vesicle cells (Kovács & Kovács 1980). VBL-induced autophagocytosis offers a good model for studying autophagocytosis in pathologically altered cells. The mechanism by which VBL induces autophagocytosis is unknown.

Other effects of vinblastine. VBL is a plant alkaloid which has been isolated from the plant *Vinca rosea* Linn. It is a highly effective oncolytic agent against certain forms of human cancer. Its effects have therefore been well studied. Toxic doses of VBL cause reduction of food intake, loss of weight and leukopenia in mice. The intraperitoneal LD_{50} dose in mouse is 3.2 ± 0.02 mg/kg and the cause of death is bacterial diseases secondary to leukopenia (Johnson *et al.* 1963). VBL arrests cell mitosis at the metaphase. This effect seems to be mediated by a reversible interaction with the subunits of the mitotic spindle (Malawista *et al.* 1968). VBL also causes precipitation of cytoplasmic microtubules, which then form paracrystals in the cytoplasm (Bensch & Malawista 1968, Krishan & Hsu 1971, Bunt 1973, Tyson & Bulger 1973). Intact microtubules are needed in the transport and secretion of the secretory products of various cells, and microtubule disruption prevents for example the transport and secretion of very low density lipoproteins (VLDL) from the liver (Orci *et al.* 1973, Stein & Stein 1973, Reaven & Reaven 1980).

AIM OF THE STUDY

The general aim of this study was to investigate the mechanism of autophagocytosis, especially the AV formation and the intralysosomal degradation of cell organelles in pathologically altered cells. For this purpose autophagocytosis was induced in mouse liver parenchymal cells by intraperitoneal injection of VBL.

The following specific problems were investigated:
1) The qualitative morphological changes occurring in hepatocytes after VBL treatment and the injurious effects of different doses of VBL.
2) The quantitative morphological changes of the volume density of AV, of AV contents and of other cell organelles and hepatocytes after VBL treatment.
3) The effect of increased autophagocytosis on the total and free activities of lysosomal hydrolases and the role of microtubules in the acquisition of acid phosphatase to AV.
4) The origin of AV membranes.
5) Possible similarities in the initiation of autophagocytosis in liver after VBL treatment as compared with some known effects of glucagon.

MATERIALS AND METHODS

Experimental animals

Male NMRI mice, 2-3 months old and weighing 27-34 g, were used in the experiments. The animals were given standard laboratory chow (R_3, Astra-Ewos, Sweden) and tap water, *ad lib.* during the experimental periods unless otherwise specified. The animals lived in controlled relative humidity (40 %) and cage temperature (21-22 ^{o}C) and in a 12-12 h controlled light-dark rhythm (lights on 6 am - 6 pm) at least two weeks before the experiments. All experiments were conducted between 10 am and 2 pm.

470 animals were used for this study.

Vinblastine administration

Vinblastine sulphate (Velbe, E. Lilly & Co., Indianapolis, U.S.A.) was dissolved in 0.9 % saline and injected as a single intraperitoneal dose of 10, 25 or 50 mg/kg body weight (VBL 2, 5 or 10 mg/ml) prior to killing. Control animals were given corresponding volume of saline.

Qualitative microscopy

Light microscopy. Hematoxylin-eosin staining was performed in order to estimate the injurious effects of VBL (10 and 50 mg/kg) on liver. Immediately after killing, pieces of liver were fixed in 10 % formalin in 0.15 M phosphate buffer, pH 7.0. The specimens were embedded in paraffin, sectioned, and the sections were stained with hematoxylin and eosin.

Periodic acid-Schiff (PAS) staining was performed in order to follow the changes in liver glycogen after VBL injection (10 mg/kg) as compared to the control. All the animals starved after VBL or saline injection before killing. Small pieces of liver were removed and frozen. Sections, 10 µm thick, were cut on an Ames Microtome-Cryostat II, preincubated in a mixture of ethanol, chloroform and acetic acid (16:3:1) and stained with the PAS technique.

Electron microscopy. Specimens for electron microscopy were taken simultaneously with those for light microscopy and biochemical assays. Specimens from at least five animals injected with VBL and from corresponding controls were examined fifteen or thirty minutes and one, two, four, twelve or twenty-four hours after the injection. Three fixing techniques were used in routine electron microscopy: In the first technique, small cubes of liver tissue were fixed by immersion in 1 % OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.4, for two hours

at +4°C. After fixation, blocks were stained with 2 % uranyl acetate, dehydrated in an ascending series of ethanol, and embedded in Epon 812. In the second technique, small cubes of liver were fixed by immersion in 3 % distilled glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for four hours either at +4°C or at room temperature (in order to preserve the microtubules), postfixed in 1 % OsO_4 in the same buffer at +4°C for one hour , stained with uranyl acetate, dehydrated, and embedded as described above. In the third technique, small cubes of liver were fixed by immersion at room temperature using the formaldehyde-glutaraldehyde fixative described by Karnovsky (1965) in order to preserve the microtubules and possible paracrystalline inclusions. Thin sections were cut with diamond knives on a Sorval Porter-Blum MT-1 ultramicrotome, stained with uranyl acetate (Watson 1958) and lead citrate (Venable & Coggeshall 1965) and examined with a JEM 100-U electron microscope.

For electron microscopic histochemistry, the animals were killed fifteen minutes (6 animals), thirty minutes (6 animals) and two hours (12 animals) after VBL injection (50 mg/kg) in order to study both the formation of AV and the newly formed AV. Six animals were included in each control group. Three histochemical techniques were used. In the first technique, acid phosphatase activity was localized in order to follow the transformation of non-enzyme containing AV to autolysosomes. Small pieces of liver tissue from the median lobe were fixed with 2 % distilled glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for four hours at +4°C. After fixation, the pieces were washed in the same buffer containing 7.5 % sucrose and frozen in isopentane chilled with liquid nitrogen. Sections, of thickness 40 µm, were cut on an Ames Microtome-Cryostat II. The sections were incubated in Gomori's (1952) lead medium containing 11 ml 0.05 M acetate buffer at pH 5 with 30 mg Na-β-glycerophosphate (final concentration 0.01 M, Sigma) as substrate and also containing 0.1 ml 12 % lead nitrate (final concentration 3.3 mM) for 20-40 min at +37°C as described by

Essner (1973). Control sections were incubated in a medium to which 10^{-2} M NaF had been added or from which the substrate was omitted. The sections were washed in cold 0.1 M sodium cacodylate buffer, pH 7.4, containing 7.5 % sucrose, postfixed in 1 % OsO_4 in the same buffer at +4°C for one hour, stained *en block* with 2 % uranyl acetate, dehydrated and embedded as described above. Sectioning and double staining were performed as already described.

In the second histochemical technique, inosine diphosphatase activity was localized in order to study the origin of the AV membranes. Fixation and sectioning of frozen sections were performed as described for the localization of acid phosphatase activity. The sections were incubated in a medium containing 10 ml 0.2 M Tris-maleate buffer at pH 7.2 and 25 mg Na-inosine diphosphate (Sigma) as substrate, 7 ml distilled water, 5 ml 0.025 M manganese chloride and 3 ml 1 % lead nitrate (final concentration 3.6 mM) (Novikoff & Goldfischer 1961) for 15 min at +37°C as described by Essner (1973). Control sections were incubated in a medium from which the substrate was omitted. After incubation, the further preparation of the specimens for electron microscopy was performed as already described.

In the third histochemical technique, OsO_4 impregnation was used in order to study further the origin of AV membranes. Pieces of liver tissue not more than 1 mm in thickness were immersed in 2 % OsO_4 in distilled water (pH 6.2-6.8) at +40°C for twenty-four hours. The OsO_4 solution was then changed and immersion was continued for twenty hours at +40°C (Friend 1969). The tissue was then drained, treated for one and a half hours with 0.5 % uranyl acetate, dehydrated and embedded in Epon 812. Sectioning and double staining were performed as already described.

Quantitative microscopy

Experimental animals, vinblastine administration and tissue preparation. In this study the VBL dose used was 25 mg/kg. This dose provoked more AV in the cytoplasm than the lowest dose used (10 mg/kg) but was not so toxic to the hepatocytes as the highest dose (50 mg/kg). The VBL was injected to twenty mice, while twenty controls were injected with the corresponding volume of saline. The mice were not fed after the injection and were killed after one, four, twelve or twenty-four hours in order to follow the glycogen disappearance from the hepatocytes. Five animals were included in each test or control group. After killing, fifteen about 1 mm^3 pieces were cut at random from the median liver lobe of each animal. The specimens were immediately fixed by immersion in 1 % OsO_4 buffered with 0.1 M sodium cacodylate, pH 7.4, at +4°C for two hours. Block staining, dehydration and embedding were performed as described in qualitative electron microscopy.

Morphometric studies. Morphometry stage I: 0.5 µm thick sections were cut with glass knives on a LKB Huxley ultramicrotome from five different blocks from each animal. The sections were stained with toluidine blue. From the best section of each block 10 micrographs were taken at random with a light microscope at 1000 × primary magnification. Fifty micrographs were thus taken from each animal. These constituted the morphometry stage I. The micrographs were transferred with mm-calibration scales to diapositive films. Counting was performed by projecting the image on the test screen with the aid of the mm-calibration scale in each slide and on the test screen.

Morphometry stages II and III: From five other blocks from each animal ultrathin sections, having an interference colour of silver to grey (about 600-900 Å thick), were cut on a Sorval Porter-Blum MT-1 ultramicrotome with diamond knives and stained as described in qualitative electron microscopy. In the electron microscope the best section from each ribbon was

selected, from which 8-10 micrographs were taken at random at 5300 × primary magnification. Additional 8-10 micrographs were taken at 10 000 × primary magnification from the same section. Randomization was performed by taking advantage of the holes in the grid. The micrographs at 5300 × magnification were taken from one corner of each grid hole and at 10 000 × magnification from the opposite corner. The micrographs at 5300 × and 10 000 × magnification constituted the morphometry stages II and III, respectively. The micrographs were transferred to diapositive films with mm-calibration scales and the counting procedure was performed as described for stage I.

The morphometric analysis was performed by the point counting method as described for liver by Rohr *et al.* (1976). Stereological estimates were expressed as densities of volume, surface area or number (Weibel & Bolender 1973). These densities are relative measurements and can be related to volume of tissue, cell or cytoplasm. Table 1 lists the compartments and primary parameters determined at the individual magnification levels at stages I, II and III.

The primary data determined at stages II and III were converted into effective values of stage I (reference system liver) by multiplying the corresponding data of the primary parameters by the appropriate conversion factors (Rohr *et al.* 1976).

The test lattices used were a 56-point multipurpose lattice at stage I, a 1:9/121:1089 double-square lattice at stage II (1:9 signifies the ratio of coarse to fine points, 121:1089 the actual number of coarse and fine points) and a 100-point multipurpose lattice at stage III (Weibel & Bolender 1973).

The mean volume ($\bar{V}$) of AV and lysosomes was obtained by dividing the volume density by the number of the corresponding organelles (Weibel 1969).

TABLE 1. Compartments examined in the morphometric study of mouse liver, and primary parameters calculated at morphometry stages I, II and III

Compartments	Primary parameters		
	Stage I x 1 000	Stage II x 5 300	Stage III x 10 000
Extracellular compartments (EC):	V_{VEC}	V_{VEC}	V_{VEC}
Kupffer cells			
Endothelial cells			
Fat storing cells			
Sinusoids			
Blood capillaries			
Bile canaliculi			
Space of Disse			
Liver parenchymal cell (C):	V_{VC}		
Nucleus (NUCL)	V_{VNUCL}, N_{VNUCL}	V_{VNUCL}	V_{VNUCL}
Cytoplasm (CYT)		V_{VCYT}	
Mitochondria (MIT)		V_{VMIT}, N_{VMIT} 1)	V_{VMIT}, S_{VOBM}, S_{VCM}
Microbodies (MB)		V_{VMB}, N_{VMB} 1)	V_{VMB}
Lysosomes (dense bodies) (LY)		V_{VLY}, N_{VLY} 2)	V_{VLY}
Autophagic vacuoles (AV)		V_{VAV_2}, V_{VAV_1}, V_{VRB}, N_{VAV} 2)	V_{VAV}
Golgi apparatus (GO)			V_{VGO}
Fat droplets (FD)		V_{VFD}, N_{VFD} 2)	V_{VFD}
VLDL-vesicles (VLDL)		V_{VVLDL}	V_{VVLDL}
Rough endoplasmic reticulum (RER)			V_{VRER}, S_{VRER}
Smooth endoplasmic reticulum (SER)			V_{VSER}, S_{VSER}
Ribosomes (RI)			V_{VRI}
Glycogen fields (GLYC)			V_{VGLYC}
Ground substance (GS)			V_{VGS}

V_V = volume density, N_V = numerical density, S_V = surface density, OBM = mitochondrial outer boundary membrane, CM = crista membrane

1) $\beta = 1.45$, 2) $\beta = 1.38$ (Rohr *et al.* 1976)

No corrections were made for systematic errors in section thickness or for compression of the tissue by sectioning. In comparative works such corrections are unnecessary since it can be assumed that all experimental groups are affected to the same extent by systematic errors (Stäubli *et al.* 1969).

Evaluation of autophagic vacuoles. The volume density of AV was estimated both as a single group and by dividing them into three different groups representing AV at different stages of development: The first group consisted of obviously newly formed AV which were in general double membrane-limited with clearly recognizable internal material (AV_2). The second group consisted of apparently older single membrane-limited AV with partly digested engulfed material (AV_1). The third group consisted of obviously old AV with unrecognizable contents showing transformation to residual bodies (RB). Examples of the three different types of AV are seen in Fig. 6.

The contents of the AV were analysed at stage II by point counting. For the analyses, the AV were classified into the following groups: 1) AV containing ERGS, comprising endoplasmic reticulum, ribosomes, Golgi complex and cytoplasmic ground substance, 2) AV containing mitochondria, 3) AV containing microbodies, 4) AV containing fat, 5) AV containing VLDL vesicles and 6) AV containing glycogen (Fig. 8). The percentages of the various cytoplasmic components in AV were calculated and compared with the corresponding mean values in the hepatocyte cytoplasm. Double or single membrane-limited AV were included in the evaluation only if their contents could be satisfactorily identified.

Enzyme assays

In these experiments the VBL doses used were 10 mg/kg or 50 mg/kg.

Liver. In the assays of the activities of the lysosomal hydrolytic enzymes the animals were starved overnight before the experiment. Immediately after the mice were killed, the livers were rapidly removed, weighed, and a 10 % (w/v) homogenate was made in ice-cold 0.25 M sucrose using a Potter Elvehjem homogenizer with a teflon pestle rotating at about 2000 rpm with three complete strokes. This homogenate was used for the estimations of the activities of lysosomal enzymes. Simultaneously, samples were taken for protein estimations.

The total activities of lysosomal enzymes were estimated from homogenates treated with 0.1 % Triton X-100 (final concentration) for 20 min before the analyses. The activities of lysosomal enzymes not bound to particles (free activity) were estimated after centrifugation of the homogenates in a MSE superspeed 50 ultracentrifuge for one hour at 100 000 g with an 8 × 25 ml rotor. The supernatant was used for the enzyme analyses.

Acid phosphatase (EC 3.1.3.2) activity was assayed by incubating homogenate in 0.1 M sodium acetate buffer, pH 5.0, at +37°C using 10 mM (final concentration) β-DL-glycerophosphate (Sigma), as substrate. After 30 min incubation, the reaction was stopped by adding 10 % trichloroacetic acid (Barrett 1972). After the precipitate had been allowed to flocculate 15 min at ±0°C, the mixture was centrifuged and the supernatant was used for the determination of inorganic phosphate according to the method described by Fiske and Subbarow (1925) at 625 nm.

β-Galactosidase (EC 3.2.1.23) activity was assayed in 0.1 M sodium citrate buffer, pH 4.2, at +37°C with 5 mM (final concentration) p-nitrophenyl-β-D-galactopyranoside (Sigma), as substrate. The enzyme activity was measured as liberation of p-nitrophenol from the substrate. The reaction was stopped after 30 min by adding 3.3 % trichloroacetic acid. The mixture was incubated for a further 5 min, centrifuged, and the supernatant was removed and mixed with 0.5 M bicarbonate-carbonate

buffer, pH 10.0. The extinction of this mixture was measured at 420 nm (Barrett 1972).

β-N-acetylglucosaminidase (EC 3.2.1.30) activity was assayed in 0.1 M sodium citrate buffer, pH 4.8, using 5 mM (final concentration) p-nitrophenyl-N-acetyl-β-glucosaminide (Sigma), as substrate. The procedure was similar to that in the β-galactosidase activity assay, except that the incubation time was 10 min (Barrett 1972).

Glycogen phosphorylase a activity (EC 2.4.1.1) was determined by measurement of the rate of liberation of inorganic phosphate from glucose 1-phosphate in the presence of glycogen, i.e. in the direction of synthesis of glycogen (Sutherland 1968, Stalmans & Hers 1975). Pieces of liver were rapidly removed and weighed and a 10 % (w/v) homogenate was made in ice-cold 0.1 M glycylglycine buffer, pH 7.4, also containing 0.15 M NaF in order to inhibit phosphorylase phosphatase activity. Homogenization was performed using a Potter-Elvehjem homogenizer with a teflon pestle. To a sample of the homogenate NaF (final concentration 0.15 M) and caffeine (final concentration 0.5 mM; inhibits phosphorylase b activity) were added. The reaction was started by adding the substrate solution, pH 6.5, which contained 1 % glycogen, 50 mM glucose 1-phosphate and 0.15 M NaF. The incubation was performed at $+37^{o}C$ for 10 min and the reaction was stopped by adding ice-cold 10 % trichloroacetic acid. The mixture was centrifuged and the supernatant was used for the determination of inorganic phosphate (Fiske & Subbarow 1925) at 625 nm.

Serum. Blood samples were taken by decapitation. The activities of acid phosphatase, β-galactosidase and β-N-acetylglucosaminidase in serum were assayed using the same methods as those described above. The sera were diluted to 10 % with 0.9 % saline before the assays. The incubation times were 10 min for β-N-acetylglucosaminidase and one hour for acid phosphatase and β-galactosidase.

Serum creatine phosphokinase (CPK, EC 2.7.3.2) activity was assayed using the reagents and instructions of Boehringer GmbH, Mannheim, FRG. The results are expressed as milli-international CPK units per ml serum (Oliver 1955).

Serum L-aspartate-2-oxogulatarate aminotransferase (GOT, EC 2.6.1.1) activity was assayed with the reagents and instructions of BDH Chemicals Ltd, England. The results are expressed as milli-international GOT units per ml serum (King & Campbell 1961).

Other biochemical methods

In these experiments the VBL doses used were the same (10 and 50 mg/kg) as in the enzyme assays.

Blood glucose. The experimental animals were divided into two groups for blood glucose estimations. The first group had free access to food during the entire experimental period. The second group was starved overnight before the blood samples were taken. Blood glucose was determined by the o-toluidine method as described by Hyvärinen (1972).

Liver glycogen. For liver glycogen estimations the experimental animals were divided into three groups. The first group had free access to food during the entire experimental period, the second was starved after the injection with VBL or saline, and the third was starved overnight prior to the injection. Small 30-40 mg pieces from the median liver lobe were rapidly removed, weighed, and glycogen was estimated using anthrone reagent as described by Brixová & Dzúriková (1972).

Cyclic AMP. For cAMP estimations, liver samples were taken from animals feeding *ad lib.* throughout the experimental periods. Cyclic AMP was determined by the binding of (^{3}H)-cAMP to a cAMP-dependent protein kinase as described by Gilman (1970), using the reagents and instructions of Boehringer GmbH,

Mannheim, FRG. Tissue extracts were prepared as follows: The median lobe of the liver was rapidly removed and frozen in isopentane chilled with liquid nitrogen. About 50 mg of tissue was weighed and homogenized with 1 ml 5 % (w/v) trichloroacetic acid in a pre-cooled mortar. The insoluble residues were centrifuged off at 3000 rpm for 10 min. HCl was added to the supernatant to obtain a final concentration of 0.05 M. The trichloroacetic acid was removed by extracting four times with 8 volumes of diethylether saturated with water. The ether was removed in a rotary evaporator and the precipitate was dissolved in 1 ml of water for determination of cAMP. Radioactivity was determined with an LKB 81 000 (Wallac) liquid scintillation counter.

Protein estimation. The protein contents of the liver homogenate samples were estimated by the method of Lowry *et al.* (1951).

Statistical methods

Standard methods were used to calculate means, standard errors and standard deviations with a Monroe 1930 electronic display calculator. Student's t-test was used for testing of the significance between the means.

RESULTS

Effects of vinblastine on the experimental animals

Shortly after VBL injection (10 mg/kg) in the experimental animals developed diarrhea. The body temperature fell about 3 ^{o}C within two hours to 35.0 ± 0.2 ^{o}C as compared to 38.1 ± 0.1 ^{o}C in the controls (n = 20). In addition, the animals were eating poorly. Their body weight decreased about 1 g within two hours, being 31.8 ± 0.6 g before and 30.7 ± 0.8 g two hours after the injection, probably due to dehydration. In spite of the fact that the VBL doses used in the experiments were higher than the LD_{50} dose, all animals survived the experimental period.

Qualitative morphological changes after vinblastine injection

Liver tissue. Hematoxylin and eosin staining: Four hours after injection with the lowest dose of VBL (10 mg/kg) very few changes were observed in the liver lobules (Fig. 1a) as compared to the controls. In a very few cases slight vacuolation and granulation of the parenchymal cells were observed. In some cases there was also a slight mesenchymal reaction and some diffusely scattered cells were observed in the tissue. With the highest VBL dose (50 mg/kg) all the parenchymal cells were vacuolated and some were also necrotic, while inflammatory cells and mesenchymal reaction were observed in the liver lobules (Fig. 1c). There also appeared to be an accumulation of fat droplets, particularly in areas near the central veins, which were dilated.

Twelve hours after VBL injection with the lowest dose, the vacuolation was prominent in all the cells. There were also a few necrotic cells with pycnotic nuclei scattered throughout the liver lobules. The mesenchymal reaction and the inflammatory cells observed four hours after injection had almost disappeared (Fig. 1b). With the highest dose, the changes were similar to those found after four hours with the same dose except for the occurrence of small, focal necrotic areas diffusely scattered in the liver lobules (Fig. 1d). Pycnotic nuclei and sometimes karyolysis were observed in the necrotic cells.

Vinblastine-induced autophagocytosis. A normal ultrastructure was observed in the hepatocytes of the control animals (Fig. 3). AV were seen only occasionally and microtubules were rare, occurring most frequently near the Golgi apparatus (Fig. 4) and the bile canaliculi.

The injection of VBL provoked a wave of autophagocytosis within one hour with all the doses used (Figs. 5 and 6). Four hours after injection the number of AV seemed to be much greater

(Fig. 7), varying however in different cells. AV were seen everywhere in the cytoplasm, although they were more numerous in the vicinity of the Golgi apparatus and the bile canaliculi. The two highest doses of VBL seemed to provoke more AV than the lowest dose. Some AV were limited by smooth, double or only occasionally by multiple membranes containing clearly recognizable engulfed material (Figs. 6 and 7). These were the newly formed AV. Their contents were variable, representing all cytoplasmic components such as ERGS, mitochondria, microbodies, fat droplets, vesicles containing VLDL, glycogen etc. (Fig. 8). Nuclear material was never seen in AV. In addition, there were many, probably older AV, limited by a single membrane, with engulfed material at different stages of degradation (Figs. 6, 7 and 10).

Twelve hours after VBL injection with the lowest dose the number of AV had decreased remarkably from the number observed after four hours. In many parenchymal cells, no AV were observed at all (Fig. 9). With the highest VBL dose there were still numerous AV in the cytoplasm, most of which were limited by a single membrane or resembled residual bodies (Fig. 10). In some cases, material resembling that found in AV seemed to be present in the space of Disse and in the sinusoidal lumen. Similar material was sometimes seen in Kupffer cell phagosomes (Fig. 11).

Twenty-four hours after injection with the two highest doses of VBL there were still many AV in the cytoplasm. The appearance of the AV was the same as twelve hours after the injection.

Other effects of vinblastine on hepatocytes. In addition to increased autophagocytosis, other ultrastructural changes were also observed in the hepatocytes after VBL injection. The most prominent of these was the apparent accumulation of secretory vesicles, containing VLDL, in the cytoplasm (Figs. 10 and 12). Their number appeared to be dose-dependent. The Golgi cisternae also appeared to be dilated and disorganized after VBL injection,

especially with the two highest doses. The Golgi cisternae usually contained a secretory product, apparently VLDL (Figs. 10 and 13). The microtubules normally observed in the cytoplasm had disappeared after VBL injection with all the doses used. Paracrystalline inclusions were never observed after VBL injection. The cisternae of the endoplasmic reticulum seemed to be sometimes partly vesiculated, especially with the highest dose of VBL (Figs. 7 and 13). Fat accumulation in the cytoplasm was observed in both the control and the VBL treated animals (Fig. 10).

Nuclei, mitochondria and microbodies had a normal ultrastructure except in a few cells in livers of animals treated with the two highest VBL doses, in which different stages of cellular necrosis were observed.

Origin of autophagic vacuole membranes. Osmium impregnation: The distribution of osmium deposits in parenchymal cells after VBL or saline injection is shown in Table 2. No differences were observed in stain distribution between the test and control groups. In almost all cells the outermost cisternae on the forming face of the Golgi apparatus and some adjacent vesicles were evenly stained (Fig. 14). After VBL injection, the cisternae were extensively dilated and the staining pattern became therefore less distinct (Figs. 15 and 16b). The heavy homogeneous staining of the cavity between the two membranes surrounding an apparently newly formed AV was a common staining pattern (Figs. 14 and 16b), as was also the staining of the cavity between the membranes of partly open vacuoles (Fig. 16a). In a few cases the cisterna surrounding an AV remained unstained (Fig. 15). In some AV the stain was scattered in the matrix (Fig. 15).

Some unidentified vesicles were heavily stained in the cytoplasm of all cells (Figs. 15 and 16). The vesicles within multivesicular bodies were also stained (Fig. 15). Staining of the endoplasmic reticulum varied from cell to cell. The

TABLE 2. Osmium impregnation and cytochemical distribution of acid phosphatase and inosine diphosphatase in mouse liver parenchymal cells after injection of VBL (50 mg/kg) or 0.9 % saline. (From the paper of Hirsimäki, P. & Reunanen, H. 1980: Studies on vinblastine-induced autophagocytosis in mouse liver. II. Origin of membranes and acquisition of acid phosphatase. - Histochemistry 67: 139-153. By permission of Springer-Verlag)

	Osmium impregnation	Acid phosphatase activity	Inosine diphosphatase activity
Lysosomes	-	+++	-
AV	++	++	-
Golgi cisternae, forming face	+++	-	-
Golgi cisternae, maturing face	-	+	+
Golgi vesicles and vacuoles	++	++	-
Cytoplasmic vesicles	++	-	-
Nuclear envelope	+	-	-
ER	++	-	+++
Mitochondria	-	-	-
Microbodies	-	-	-

AV Autophagic vacuole
ER Endoplasmic reticulum
+++ Stained in all cells
++ Stained in most cells
+ Stained in a few cells
- Not stained

most common pattern was unstained or negligibly stained smooth and rough surfaced endoplasmic reticulum (Fig. 17a). In a few cells the endoplasmic reticulum was stained extensively black (Fig. 17b). In the stained cells the endoplasmic reticulum was often dilated and the mitochondria were swollen (Figs. 17a and b). The nuclear envelope was only occasionally lightly stained. The deposits within the cisternae of the endoplasmic reticulum and the nuclear envelope were mostly coarse and granular.

Localization of inosine diphosphatase activity: Inosine diphosphatase activity was localized in the rough and smooth surfaced endoplasmic reticulum of the parenchymal cells in both saline and VBL treated mice (Table 2). Enzyme reaction product was present within the cisternae and on the membranes of rough and smooth surfaced endoplasmic reticulum (Figs. 18a, b and c). The membranes enclosing AV did not show any inosine diphosphatase activity (Figs. 18a, b and c). Weak enzyme activity was sometimes observed in the engulfed membranes within the AV (Fig. 18b). In a few cases, the innermost cisterna on the maturing face of the Golgi apparatus showed inosine diphosphatase activity.

Acquisition of acid phosphatase. The hepatocytes of both VBL treated and control mice showed a similar cytochemical staining pattern with the Gomori method (Table 2). Weak lead deposits were occasionally observed in the innermost cisternae on the maturing face of the Golgi apparatus, in some adjacent vesicles and in the lysosomes of the parenchymal cells. The amount of GERL membranes associated with the maturing face of the Golgi apparatus appeared to be very small and the membranes were only occasionally acid phosphatase positive. There were three types of AV discernable after VBL injection: One group, showing no enzyme activity at all, was limited by a double membrane and the engulfed material was clearly recognizable (Fig. 19a). The second group of AV was limited by a double membrane and the reaction product of acid phosphatase activity was localized in the space between the membranes (Figs. 19a and b). The third group was limited by a single membrane, and the lead deposits were scattered throughout the matrix of these AV (Fig. 19c). In a few cases, these AV had no acid phosphatase activity. Fusion of AV with primary or secondary lysosomes was difficult to discern (Fig. 19a). The cisternae of the rough and smooth surfaced endoplasmic reticulum did not show any acid phosphatase activity.

TABLE 3. Combined results of morphometry stages I, II and III in mouse liver 1, 4, 12 and 24 hours after VBL injection (25 mg/kg) as compared to controls

Symbol of parameter	Dimension	Treatment	Time since injection (h) 1	4	12	24
V_{VEC}	$\mu m^3/\mu m^3$	VBL	0.163±0.006	0.221±0.008	0.218±0.013	0.241±0.008
		Saline	0.164±0.007	0.222±0.013	0.182±0.011	0.258±0.012
V_{VNUCL}	$\mu m^3/\mu m^3$	VBL	0.055±0.003	0.058±0.002	0.061±0.001	0.060±0.002
		Saline	0.061±0.003	0.058±0.003	0.056±0.002	0.060±0.002
V_{VCYT}	$\mu m^3/\mu m^3$	VBL	0.782±0.006	0.721±0.007	0.721±0.013	0.699±0.005
		Saline	0.775±0.003	0.720±0.017	0.762±0.005	0.682±0.008
V_{VMIT}	$\mu m^3/\mu m^3$	VBL	0.147±0.008	0.136±0.004	0.137±0.007	0.135±0.004
		Saline	0.160±0.007	0.132±0.006	0.154±0.003	0.131±0.003
V_{VMB}	$\mu m^3/\mu m^3$	VBL	0.010±0.001	0.010±0.001	0.010±0.001	0.009±0.001
		Saline	0.014±0.001	0.010±0.001	0.013±0.001	0.007±0.001
V_{VLY}	$\mu m^3/\mu m^3$	VBL	0.011±0.001***	0.014±0.001*	0.014±0.002**	0.013±0.002
		Saline	0.006±0.001	0.010±0.001	0.007±0.001	0.009±0.001
V_{VAV}	$\mu m^3/\mu m^3$	VBL	0.028±0.004**	0.032±0.001***	0.025±0.006*	0.029±0.004**
		Saline	0.006±0.001	0.006±0.001	0.003±0.001	0.002±0.001
V_{VGO}	$\mu m^3/\mu m^3$	VBL	0.005±0.001	0.003±0.001	0.001±0.001*	0.004±0.001
		Saline	0.008±0.001	0.005±0.001	0.005±0.001	0.004±0.001
V_{VFD}	$\mu m^3/\mu m^3$	VBL	0.008±0.026	0.012±0.004	0.055±0.008	0.078±0.010***
		Saline	0.005±0.001	0.017±0.002	0.088±0.012	0.142±0.006
V_{VVLDL}	$\mu m^3/\mu m^3$	VBL	0.021±0.003**	0.031±0.004**	0.027±0.007*	0.007±0.002*
		Saline	0.004±0.001	0.001±0.001	0.002±0.001	0.002±0.001
V_{VRER}	$\mu m^3/\mu m^3$	VBL	0.174±0.009	0.199±0.008	0.157±0.011	0.146±0.008
		Saline	0.217±0.026	0.195±0.007	0.200±0.014	0.175±0.017
V_{VSER}	$\mu m^3/\mu m^3$	VBL	0.096±0.007	0.101±0.005	0.058±0.011	0.071±0.005
		Saline	0.096±0.011	0.137±0.012	0.052±0.005	0.069±0.005
V_{VRI}	$\mu m^3/\mu m^3$	VBL	0.070±0.005	0.028±0.002**	0.068±0.001	0.048±0.005
		Saline	0.082±0.010	0.046±0.004	0.078±0.006	0.030±0.007
V_{VGLYC}	$\mu m^3/\mu m^3$	VBL	0.006±0.003*	0.004±0.001*		
		Saline	0.016±0.001	0.011±0.002	0.001±0.000	
V_{VGS}	$\mu m^3/\mu m^3$	VBL	0.206±0.010	0.151±0.007	0.169±0.011	0.150±0.005*
		Saline	0.161±0.023	0.150±0.007	0.160±0.006	0.111±0.008

Results are expressed as volume densities (V_V) per liver tissue. For symbols see Table 1. Values given are the means from five animals ± standard error. Levels of significance as compared to control:

* $p < 0.050$
** $p < 0.010$
*** $p < 0.001$

Quantitative morphological changes after vinblastine injection

Liver tissue. No changes were observed in the volume densities of the extracellular compartments or in the volume densities of the hepatocyte nuclei or cytoplasm after VBL injection as compared to the respective controls (Table 3). The numerical densities of hepatocyte nuclei were also unchanged during the entire experimental period (Table 4).

TABLE 4. Numerical densities (N_V) of mouse hepatocyte nuclei (NUCL) in liver tissue, mitochondria (MIT), microbodies (MB), lysosomes (LY), autophagic vacuoles (AV) and fat droplets (FD) per μm^3 hepatocyte, and mean volume ($\bar{V}$) of hepatocyte lysosomes and autophagic vacuoles 1, 4, 12 and 24 hours after VBL injection (25 mg/kg) as compared to controls

Symbol of parameter	Dimension	Treatment	Time since injection (h)			
			1	4	12	24
N_{VNUCL}	$10^6 \cdot /cm^3$	VBL	145.2±21.7	149.4±18.1	138.0± 8.8	143.8±11.3
		Saline	147.1±10.5	159.9±15.3	156.3±12.4	141.9±13.5
N_{VMIT}	$/\mu m^3$	VBL	0.279±0.037	0.310±0.045	0.286±0.036	0.323±0.025
		Saline	0.309±0.022	0.434±0.038	0.294±0.031	0.273±0.033
N_{VMB}	$/\mu m^3$	VBL	0.123±0.012	0.085±0.010	0.090±0.009	0.096±0.015
		Saline	0.136±0.010	0.082±0.002	0.102±0.009	0.127±0.010
N_{VLY}	$/\mu m^3$	VBL	0.265±0.012	0.218±0.026	0.355±0.060	0.350±0.038*
		Saline	0.213±0.039	0.219±0.017	0.268±0.033	0.221±0.027
$\bar{V}_{LY}$	μm^3	VBL	0.042±0.006	0.064±0.008	0.039±0.003	0.037±0.011
		Saline	0.028±0.050	0.046±0.005	0.026±0.004	0.041±0.004
N_{VAV}	$/\mu m^3$	VBL	0.040±0.007	0.063±0.014*	0.070±0.0001**	0.051±0.007**
		Saline	0.020±0.002	0.012±0.001	0.015±0.007	0.009±0.002
V_{AV}	μm^3	VBL	0.70 ±0.128*	0.508±0.152	0.357±0.074	0.569±0.059
		Saline	0.30 ±0.093	0.50 ±0.089	0.20 ±0.142	0.222±0.156
N_{VFD}	$/\mu m^3$	VBL	0.017±0.003	0.042±0.008	0.129±0.023	0.166±0.022
		Saline	0.017±0.005	0.051±0.005	0.117±0.013	0.231±0.034

Values given are the mean values from five animals ± standard error. Levels of significance compared to control:

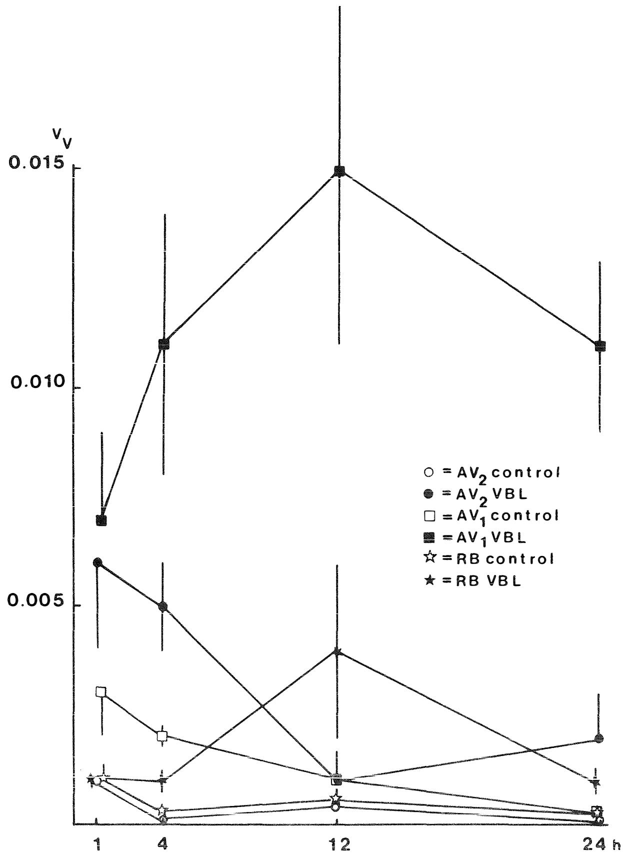
* $p < 0.050$
** $p < 0.010$

Vinblastine-induced autophagocytosis. The combined results of various primary parameters at morphometry stages I, II and III are presented in Table 3. A remarkable change occurred in the volume

densities of the lysosomal system including the AV, the lysosomes and the Golgi apparatus after VBL injection. The volume density of the AV increased during the entire experimental period (Table 3), being 4.7 times greater one hour ($p < 0.010$), 5.3 times greater four hours ($p < 0.001$), 8.3 times greater twelve hours ($p < 0.025$) and 14.5 times greater twenty-four hours after injection ($p < 0.005$) than in the corresponding controls. This increase of the volume densities of the AV was to the greatest extent due to the increase in their number, which was two times greater one hour, 5.3 times greater four hours ($p < 0.025$), 4.7 times greater twelve hours ($p < 0.005$) and 5.7 times greater twenty-four hours after VBL injection ($p < 0.010$) than in the controls (Table 4). One hour after VBL injection the mean volume of the AV was 2.3 times greater ($p < 0.050$) than the control value (Table 4).

The appearance of the AV at different stages of development during the experiment is presented in Text-fig. 2. The volume density of the single membrane-limited AV_1 was the greatest of the three types of AV throughout the experimental period in VBL treated mice. Their volume density differed significantly from the volume densities of the RB one hour ($p < 0.050$) and four hours ($p < 0.025$) after VBL injection, and from the AV_2 and RB twelve hours ($p < 0.050$ and $p < 0.050$ respectively) and twenty-four hours ($p < 0.005$ and $p < 0.005$ respectively) after injection. The volume density of the AV_2 was at its greatest at the beginning of the experiment.

The percentages of the contents of the AV in the VBL injected mice are compared with the percentages of the corresponding cytoplasmic compartments in Table 5. There were only a few significant changes regarding the contents of the AV as compared to the corresponding cytoplasmic compartments: The percentage of vesicles containing VLDL particles was higher ($p < 0.010$) in the cytoplasm one hour after VBL injection than in AV, while the amount of fat in AV was lower twenty-four hours ($p < 0.010$) after VBL injection as compared to the cytoplasmic fat.



Text-fig. 2. Volume densities (V_V; $\mu m^3/\mu m^3$) of autophagic vacuoles (AV) at various stages of development in mouse hepatocytes after VBL injection (25 mg/kg) as compared to controls. AV_2: An AV surrounded by a double membrane. AV_1: An AV surrounded by a single membrane. RB: residual body. Each symbol is the mean value from five animals and the bars indicate the standard error.

Other effects of vinblastine on hepatocytes. The volume density of lysosomes (dense bodies) was 1.8 times greater one hour ($p < 0.001$), 1.4 times greater four hours ($p < 0.050$) and 2 times greater twelve hours ($p < 0.010$) after VBL injection than in the corresponding controls (Table 3). However, their number was only slightly but not significantly greater from the corresponding controls until twenty-four hours after VBL injection when it was significantly increased ($p < 0.050$). No differences were observed in the mean volumes of the lysosomes in the VBL injected animals (Table 4).

The volume density of the Golgi apparatus was significantly smaller ($p < 0.025$) twelve hours after VBL injection than in the control (Table 3).

The volume densities of the vesicles containing VLDL particles were greater in the VBL injected mice during the entire experimental period: 5.3 times greater one hour ($p < 0.005$), 31 times greater four hours ($p < 0.005$), 13.5 times greater twelve hours ($p < 0.025$) and 3.5 times greater twenty-four hours ($p < 0.050$) after injection (Table 3) than in the controls.

The glycogen granules disappeared from the hepatocytes of both the control and VBL injected mice during the experimental period. The disappearance was faster in the VBL injected mice, the volume densities of the glycogen areas being smaller one hour ($p < 0.050$) and four hours ($p < 0.050$) after injection than in the controls. Twelve hours after injection, no glycogen could be discerned in the hepatocytes of the VBL injected mice (Table 3). In several sections, glycogen was stained weakly, interfering the estimation of glycogen areas.

There were some single significant changes in the volume densities of certain cytoplasmic compartments during the experimental period: The volume density of fat droplets was significantly smaller twenty-four hours after VBL injection ($p < 0.001$), the volume density of ribosomes was significantly smaller four

TABLE 5. The percentages of the volume densities (V_V) of cytoplasmic compartments in hepatocyte cytoplasm and in autophagic vacuoles (AV) 1, 4, 12 and 24 hours after VBL injection (25 mg/kg)

Cytoplasmic compartments	1 h		4 h		12 h		24 h	
	V_V cytoplasm	V_V in AV	V_V in cytoplasm	V_V in AV	V_V in cytoplasm	V_V in AV	V_V in cytoplasm	V_V in AV
Mitochondria	19.49±1.10	20.17±2.99	19.74±0.57	17.28±3.46	19.68±1.03	11.24±7.77	20.15±0.59	19.36±8.43
Microbodies	1.33±0.15	1.97±0.51	1.45±0.21	2.32±0.57	1.44±0.19	1.66±1.66	1.34±0.15	2.83±2.05
Fat droplets	1.06±0.35	0.99±0.50	1.74±0.61	1.80±0.78	7.90±1.17	2.74±2.04	11.65±1.64	3.80±1.61**
VLDL-vesicles	2.79±0.35	0.86±0.41**	4.50±0.58	3.68±0.83	3.88±1.02	1.60±1.01	1.04±0.29	3.36±1.84
Glycogen	0.80±0.46	3.37±2.22	0.58±0.15	0.52±0.47	-	-	-	-
ERGS	74.53±4.41	72.64±3.80	71.99±1.97	74.40±3.83	67.10±5.23	82.76±9.71	65.82±3.88	70.65±8.86
Total (%)	100	100	100	100	100	100	100	100

Values given are means for five animals ± standard error. Level of significance compared to corresponding cytoplasmic value:
** $p < 0.010$

hours after VBL injection ($p < 0.010$) and the volume density of ground substance was significantly greater twenty-four hours after VBL injection ($p < 0.025$) than in the corresponding controls (Table 3).

The volume densities of mitochondria, microbodies and rough surfaced and smooth surfaced endoplasmic reticulum after VBL injection did not differ significantly from the corresponding control values (Table 3).

The surface density of the rough surfaced endoplasmic reticulum was significantly greater twelve hours after VBL injection ($p < 0.050$) than in the control. The surface density of the smooth surfaced endoplasmic reticulum was significantly smaller ($p < 0.025$) four hours after VBL injection than in the corresponding control (Table 6). The surface density of mitochondrial outer boundary membrane was significantly smaller one hour after VBL injection ($p < 0.050$) than in the control. The surface densities of the crista membranes or the inner mitochondrial membranes did not differ significantly from the corresponding control values (Table 6).

TABLE 6. Surface densities (S_V) of rough surfaced (RER) and smooth surfaced (SER) endoplasmic reticulum, mitochondrial outer boundary membranes (OBM), inner mitochondrial membranes (IMM) and crista membranes (CM) in mouse hepatocytes 1, 4, 12 and 24 hours after VBL injection (25 mg/kg) as compared to controls

Symbol of parameter	Dimension	Treatment	Time since injection (h) 1	4	12	24
S_{VRER}	$\mu m^2/\mu m^3$	VBL	3.280±0.150	2.795±0.088	4.028±0.240*	3.130±0.145
		Saline	3.977±0.370	3.096±0.107	3.110±0.249	3.075±0.152
S_{VSER}	$\mu m^2/\mu m^3$	VBL	1.436±0.070	1.262±0.059*	1.315±0.325	0.955±0.079
		Saline	1.627±0.170	1.905±0.162	0.924±0.151	1.163±0.073
S_{VOBM}	$\mu m^2/\mu m^3$	VBL	1.049±0.065*	1.040±0.066	1.237±0.073	1.062±0.049
		Saline	1.301±0.051	1.118±0.042	1.047±0.102	1.051±0.032
S_{VIMM}	$\mu m^2/\mu m^3$	VBL	5.043±0.662	4.450±0.227	5.367±0.219	4.637±0.358
		Saline	5.414±0.235	4.471±0.178	4.509±0.431	4.580±0.107
S_{VCM}	$\mu m^2/\mu m^3$	VBL	3.995±0.673	3.410±0.172	4.130±0.169	3.575±0.310
		Saline	4.113±0.228	3.353±0.143	3.463±0.331	3.584±0.073

Results are expressed as surface densities per μm^3 liver tissue. Values given are the means from five animals ± standard error. Levels of significance as compared to control:
* $p < 0.050$

Lysosomal hydrolase activities in liver

The activities of the three hepatic lysosomal hydrolases four and twelve hours after VBL injection are presented in Tables 7, 8 and 9. As indicated in the tables, there were no significant changes in the total activities of either acid phosphatase (Table 7), β-galactosidase (Table 8) or β-N-acetylglucosaminidase (Table 9) in liver homogenates after injection with two doses (10 mg/kg and 50 mg/kg) of VBL. The free activities of the enzymes did not differ significantly from the control values until twelve hours after injection with the highest VBL dose, when the free activity of acid phosphatase was significantly lower ($p < 0.005$) than that of the controls (Table 7). By contrast, the free activity of β-N-acetylglucosaminidase was increased significantly ($p < 0.001$) as compared to the control value (Table 9).

TABLE 7. Acid phosphatase activity in mouse liver homogenate (total activity) and supernatant (100,000 xg 1 h, free activity) after VBL injection. (From the paper of Hirsimäki, P., Trump, B.F. & Arstila, A.U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

VBL dose	Time since injection (h)	Treatment	n	Total activity (nmoles/min/mg prot.)	Free activity (nmoles/min/mg prot.)
10 mg/kg	4	VBL	8	5.76±0.43	
		Saline	8	6.37±0.43	
	12	VBL	8	5.18±0.43	
		Saline	8	5.59±0.42	
50 mg/kg	4	VBL	12	5.10±0.23	0.81±0.04
		Saline	12	5.38±0.24	0.89±0.04
	12	VBL	12	5.33±0.63	0.81±0.03**
		Saline	12	6.23±0.43	0.98±0.03

Results are expressed as nmoles liberated inorganic phosphate/min/mg protein. Values given are means ± standard error. Levels of significance as compared to control:
** $p < 0.010$

TABLE 8. β-Galactosidase activity in mouse liver homogenate (total activity) and supernatant (100,000 xg 1 h, free activity) after VBL injection. (From the paper of Hirsimäki, P., Trump, B.F. & Arstila, A.U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

VBL dose	Time since injection (h)	Treatment	n	Total activity (nmoles/min/mg prot.)	Free activity (nmoles/min/mg prot.)
10 mg/kg	4	VBL	12	0.98±0.09	
		Saline	12	1.00±0.11	
		VBL	9	0.92±0.09	
		Saline	9	0.96±0.07	
50 mg/kg	4	VBL	12	1.03±0.12	0.06±0.01
		Saline	12	1.23±0.17	0.04±0.01
	12	VBL	11	0.88±0.12	0.06±0.01
		Saline	11	1.07±0.15	0.05±0.01

Results are expressed as nmoles liberated p-nitrophenol/min/mg protein. Values given are means ± standard error.

TABLE 9. β-N-Acetylglucosaminidase activity in mouse liver homogenate (total activity) and supernatant (100,000 xg 1 h, free activity) after VBL injection. (From the paper of Hirsimäki, P., Trump, B.F. & Arstila, A.U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

VBL dose	Time since injection (h)	Treatment	n	Total activity (nmoles/min/mg prot.)	Free activity (nmoles/min/mg prot.)
10 mg/kg	4	VBL	12	16.51±1.58	
		Saline	10	14.22±1.20	
	12	VBL	12	13.14±0.80	
		Saline	12	14.30±0.68	
50 mg/kg	4	VBL	12	13.56±1.49	0.19±0.02
		Saline	12	11.98±1.29	0.15±0.02
	12	VBL	12	16.04±1.23	0.30±0.02***
		Saline	12	14.53±0.53	0.18±0.02

Results are expressed as nmoles liberated p-nitrophenol/min/mg protein. Values given are means ± standard error. Levels of significance as compared to control:
*** $p < 0.001$

Enzyme activities in serum

The activities of the three lysosomal hydrolases in serum four and twelve hours after VBL injection are presented in Table 10. There were no changes in the enzyme activities after injection with the lowest VBL dose. However, with the highest VBL dose the activities of the three enzymes were significantly increased after injection as compared to the controls. Acid phosphatase activity was significantly increased four hours ($p < 0.025$) and twelve hours ($p < 0.005$) after injection, while β-galactosidase and β-N-acetylglucosaminidase activities were significantly increased only twelve hours after injection ($p < 0.010$ and $p < 0.010$, respectively).

TABLE 10. Acid hydrolase activities in mouse serum after VBL injection. (From the paper of Hirsimäki, P., Trump, B.F. & Arstila, A.U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

VBL dose	Time since injection (h)	Treatment	n	Acid phosphatase (nmoles/min/ml)	β-Galactosidase (nmoles/min/ml)	β-N-Acetylglucosaminidase (nmoles/min/ml)
10 mg/kg	4	VBL	8	8.29±1.01	2.41±0.50	121±27.1
		Saline	8	10.21±2.66	1.69±0.52	130±53.0
	12	VBL	8	10.67±0.50	2.99±0.44	144±28.8
		Saline	8	8.22±0.95	3.00±0.71	123±37.2
50 mg/kg	4	VBL	12	14.82±1.99*	3.22±0.37	199±21.7
		Saline	12	8.43±1.63	2.50±0.56	180±17.8
	12	VBL	12	19.88±2.73**	4.63±0.64**	287±27.8**
		Saline	12	8.52±1.58	2.25±0.37	166±22.9

Results are expressed as nmoles inorganic phosphate liberated/min/ml in the case of acid phosphatase and as nmoles nitrophenol liberated/min/ml in the cases of β-galactosidase and β-N-acetylglucosaminidase. Values given are means ± standard error. Levels of significance as compared to control:

* $p < 0.050$
** $p < 0.010$

The serum GOT activities four and twelve hours after injection with two doses of VBL (10 mg/kg and 50 mg/kg) are presented in Table 11. The GOT activity increased significantly with both doses of VBL both four hours and twelve hours after injection.

The serum CPK activity increased significantly four hours after VBL injection with both doses used. No significant differences were observed twelve hours after injection (Table 11).

TABLE 11. GOT and CPK activities in mouse serum after VBL injection. (From the paper of Hirsimäki, P., Trump, B.F. & Arstila, A.U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

VBL dose	Time since injection (h)	Treatment	n	GOT (mU/ml)	n	CPK (mU/ml)
10 mg/kg	4	VBL	6	160.8±10.45***	8	3 462±140***
		Saline	6	81.0±11.45	8	2 307±203
	12	VBL	6	157.1± 9.91***	8	2 151±216
		Saline	6	77.1± 6.31	8	2 005±219
50 mg/kg	4	VBL	8	381.3±50.08**	8	3 103±425*
		Saline	8	109.1± 5.51	8	2 011±309
	12	VBL	8	930.6±45.93***	8	2 262±147
		Saline	8	103.8± 9.02	8	2 439±152

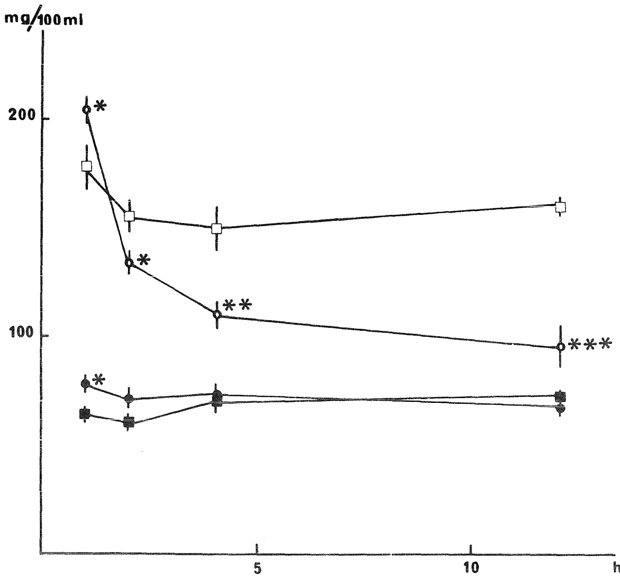
Results are expressed as milli-international GOT units/ml in the case of GOT and as milli-international CPK units/ml in the case of CPK. Values given are means ± standard error. Levels of significance as compared to control:

* $p < 0.050$
** $p < 0.010$
*** $p < 0.001$

Blood glucose and liver glycogen

Blood glucose was significantly elevated one hour after VBL injection (10 mg/kg) both in the mice which had free access to food throughout the experimental period and in those which were starved overnight ($p < 0.050$ and $p < 0.050$ respectively) as compared to the corresponding controls (Text-fig. 3). This ele-

vation was reversed after two hours in the former group, so that the blood glucose level of the VBL injected animals was significantly lower ($p < 0.050$) than in the controls. This significant difference remained and became even more apparent at four and twelve hours ($p < 0.010$ and $p < 0.001$ respectively). In the starved groups, no significant differences were found between VBL injected and control groups two, four or twelve hours after injection (Text-fig. 3).

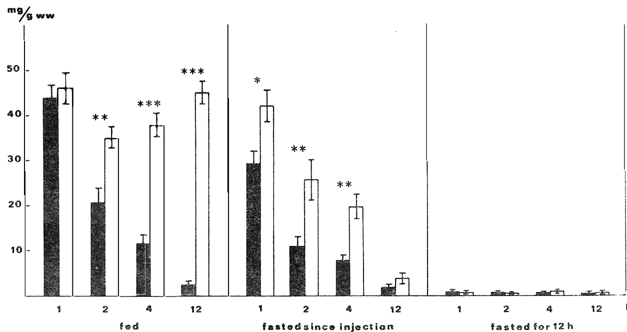


Text-fig. 3. Blood glucose levels in mice after vinblastine (1.0 mg/kg; circles) or saline (squares) injection. Open symbols denote that the animals had free access to food throughout the experiment. Filled symbols denote that the animals were starved overnight before killing. Each symbol is the mean value from four animals and the vertical bars indicate the standard error. Levels of significance as compared to controls:

* $p < 0.050$
** $p < 0.010$
*** $p < 0.001$

No differences were observed in the PAS staining in the livers of the animals one hour after VBL injection as compared to the controls. Almost every hepatocyte contained large amounts of PAS-positive material presumed to be glycogen (Fig. 2a). Two hours after injection, the livers of the VBL treated animals showed clearly smaller amounts of PAS-positive material, especially around the central veins (Fig. 2d), than the controls (Fig. 2c). This difference became even more pronounced four hours after injection (Figs. 2e and f), when the VBL injected animals had very little PAS-positive material in the liver. The heaviest staining was seen in the portal areas, while the areas around the central veins were unstained. Twelve hours after injection, no PAS-positive material could be discerned in either the VBL treated or in the control livers (Fig. 2b).

Liver glycogen values one, two, four and twelve hours after VBL injection (10 mg/kg) are presented in Text-fig. 4. In the



Text-fig. 4. Mouse liver glycogen levels 1, 2, 4 and 12 hours after VBL (10 mg/kg, black columns) or saline (white columns) injection. Each column represents the mean value from four animals and the vertical bars indicate the standard error. Levels of significance as compared to controls:

*	$p < 0.050$
**	$p < 0.010$
***	$p < 0.001$

first, fed group of animals, the glycogen content was significantly decreased two hours after VBL injection when compared to the control ($p < 0.005$). The difference became more pronounced with greater elapse of time since the injection. In the group of animals which was starved after the injection, the liver glycogen was already lower ($p < 0.050$) one hour after VBL injection than in the corresponding control. This difference persisted up to twelve hours, when the difference between the test and control groups was no longer significant. No differences were observed in the glycogen contents between VBL injected and control animals in the groups which were starved overnight (Text-fig. 4).

Cyclic AMP and glycogen phosphorylase a

The liver cAMP level did not differ significantly from the control value 15, 30 or 60 min after VBL injection (10 mg/kg, Table 12).

No differences were observed in glycogen phosphorylase a activities 15, 30 or 60 min after VBL injection (10 mg/kg, Table 13) as compared to controls.

TABLE 12. Cyclic AMP content of mouse liver 15, 30 and 60 min after VBL injection (10 mg/kg) as compared to controls

		Time since injection (min)		
		15	30	60
Treatment	Saline	VBL	VBL	VBL
pmoles/mg ww	0.423±0.007	0.386±0.039	0.408±0.020	0.416±0.039
n	6	6	6	6

Results are expressed as pmoles cAMP/mg wet weight of liver tissue. Values given are means ± standard error

TABLE 13. Glycogen phosphorylase a activity in mouse liver 15, 30 and 60 min after VBL injection (10 mg/kg) as compared to controls

		Time since injection (min)		
		15	30	60
Treatment	Saline	VBL	VBL	VBL
μmol/min/g prot	122±6.56	113±6.39	119±3.91	120±5.27
n	8	8	8	8

Results are expressed as micromoles liberated inorganic phosphate/min/g protein. Values given are means ± standard error

DISCUSSION

Effects of vinblastine on liver

The present study showed that all the doses of VBL used had an injurious effect on liver. Tissue injury could be detected by both light and electron microscopy and unspecifically also by biochemical studies. The effect of VBL appeared to be dose-dependent. In the light microscopical morphometrical study, however, no significant differences were observed in the volume densities of the extracellular compartments or hepatocytes as compared to controls when the middle VBL dose was used.

Both serum GOT and CPK activities were elevated after VBL injection. Elevated serum GOT activity has been shown to be a fairly good indicator of liver injuries (Korsrud *et al.* 1972). The elevated serum CPK activity need not, however, be entirely due to the liver injury since vinca alkaloids cause for example skeletal muscle injury (Clarke *et al.* 1972). The elevated activities of the three lysosomal hydrolases in the serum twelve hours after VBL injection with the highest dose might have originated from the necrotic parenchymal cells and/or from other tissues. To what extent the elevated activities were due to the possible exocytosis of AV contents from parenchymal cells remains to be solved.

Effects of vinblastine on hepatocytes

Induction of autophagocytosis. The results show that injection of VBL induced a wave of autophagocytosis in the hepatocytes during twenty-four hours. The number of AV appeared to be dose dependent in qualitative studies. The morphometric analysis confirmed that the significant increase of the volume density of AV was mainly due to the significant increase in their number from four hours to twenty-four hours after injection. Pfeifer (1979) summarized several reports which show that changes in the number of AV observed morphometrically correlate with changes in protein degradation measured biochemically. This observation is also in agreement with the concept of VBL-induced autophagocytosis, since it has been shown that VBL causes an increased degradation of liver proteins and lipids measured *in vitro* by the release of trichloroacetic acid-soluble products (Marzella & Glaumann 1980a). These results and the results of the present study suggest that VBL injection causes an increased formation of AV within four hours. This in turn leads to an increased degradation of the cytoplasmic components of the cell.

Appearance of autophagic vacuoles and acquisition of acid phosphatase. The apparently newly formed AV were mainly surrounded by smooth double membranes resembling those of the smooth surfaced endoplasmic reticulum, as has been previously described in rat liver after glucagon administration (Arstila & Trump 1968). These AV had clearly recognizable contents containing all cytoplasmic components except nuclear material as also previously has been described (Ericsson 1969, Pfeifer 1978) and usually showed no acid phosphatase activity. The lack of enzyme activity suggests the existence of a pre-lysosomal phase as has been described by de Duve and Wattiaux (1966). Some double membrane-limited AV contained acid phosphatase activity between the limiting membranes. The single membrane-limited older AV were also in general acid phosphatase positive, containing enzyme activity in their matrix. The latter two types of AV with acid

phosphatase activity can be considered as autolysosomes (de Duve & Wattiaux 1966).

It was apparent from the results obtained in the present work that the acid phosphatase activity appeared in a stepwise manner from the space between the double membranes to the matrix of the AV. During this process the inner membrane disappeared, obviously being digested by the lysosomal enzymes. A similar mechanism has also been suggested by other authors (Arstila & Trump 1968, Arstila *et al.* 1972, Pfeifer 1976, Paavola 1978b).

The process in which lytic enzymes become associated with the AV is a separate question. It has been proposed that both primary and secondary (Arstila & Trump 1968) or secondary lysosomes (Deter & de Duve 1967, Deter *et al.* 1967) fuse with AV. Biochemical and morphometrical studies on liver fractions (Deter 1971) and previous labelling of secondary lysosomes with iron (Deter 1975b) support the latter alternative. In the present study the fusion of lysosomes with AV was difficult to discern in the histochemical studies on the localization of acid phosphatase activity.

It has been speculated that VBL may interfere with the fusion of lysosomes with AV (Pfeifer 1976), suggesting a dependence of lysosomal movements on intact microtubules. The histochemical results with localization of acid phosphatase activity suggest that disruption of microtubules by VBL did not prevent the fusion of lysosomes and AV, since acid phosphatase positive AV were frequently found after VBL injection. Further studies are, however, needed to determine whether this process is slowed by VBL.

The morphometric analysis showed that the volume density of the apparently newly formed AV was highest at the beginning of the experiment (one hour after VBL injection), decreasing thereafter to a relatively low level. This double membrane stage is obviously the shortest period in the "life cycle" of an AV.

Later on, twelve hours after VBL injection, the volume density of the single membrane-limited AV was elevated, probably reflecting their accumulation in the cytoplasm. Similar accumulation of "older" AV in the cytoplasm has been described in mouse seminal vesicle cells after VBL treatment *in vitro* (Kovács & Kovács 1980). Pfeifer (1978) has calculated that the average half-life of AV in the liver is 8-9 min. In VBL-induced autophagocytosis it may however be longer, since in *in vitro* experiments on liver mitochondrial-lysosomal fraction VBL caused retarded degradation of the AV contents for two hours. It was proposed that this was due to an overloading of the lysosomal compartment (Marzella & Glaumann 1980a). These results could be one explanation for the increase in the volume density of the single membrane-limited AV twelve hours after VBL injection observed in the present study. Other explanations are the disturbance of fusion of the older autolysosomes with other AV and/or the interference of their transport to the sinusoidal border and the bile canaliculi, for the possible exocytosis of their contents due to the disruption of microtubules by VBL. This kind of exocytosis has been demonstrated in rat liver after VBL treatment (Marzella & Glaumann 1980b). In the present study, however, destroyed cellular material was seen in the sinusoidal lumen but exocytosis of AV contents was difficult to observe in the electron microscope preparations.

The mean volumes of the AV (0.357 ± 0.074 - 0.70 ± 0.128 $m\mu^3$) after VBL injection in the present study correlated fairly well with Deter's (1971) observations (0.364 ± 0.010 - 0.493 ± 0.34 $m\mu^3$) 50 min after glucagon administration in rat liver. The corresponding values after phenobarbital treatment (Bolender & Weibel 1973) or partial hepatectomy (Pfeifer 1979) in rat liver were about one third of the results of the present study.

The mean volume of the AV increased significantly one hour after VBL injection in the present study. An increase in the mean volume of the AV in increased autophagocytosis has also been described in rat liver (Deter 1971, Bolender & Weibel 1973).

No analysis of the mean volumes of the AV at different stages of development was made in the present study. It can, however, be assumed that the reason for the increase in the mean volume of AV was that the volume density of the newly formed, large AV was at its greatest one hour after VBL injection. Later on, when the volume density of the smaller, apparently older AV with partly degraded contents was greater, the mean volume became smaller.

Origin of autophagic vacuole membranes. Staining with unbuffered OsO_4 has been used as a marker for the Golgi apparatus in many tissues. As a rule OsO_4 stains the outer cisternae and adjacent tubules and vesicles on the forming face of the Golgi apparatus (Dalton & Felix 1956, Friend & Murray 1965, Friend 1969, Kalimo 1971, McDowell 1974). In addition, staining of the cisternae limiting an AV has been described (Friend 1969, Arstila *et al.* 1972, McDowell 1974, Hirsimäki & Reunanen 1978) and also of the vesicles within multivesicular bodies (Friend 1969, Kalimo 1971, McDowell 1974). By contrast, lysosomes have been reported not to be stained in several tissues (Friend & Brassil 1970, Kalimo 1971, McDowell 1974). The cisternae of the endoplasmic reticulum are stained in some tissues (Dalton & Felix 1956, Friend & Murray 1965, Friend & Brassil 1970, Kalimo 1971, Arstila *et al.* 1972, McDowell 1974).

In the present study unbuffered OsO_4 stained the outermost cisternae and vesicles on the forming face of the Golgi apparatus and also the cisternae of most forming and newly formed AV, the matrix of most apparently older AV, small cytoplasmic vesicles and vesicles within multivesicular bodies. The staining of endoplasmic reticulum varied from cell to cell.

The exact nature of the staining with unbuffered OsO_4 is unknown, but is has been suggested that sites of high concentrations of intermediates of cholesterol and/or other steroids are evenly stained with unbuffered OsO_4, whereas cholesterol itself and neutral lipids are not stained (Friend & Brassil 1970,

Griffiths & Beck 1977). The heavy staining of the cisternae on the forming face of the Golgi apparatus, AV cisternae in all cells and the endoplasmic reticulum cisternae of some cells observed in the present work might well result from high concentrations of unsaturated precursors of cholesterol or other steroids synthetized from cholesterol.

There are two supposed sites of membrane transformation in the cell: The Golgi apparatus and the membranes limiting a newly formed AV. Membranes of the Golgi cisternae thicken across the stack from forming face to maturing face (Morré *et al.* 1971, Whaley 1975). The transformation process from the thinner endoplasmic reticulum-like, endoplasmic, to thicker more plasma-membrane-like, exoplasmic, membranes may be a function of the Golgi apparatus (de Duve 1969, Morré *et al.* 1971). Endoplasmic reticulum membrane is thought to be important in the maintenance and continuity of Golgi cisternae (Beams & Kessel 1968, Favard 1969, Morré *et al.* 1971). Precursor material for this membrane thickening is apparently synthetized within the endoplasmic reticulum, transported to the Golgi apparatus and stored there in high concentrations (McDowell 1974).

The cisterna around an AV has been thought to be derived from the smooth surfaced endoplasmic reticulum in rat liver (Arstila & Trump 1968, 1969). The thickening of the outer membrane in the latter stage of AV formation from endoplasmic to exoplasmic type has been suggested to originate from the addition of exoplasmic membrane resulting from fusion with primary and/or secondary lysosomes. At this stage dense osmium deposits have been observed in the space between the double limiting membranes (Arstila *et al.* 1972). These deposits gradually penetrated into the matrix of the AV. However, the staining pattern of AV membranes with unbuffered OsO_4 observed in the present study differed from that described by Arstila *et al.* (1972) so that the cisterna was already heavily stained in the very early stages of AV formation, before the vacuole completely surrounded the engulfed internal organelles. This stage of AV formation precedes

the fusion with lysosomes (de Duve & Wattiaux 1966, Arstila & Trump 1968, 1969). Thus the results of the present study show that the staining of the space between the AV membranes is evidently not a result of fusion of AV with the lysosomes. Similar results have also been reported in rat kidney tubule cells (McDowell 1974).

In the present work the source of AV membranes stained with unbuffered OsO_4 appears to be the smooth surfaced cisternae of the endoplasmic reticulum. The thickening of the membranes limiting an AV can be considered as analogous to that occurring in the forming face of the Golgi apparatus. The similar staining pattern of these two membrane populations suggests that analogous metabolic processes occur within them. The transformation process in AV membranes occurs independently of lysosomal fusion. Similar results have also been reported by McDowell (1974) in kidney tubule cells.

Some newly formed AV were not stained with unbuffered OsO_4. One explanation could be that their membranes have a different origin to that proposed above. A possible origin could be the GERL membranes, which are not stained with unbuffered OsO_4 (Novikoff *et al.* 1971, Paavola 1978a, 1978b). The reason for the paucity of this kind of newly formed AV would be that the GERL seemed to be weakly developed in mouse hepatocytes. In the present study the corresponding membranes showed only faint or no acid phosphatase activity, suggesting a very small size of the GERL. GERL has been shown to be an acid phosphatase positive network of membranes in cells in which it is well developed (Holtzman *et al.* 1967, Novikoff *et al.* 1971, Paavola 1978b). Similar observations of the size of the GERL as in the present study have been reported by Essner and Oliver (1974) for normal mouse liver.

Variations in the staining of the endoplasmic reticulum with unbuffered OsO_4 are normally seen within a single cell or between adjacent cells in many tissues (Friend 1969, Friend & Brassil

1970, Kalimo 1971, Novikoff *et al.* 1971, McDowell 1974). This has been supposed to be due to technical factors or to be a reflection of the different physiological state of cells at the time of fixation (Friend & Murray 1965). In hepatocytes participating in bile acid synthesis the endoplasmic reticulum may be stained by unbuffered OsO_4 (Friend & Brassil 1970). In the present study the ultrastructural changes in mitochondria and endoplasmic reticulum observed in the cells in which the endoplasmic reticulum was stained suggest that technical factors may also have affected the cells during incubation.

Inosine diphosphatase activity has been considered as a useful marker for the endoplasmic reticulum (Essner 1973). In the Golgi apparatus reaction product of inosine diphosphatase activity is found within the cavities of the innermost cisternae of the maturing face (Novikoff *et al.* 1971, Essner 1973). In the present study, enzyme activity was observed within the cisternae and on the surface of the endoplasmic reticulum and only occasionally within the inner Golgi cisterna. A similar localization of this enzyme has been observed in rat liver (Arstila & Trump 1968), in human liver (Ma & Biempica 1971) and in rat neurons (Holtzman *et al.* 1967).

In the present study, no inosine diphosphatase activity was observed in the membranes of either newly formed double membrane-limited AV or older single membrane-limited AV. However, some AV contained fragments of engulfed endoplasmic reticulum having this enzyme activity. The results of the present study differ from the results of Arstila and Trump (1968) who reported inosine diphosphatase activity between the membranes of newly formed AV in rat liver. This was proposed to indicate an origin of the AV from the endoplasmic reticulum membranes.

From the results of the present study two possibilities concerning AV formation can be proposed: 1) Some kind of transformation occurs in endoplasmic reticulum membranes during AV formation, as a result of which the inosine diphosphatase

activity of these membranes disappears. The results with unbuffered OsO_4 staining support this alternative. 2) AV membranes could also originate from the GERL membranes which do not have inosine diphosphatase activity (Novikoff *et al.* 1971, Essner & Oliver 1974). Because the amount of GERL membranes appeared to be very small in mouse liver in the present study, the first alternative seems more probable.

Contents of autophagic vacuoles. Autophagocytosis has been proposed to be a random process (de Duve & Wattiaux 1966). However, some quantitative evidence exists which suggests that this is not the case. In a morphometric study of the removal of phenobarbital-induced membranes from hepatocytes after cessation of treatment it appeared that AV were formed in a non-random fashion by specific removal of the excess endoplasmic reticulum membranes (Bolender & Weibel 1973). Selective autophagocytosis of mitochondria and microbodies has been described by Pfeifer (1978) in normal rat liver. In the present study the most reliable results of the AV content analysis were those obtained one and four hours after VBL injection because the number of newly formed AV with clearly recognizable contents was the greatest in these samples. Later their number decreased. One hour after VBL injection the percentage of the volume density of vesicles containing VLDL was significantly greater in the cytoplasm than in the AV but this difference disappeared four hours after injection. No selective autophagocytosis of the other cytoplasmic compartments was observed during the first twelve hours. Although the analysis was less reliable twenty-four hours after VBL injection, it was interesting to note that the percentage of the volume density of the fat droplets was significantly greater in the cytoplasm than in AV. The results indicate that the VLDL vesicles one hour and the fat droplets twenty-four hours after VBL injection are segregated in a smaller quantity than the other cytoplasmic components, suggesting that autophagocytosis is to some extent a selective process. The selectivity may depend on the physiological or pathological state of the cell, as has been indicated by Locke and

McMahon (1971), Bolender and Weibel (1973) and Pfeifer (1978).

Lysosomal hydrolase activities. The total activities of the three lysosomal enzymes, acid phosphatase, β-galactosidase and β-N-acetylglucosaminidase, were estimated in order to investigate whether the increased autophagocytosis also had the effect of increasing their activities. No increase was, however, observed after VBL treatment as compared to the controls. The results show that the amount of lysosomal hydrolases was apparently sufficient for the increased degradation of cytoplasmic components during the experimental period (twelve hours). These results are in general in agreement with the total activities of lysosomal hydrolases obtained in other short-term experiments after VBL treatment (Marzella & Glaumann 1980a) and also after glucagon treatment (Arstila & Trump 1968, Deter 1971) in rat liver.

Although the total activities of the lysosomal enzymes did not change, the volume density of the lysosomes increased after VBL injection. This might reflect a swelling of the lysosomes. A direct fusion of lysosomes with vesicles containing VLDL (crinophagy) has been described by Reaven and Reaven (1980). This may lead to an increase in the volume density of the lysosomal compartment of the cell. However, no significant increase in the mean volume of lysosomes was observed although it increased slightly during the twelve hours after VBL injection. The increase in the number of lysosomes twenty-four hours after VBL injection might reflect the increased turnover of AV to telolysosomes due to the increased autophagocytosis.

The labilization of the autolysosomal membranes after VBL treatment, as measured by the free activity of the lysosomal enzymes, was not as clear as has been shown 50 min after glucagon treatment in the liver (Deter 1971). In the present study only the free activity of β-N-acetylglucosaminidase was increased twelve hours after VBL injection with the highest dose used, while at the same time the free activity of acid phosphatase was lower

than that of the control. Whether these results might reflect the effect of VBL on the lysosomes or the different localization of the investigated enzymes inside the lysosomes (Beck & Tappel 1968) is not clear.

Other changes in hepatocytes. In addition to increased autophagocytosis VBL administration also affected parenchymal cells in other respects. The most pronounced ultrastructural changes in all cells were the disappearance of microtubules, accumulation of VLDL filles vesicles and the disorganization of the Golgi cisternae. Although the disappearance of microtubules was complete after VBL treatment, it was striking that no paracrystalline inclusions were observed as has been reported for some other tissues (Bensch & Malawista 1968, Krishan & Hsu 1971, Bunt 1973, Tyson & Bulger 1973). Similar results to those obtained in the present study have also been reported by other authors. Stein *et al.* (1974) did not observe paracrystals in the parenchymal cells in vincristine- or vinblastine-incubated rat liver slices. Le Marchand *et al.* (1973) have described amorphous masses in vincristine perfused mouse liver, which may have been precipitated microtubular proteins. Such masses were not observed in the present study. One explanation for the lack of paracrystalline inclusions in the liver may be the paucity of microtubules in the liver tissue (Bruni & Porter 1965).

Hepatocytes synthetize and secrete triglycerides primarily in the form of VLDL. The increase in the volume density of the secretory vesicles containing VLDL in the hepatocytes in the present study indicated that VBL interfered with the secretion of VLDL from the liver. The reason might be the disaggregation of the microtubular protein caused by VBL. Microtubules are needed in the transport and release of VLDL from the parenchymal cells (Le Marchand *et al.* 1973, Orci *et al.* 1973, Stein & Stein 1973, Stein *et al.* 1974, Reaven & Reaven 1980).

The disorganization of the Golgi complex appeared as a swelling of the cisternae. The number and/or the size of the cisternae

was reduced, since the volume density of the Golgi apparatus was significantly decreased twelve hours after VBL injection. Similar observations have been made in rat liver after colchicine injection (Reaven & Reaven 1980) and with VBL and colchicine in other cell types (Moskalewski *et al.* 1975, 1976). The disorganization of the Golgi apparatus observed after treatment with antimicrotubular agents might result from the disruption of the microtubules, suggesting that microtubules may be important in the maintenance of the Golgi apparatus (Reaven & Reaven 1980).

The other changes observed in the qualitative and quantitative morphological studies of the hepatocytes indicated the injurious effects of VBL. These changes included partial vesiculation of the endoplasmic reticulum and both decrease in the surface density of the smooth surfaced endoplasmic reticulum four hours after injection and increase in the surface density of the rough surfaced endoplasmic reticulum twelve hours after injection. The volume density of cytoplasmic ribosomes was also decreased four hours after VBL injection. On the other hand, the volume density of ground substance was significantly greater than the control value twenty-four hours after VBL injection. The reason for this might be the significant increase in the volume density of fat droplets observed in control animals at the same time, which in turn might result from the starving of the animals during the experiment. Fat accumulation in hepatocytes has been described for example in starved cows (Reid 1973). VBL also affected mitochondria: The surface density of the mitochondrial outer boundary membrane was significantly decreased one hour after injection. Since the changes described were obviously of minor interest from the point of view of autophagocytosis they will not be discussed here in more detail.

Effects of vinblastine on liver glycogen, cyclic AMP, glycogen phosphorylase a and blood glucose

Liver glycogen content, cAMP level and glycogen phosphorylase a activity were estimated in order to compare the effects of VBL

with the known effects of glucagon on liver glycogen degradation. The blood glucose level was estimated simultaneously.

The injection of VBL was followed by a loss of glycogen from the liver. This was observed in PAS staining, in quantitative morphological studies and in the biochemical estimations of glycogen. The greatest difference in liver glycogen content between the VBL treated and the control animals in biochemical estimations was in the group which was fed during the entire experimental period. This difference could at least partly have arisen from the indisposition of the VBL treated mice, which did not show normal eating behaviour. However, the difference was also evident in those experiments in which all the animals were starved after VBL or saline injection. On the other hand, starvation for twelve hours lowered the liver glycogen content of the control animals so much that no differences between VBL treatment and controls could be discerned. In the quantitative morphological studies the volume density of liver glycogen was significantly lowered one and four hours after VBL injection. Twelve hours after VBL injection no glycogen at all was observed in the liver. This result and also the relatively low volume densities of liver glycogen observed in the present study (about 2 % in the cytoplasm of the control animals one hour after saline injection) as compared to the corresponding value (about 21 %) in rat liver cytoplasm (Pfeifer 1978) might be a reflection of the variations in glycogen staining observed in the thin sections during the present study.

The blood glucose values were elevated half an hour after VBL injection. The elevation resembled the sharp rise in blood glucose typical for glucagon stimulated glycogenolysis (Miller *et al.* 1959). One hour after VBL injection, the blood glucose level of the fed group was lower than the control value. This difference was retained during the entire experimental period. A similar hypoglycemic effect of vinca alkaloids has been described by Svoboda *et al.* (1964).

There are two principal enzymatic pathways by which glycogen can be degraded to glucose in the liver; by glycogen phosphorylase in the cytoplasm and by α-glucosidase in the lysosomal system. Phosphorolysis is involved in the rapid breakdown of glycogen when glucose or glucose phosphates are required for the energy needs of the body. The lysosomal α-glucosidase is necessary when glycogen is engulfed in an AV (Hers & de Barsy 1973). In the hereditary Pompe's disease this enzyme is lacking, and glycogen therefore accumulates inside AV (Hers 1963, Baudhuin *et al.* 1964, Hers 1965).

According to the results of the present study, increased glycogen degradation due to the activation of glycogen phosphorylase appeared to be improbable, because this enzyme is activated by elevated cAMP levels in the liver. Glucagon increases the cAMP content in isolated perfused rat liver within a few minutes (Exton & Park 1967, Exton *et al.* 1971a, 1971b). In the present study, however, neither the cAMP level nor the glycogen phosphorylase a activity changed after VBL injection. Furthermore, Bos and Emmelot (1974) have found that VBL and colchicine inhibit glucagon-stimulated adenylate cyclase and cAMP-phosphodiesterase in rat liver plasma membrane. Unchanged cAMP levels have also been observed in autophagocytosis induced by amino-acid deprivation in perfused rat liver. Simultaneously glycogen was frequently found in AV (Mortimore & Schworer 1977).

According to the results of the present study the increased degradation of glycogen after VBL injection may occur in the lysosomal system by means of increased autophagocytosis. The results also show that VBL induces autophagocytosis independently of alterations in the level of liver cAMP.

SUMMARY AND CONCLUSIONS

Vinblastine-induced autophagocytosis and other injurious effects were studied in mouse liver parenchymal cells during twenty-four hours after intraperitoneal injection with three doses of vinblastine (10, 25 and 50 mg/kg). Both qualitative (all doses) and quantitative (25 mg/kg) light and electron microscopical methods and biochemical (10 and 50 mg/kg) methods were used. The main results obtained were:

1) Vinblastine induced an increased formation of autophagic vacuoles with all the doses used. The quantitative studies confirmed that the increase in the volume density of autophagic vacuoles from four hours to twenty-four hours after injection was due to the significant increase in their number, which was 5.3-fold four hours, 4.7-fold twelve hours and 5.7-fold twenty-four hours after injection as compared to the controls. The mean volume of the autophagic vacuoles increased significantly one hour after vinblastine injection, at which time the formation of new autophagic vacuoles was at its greatest. The number of autophagic vacuoles appeared to be dose-dependent. The appearance and contents of the newly formed autophagic vacuoles was similar to that described earlier in the hepatocytes. There was an accumulation of single membrane-limited, obviously older autophagic vacuoles in the cytoplasm. Their volume density was at its maximum twelve hours after injection, suggesting a retarded turnover of autophagic vacuoles. There may be exocytosis of autolysosomal material into the sinusoidal lumen and the space of Disse.

2) Using osmium impregnation, very intense staining was frequently observed in the cavity between the double membranes destined to form autophagic vacuoles, in the cavity between the double membranes of apparently newly formed autophagic vacuoles as well as in the outer cisternae on the forming face of the Golgi apparatus. The results suggested that the membranes of most autophagic vacuoles might be derived from smooth surfaced

cisternae of the endoplasmic retuculum, in which a kind of transformation process occurs during autophagic vacuole formation. The transformation may be analogous to that observed at the Golgi cisternae and occurs independently of fusion with lysosomes. The membranes of the autophagic vacuoles did not contain inosine diphosphatase activity, which is a generally used marker of the endoplasmic reticulum.

3) The segregation of cytoplasmic components appeared to be selective to some extent after vinblastine injection. Vesicles containing very low density lipoprotein particles one hour after injection and lipid droplets twenty-four hours after injection were segregated in smaller quantities than the other cytoplasmic components.

4) Acid phosphatase activity was localized between the double membranes of a few apparently newly formed autophagic vacuoles and within most obviously older single membrane-limited autophagic vacuoles. The absence of enzyme activity from several newly formed autophagic vacuoles suggested the existence of a pre-lysosomal phase. Although the microtubules disappeared from the cytoplasm after injection, vinblastine did not prevent the fusion of newly formed autophagic vacuoles with the preexisting lysosomes.

5) The total activities of the three lysosomal hydrolases, acid phosphatase, β-galactosidase and β-N-acetylglucosaminidase, did not change four and twelve hours after vinblastine injection, suggesting that the amount of lysosomal hydrolases was adequate in the liver in spite of increased degradation of cytoplasmic components by autophagocytosis. The labilization of the autolysosomal membranes, as measured by the free activities of these enzymes, was not so clear as earlier described in rat liver after glucagon treatment.

6) The level of liver cyclic adenosine monophosphate did not change after vinblastine injection, suggesting that the induc-

tion of autophagocytosis was not mediated via an elevated level of intracellular cyclic adenosine monophosphate. Although liver glycogen disappeared from vinblastine injected animals faster than from the controls, glycogen phosphorylase a activity did not change. The loss of glycogen might occur via increased autophagocytosis.

7) The injurious effects of vinblastine were evident both in light and electron microscopical and in biochemical studies and seemed to be dose-dependent. Diffuse necrosis of the liver lobules and inflammatory cells were observed, especially with the highest dose used, within twelve hours. In the parenchymal cells the microtubules disappeared, while the Golgi cisternae were dilated and disorganized and the volume density of the Golgi apparatus was significantly decreased twelve hours after vinblastine injection. The volume density of lysosomes was increased during the twelve hours after vinblastine injection. Vesicles containing very low density lipoprotein particles accumulated in the cytoplasm so that their volume density was significantly increased during the entire experimental period. Vinblastine apparently interfered with the transport and secretion of the very low density lipoproteins from the parenchymal cells. Furthermore, the endoplasmic reticulum was partially dilated and vesiculated after vinblastine injection. The elevated activities of serum L-aspartate-2-oxoglutarate aminotransferase, creatine phosphokinase and the lysosomal hydrolases acid phosphatase, β-galactosidase and β-N-acetylglucosaminidase, especially with the highest dose, also provided evidence for the injurious effects of vinblastine.

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REFERENCES

Abraham, R., Golberg, L. & Grasso, P. 1967: Hepatic response to lysosomal effect of hypoxia, neutral red and chloroquine. - Nature (London) 215: 194-196.

Arstila, A. U., Nuuja, I. J. M. & Trump, B. F. 1974: Studies on cellular autophagocytosis. Vinblastine-induced autophagy in the rat liver. - Exp. Cell. Res. 87: 249-252.

Arstila, A. U., Shelburne, J. D. & Trump, B. F. 1972: Studies on cellular autophagocytosis. A histochemical study on sequential alterations of mitochondria in the glucagon-induced autophagic vacuoles of rat liver. - Lab. Invest. 27; 317-323.

Arstila, A. U. & Trump, B. F. 1968: Studies on cellular autophagocytosis. The formation of autophagic vacuoles in the liver after glucagon administration. - Amer. J. Pathol. 53: 687-733.

Arstila, A. U. & Trump, B. F. 1969: Autophagocytosis: Origin of membrane and hydrolytic enzymes. - Virchows Arch. B Cell Path. 2: 85-90.

Ashford, T. P. & Porter, K. R. 1962: Cytoplasmic components in hepatic cell lysosomes. - J. Cell Biol. 12: 198-202.

Ballard, J. F. 1977: Intracellular protein degradation. In: Campbell, P. N. & Aldridge, W. N. (eds.), Essays in biochemistry, vol. 13: 1-37 pp. Academic Press, London, New York.

Barrett, A. J. 1972: Lysosomal enzymes. In: Dingle, J. T. (ed.), Lysosomes, a laboratory handbook: 46-135 pp. North-Holland Publ. Co., Amsterdam, London.

Baudhuin, P., Hers, H. G. & Loeb, H. 1964: An electron microscopic and biochemical study of type II glycogenosis. - Lab. Invest. 13: 1139-1152.

Beams, H. W. & Kessel, R. G. 1968: The Golgi apparatus: Structure and function. - Int. Rev. Cytol. 23: 209-276.

Beck, C. & Tappel, A. L. 1968: Rat-liver lysosomal β-glucosidase: A membrane enzyme. - Biochim. biophys. Acta (Amst.) 151: 159-164.

Bensch, K. G. & Malawista, S. E. 1968: Microtubule crystals: A new biophysical phenomenon induced by vinca alkaloids. - Nature (London) 218: 1176-1177.

Bolender, R. P. & Weibel, J. T. 1973: A morphometric study of the removal of phenobarbital-induced membranes from hepatocytes after cessation of treatment. - J. Cell Biol. 56: 746-761.

Bos, C. J. & Emmelot, P. 1974: Studies on plasma membranes XXI. Inhibition of liver plasma membrane enzymes by tumour-promoting phorbolester, mitotic inhibitors and cytochalasin B. - Chem.-Biol. Interactions 8: 349-361.

Brandes, D. 1965: Lysosomes and aging pigment. In: Krohn, P. L. (ed.) Topics in the biology of aging: 149-158 pp. Interscience, New York.

Brixová, E. & Dzūriková, V. 1972: Determination of glycogen, lipids and proteins in hepatic needle biopsy. - Clin. chim. Acta 36: 543-548.

Bruni, C. & Porter, K. R. 1965: The fine structure of the parenchymal cell of the normal rat liver. I. General observations. - Amer. J. Pathol. 46: 691-755.

Bunt, A. H. 1973: Paracrystalline inclusions in optic nerve terminals following intraocular injection of vinblastine. - Brain Res. 53: 29-39.

Clarke, J. T. R., Karpati, G., Carpanter, S. & Wolfe, L. S. 1972: The effect of vincristine on skeletal muscle in the rat. A correlative histochemical, ultrastructural and chemical study. - J. Neuropathol. exp. Neurol. 31: 247-265.

Coffey, J. W. & de Duve, C. 1968: Digestive activity of lysosomes. I. The digestion of proteins by extracts of rat liver lysosomes. - J. biol. Chem. 234: 3255-3263.

Dalton, A. J. & Felix, M. D. 1956: A comparative study of the Golgi complex. - J. biophys. biochem. Cytol. 2, suppl.: 79-84.

Deter, R. L. 1971: Quantitative characterization of dense body, autophagic vacuole, and acid phosphatase-bearing particle populations during the early phases of glucagon-induced autophagy in rat liver. - J. Cell Biol. 48: 473-487.

Deter, R. L. 1975a: Analog modeling of glucagon-induced autophagy in rat liver. I. Conceptual and mathematical model of telolysosome - autophagosome - autolysosome interaction. - Exp. Cell Res. 94: 122-126.

Deter, R. L. 1975b: Analog modeling of glucagon-induced autophagy in rat liver. II. Evaluation of iron labeling as a means for identifying telolysosome, autophagosome and autolysosome populations. - Exp. Cell Res. 94: 127-139.

Deter, R. L., Baudhuin, P. & de Duve, C. 1967: Participation of lysosomes in cellular autophagy induced in rat liver by glucagon. - J. Cell Biol. 35: 611.

Deter, R. L. & de Duve, C. 1967: Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes. - J. Cell Biol. 33: 437-449.

de Duve, C. 1959: Lysosomes, a new group of cytoplasmic particles. In: Hayashi, T. (ed.), Subcellular particles: 128-159 pp. Ronald Press, New York.

de Duve, C. 1963: The lysosome concept. In: De Reuck, A. V. S. & Cameron, M. P. (eds.), Ciba foundation symposium on lysosomes: 1-31 pp. Churchill, London.

de Duve, C. 1969: The lysosome in retrospect. In: Dingle, J. T. & Fell, H. B. (eds.), Lysosomes in biology and pathology, vol. 1: 3-40 pp. North-Holland Publ. Co., Amsterdam, London.

de Duve, C. & Wattiaux, R. 1966: Functions of lysosomes. - Ann. Rev. Physiol. 28: 435-492.

Ericsson, J. L. E. 1969: Mechanism of cellular autophagy. In: Dingle, J. T. & Fell, H. B. (eds.), Lysosomes in biology and pathology, vol. 2: 345-394 pp. North-Holland Publ. Co., Amsterdam, London.

Ericsson, J. L. E. & Glinsmann, W. H. 1966: Observations on the subcellular organization of hepatic parenchymal cells. I. Golgi apparatus, cytosomes and cytosegresomes in normal cells. - Lab. Invest. 15: 750-761.

Ericsson, J. L. E., Trump, B. F. & Weibel, J. 1965: Electron microscopic studies of the proximal tubule of the rat kidney. Cytosegresomes and cytosomes: their relationships to each other and to the lysosome concept. - Lab. Invest. 14: 1341-1365.

Essner, E. 1973: Phosphatases. In: Hayat, M. A. (ed.), Electron microscopy of enzymes, vol. 1: 44-76 pp. Van Nostrand Reinhold Co., Cincinnati, Toronto, London, Melbourne.

Essner, E. & Novikoff, A. B. 1960: Human hepatocellular pigments and lysosomes. - J. Ultrastruct. Res., 3: 374.

Essner, E. & Oliver, C. 1974: Lysosome formation in hepatocytes of mice with Chédiak - Higashi syndrome. - Lab. Invest. 30: 596-607.

Exton, J. H., Lewis, S. B., Ho, R. J., Robison, C. A. & Park, C. R. 1971a: The role of cyclic AMP in the interaction of glucagon and insulin in the control of liver metabolism. - Ann. NY Acad. Sci. 185: 85-100.

Exton, H. J. & Park, C. R. 1967: The role of cyclic AMP in the control of liver metabolism. - Adv. Enzyme Regul. 6: 391-407.

Exton, H. J., Robison, G. A., Sutherland, E. W. & Park, C. R. 1971b: Studies on the role of adenosine 3', 5'-monophosphate in the hepatic actions of glucagon and catecholamines. - J. biol. Chem. 246: 6166-6177.

Favard, P. 1969: The Golgi apparatus. In: Lima-de-Faria, A. (ed.), Handbook of molecular cytology, 1130-1155 pp. North-Holland Publ. Co., Amsterdam, London.

Fiske, C. H. & Subbarow, Y. 1925: The colorimetric determination of phosporus. - J. biol. Chem. 66: 375-400.

Frank, A. L. & Christensen, A. J. 1968: Localization of acid phosphatase in lipofuscin granules and possible autophagic vacuoles in interstitial cells of the guinea pig testis. - J. Cell Biol. 36: 1-13.

Friend, D. S. 1969: Cytochemical staining of multivesicular body and Golgi vesicles. - J. Cell Biol. 41: 269-279.

Friend, D. S. & Brassil, G. E. 1970: Osmium staining of endoplasmic reticulum and mitochondria in the rat adrenal cortex. - J. Cell Biol. 46: 252-266.

Friend, D. S. & Murray, M. J. 1965: Osmium impregnation of the Golgi apparatus. - Amer. J. Anat. 117: 135-150.

Gilman, A. G. 1970: A protein binding assay for adenosine 3': 5'-cyclic monophosphate. - Proc. Nat. Acad. Sci. USA 67: 305-312.

Glaumann, H., Berezesky, I. K., Ericsson, J. L. E. & Trump, B. F. 1975a: Lysosomal degradation of cell organelles. I. Ultrastructural analysis of uptake and digestion of intravenously injected mitochondria by Kupffer cells. - Lab. Invest. 33: 239-251.

Glaumann, H., Berezesky, I. K., Ericsson, J. L. E. & Trump, B. F. 1975b: Lysosomal degradation of cell organelles. II. Ultrastructural analysis of uptake and digestion of intravenously injected microsomes and ribosomes by Kupffer cells. - Lab. Invest. 33: 252-261.

Glaumann, H. & Trump, B. F. 1975: Lysosomal degradation of cell organelles. III. Uptake and disappearance in Kupffer cells of intravenously injected isotope-labeled mitochondria and microsomes *in vivo* and *in vitro*. - Lab. Invest. 33: 262-272.

Glinsmann, W. H. & Ericsson, J. L. E. 1966: Observations on the subcellular organization of hepatic parenchymal cells. II. Evolution of reversible alterations induced by hypoxia. - Lab. Invest. 15: 762-777.

Gomori, G. 1952: Microscopic histochemistry. Principles and practice, 189-194 pp. - University of Chicago Press.

Griffiths, G. W. & Beck, S. D. 1977: Effect of dietary cholesterol on the pattern of osmium deposition in the symbiote-containing cells of the pea aphid. - Cell Tiss. Res. 176: 191-203.

Helminen, H. J. & Ericsson, J. L. E. 1971: Ultrastructural studies on prostatic involution in the rat. Mechanism of autophagy in epithelial cells, with special reference to the rough-surfaced endoplasmic reticulum. - J. Ultrastruct. Res. 36: 708-724.

Hers, H. G. 1963: Alpha-glucosidase deficiency in generalized glycogen storage disease (Pompe's disease). - Biochem. J. 86: 11-16.

Hers. H. G. 1965: Inborn lysosomal diseases. - Gastroenterology 48: 625-633.

Hers, H. G. & de Barsy, T. 1973: Type II glycogenosis (Acid maltase deficiency). In: Hers, H. G. & van Hoof, F. (eds.), Lysosomes and storage diseases: 197-216 pp. Academic Press, New York, London.

Hirsimäki, P. & Pilström, L. 1979: A morphometric study of vinblastine-induced autophagocytosis in mouse hepatocytes. - J. Ultrastruct. Res. 69: 149-150.

Hirsimäki, P. & Reunanen, H. 1978: Histochemical studies on vinblastine-induced autophagocytosis in the mouse liver. - J. Ultrastruct. Res. 63: 95.

Hirsimäki, Y., Arstila, A. U. & Trump, B. F. 1975: Autophagocytosis: *In vitro* induction by microtubule poisons. - Exp. Cell Res. 92: 11-14.

Holtzman, E., Novikoff, A. B. & Villaverde, H. 1967: Lysosomes and GERL in normal and chromatolytic neurons of the rat ganglion nodosum. - J. Cell Biol. 33: 419-435.

Hruban, Z., Spargo, B., Swift, H., Wissler, R. W. & Kleinfeld, R. G. 1963: Focal cytoplasmic degradation. - Amer. J. Pathol. 42: 657-683.

Hugon, J. & Borgers, M. 1966: Ultrastructural and cytochemical studies on karyolytic bodies in the epithelium of the duodenal crypts of whole body x-irradiated mice. - Lab. Invest. 15: 1528-1543.

Hyvärinen, A. 1972: Veren sokerin määritys. In: Hyvärinen, A., Jännes, J., Nikkilä, E., Saris, N.-E. & Vuopio, P. (eds.), Kliiniset laboratoriotutkimukset: 204-206 pp. Werner Söderström Oy, Porvoo, Helsinki.

Jacques, P. J. 1975: The endocytic uptake of macromolecules. In: Trump, B. F. & Arstila, A. U. (eds.), Pathobiology of cell membranes, vol. 1: 255-279 pp. Academic Press, New York, San Francisco, London.

Johnson, I. S., Armstrong, J. G., Gorman, M. & Burnett, J. P. 1963: The vinca alkaloids: A new class of oncolytic agents. - Cancer Res. 23: 1390-1427.

Kalimo, H. 1971: Ultrastructural studies on the hypothalamic neurosecretory neurones of the rat. I. The paraventricular neurones of the non-treated rat. - Z. Zellforsch. 122: 283-300.

Karnovsky, M. J. 1965: A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. - J. Cell Biol. 27: 137A-138A.

Kerr, J. F. R. 1970: Liver cell defaecation: An electron-microscope study of the discharge of lysosomal residual bodies into the intercellular space. - J. Path. 100: 99-103.

King, E. J. & Campbell, D. M. 1961: International enzyme units. An attempt at international agreement. - Clin. chim. Acta 6: 301-306.

Korsrud, G. O., Grice, H. C. & McLaughlan, J. M. 1972: Sensitivity of several serum enzymes in detecting carbon tetrachloride-induced liver damage in rats. - Toxicol. appl. Pharmacol. 22: 474-483.

Kovács, A. L. & Kovács, J. 1980: Autophagocytosis in mouse seminal vesicle cells *in vitro*. Temperature dependence and effects of vinblastine and inhibitors of protein synthesis. - Virchows Arch. B Cell Path. 32: 97-104.

Kovács, J. & Réz, G. 1974: Prevention of induced autophagy by ementine in exocrine cells of mouse pancreas and seminal vesicle. - Virchows Arch. B Cell Path. 15:209-216.

Kovács, J., Réz, G. & Kiss, A. 1975: Vinblastine-induced autophagocytosis and its prevention by cycloheximide and ementine in mouse pancreatic acinar cells *in vivo*. - Cytobiologie: 11: 309-313.

Krishan, A. & Hsu, D. 1971: Binding of colchicine - ^{3}H to vinblastine- and vincristine-induced crystals in mammalian tissue culture cells. - J. Cell Biol. 48: 407-410.

LeMarchand, Y., Singh, A., Assimacopoulos-Jeannet, F., Orci, L., Rouiller, C. & Jeanrenaud, B. 1973: A role for the microtubular system in the release of very low density lipoproteins by perfused mouse livers. - J. biol. Chem. 248: 6862-6870.

Locke, M. & McMahon, J. T. 1971: The origin and fate of microbodies in the fat body of an insect. - J. Cell Biol. 48: 61-78.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. 1951: Protein measurement with the Folin phenol reagent. - J. biol. Chem. 193: 265-275.

Ma, M. H. & Biempica, L. 1971: The normal human liver cell. - Amer. J. Pathol. 62: 353-390.

Malawista, S. E., Sato, H. & Bensch, K. G. 1968: Vinblastine and griseofulvin reversibly disrupt the living mitotic spindle. - Science 160: 770-772.

Marzella, L. & Glaumann, H. 1980a: Increased degradation in rat liver induced by vinblastine. I. Biochemical characterization. - Lab. Invest. 42: 8-17.

Marzella, L. & Glaumann, H. 1980b: Increased degradation in rat liver induced by vinblastine. II. Morphologic characterization. - Lab. Invest. 42: 18-27.

McDowell, E. M. 1974: Unbuffered osmium staining in pars recta of the proximal tubule from rat kidney studied by thin and semi-thin section cytochemistry. - Histochemistry 39: 335-344.

Miller, L. L., Sokal, J. E. & Sarcione, E. J. 1959: Effects of glucagon and tolbutamine on glycogen in isolated perfused rat liver. - J. Physiol. 197: 286-288.

Morré, D. J., Mollenhauer, H. H. & Bracker, C. E. 1971: Origin and continuity of Golgi apparatus. In: Reinert, J. & Ursprung, H. (eds.), Origin and continuity of cell organelles. Results and problems in cell differentation, vol. 2: 82-126 pp. Springer-Verlag, Berlin, Heidelberg, New York.

Mortimore, G. E. & Schworer, C. M. 1977: Induction of autophagy by amino-acid deprivation in perfused rat liver. - Nature (London) 270: 174-176.

Moskalewski, S., Thyberg, J. & Friberg, V. 1976: *In vitro* influences of colchicine on the Golgi complex in A- and B-cells of guinea pig pancreatic islets. - J. Ultrastruct. Res. 54: 304-317.

Moskalewski, S., Thyberg, J., Lohmander, S. & Friberg, V. 1975: Influence of colchicine and vinblastine on the Golgi complex and matrix deposition in chondrocyte aggregates. - Exp. Cell Res. 95: 440-454.

Nevalainen, T. J. 1975: Cytotoxicity of vinblastine and vincristine to pancreatic acinar cells. - Virchows Arch. B Cell Path. 18: 119-127.

Novikoff, A. B. 1959: The proximal tubule cell in experimental hydronephrosis. - J. biophys. biochem. Cytol. 6: 136-138.

Novikoff, A. B., Essner, E. & Quintana, N. 1964: Golgi apparatus and lysosomes. - Fed. Proc. 23: 1010-1022.

Novikoff, A. B. & Goldfischer, S. 1961: Nucleosidediphosphatase activity in the Golgi apparatus and its usefulness in cytological studies. - Proc. Nat. Acad. Sci. USA 47: 802-810.

Novikoff, P. M., Novikoff, A. B., Quintana, N. & Hauw, J.-J. 1971: Golgi apparatus, GERL, and lysosomes of neurons in rat dorsal root ganglia, studied by thick section and thin section cytochemistry. - J. Cell Biol. 50: 859-886.

Novikoff, A. B. & Shin, W.-Y. 1964: The endoplasmic reticulum in the Golgi zone and its relations to microbodies, Golgi apparatus and autophagic vacuoles in rat liver cells. - J. Microsc. (Paris) 3: 187-206.

Oliver, I. T. 1955: A spectrophotometric method for the determination of creatine phosphokinase and myokinase. - Biochem. J. 61: 116-122.

Orci, L., Le Marchand, Y., Sigh, A., Assimacopoulos-Jeannet, F., Rouiller, Ch. & Jeanrenaud, B. 1973: Role of microtubules in lipoprotein secretion by the liver. - Nature (London) 244: 30-32.

Paavola, L. G. 1978a: The corpus lutem of the guinea pig. II. Cytochemical studies on the Golgi complex, GERL, and lysosomes in luteal cells during maximal progesterone secretion. - J. Cell Biol. 79: 45-58.

Paavola, L. G. 1978b: The corpus lutem of the guinea pig. III. Cytochemical studies on the Golgi complex and GERL during normal postpartum regression of luteal cells, emphasizing the origin of lysosomes and autophagic vacuoles. - J. Cell Biol. 79: 59-73.

Pfeifer, U. 1971: Probleme der cellulären Autophagie. Morphologische, entzymcytochemische und quantitative Untersuchungen an normalen und alterierten Leberepithelien der Ratte. - Erg. Anat. Entwgesch., 44: 1-74.

Pfeifer, U. 1972: Morphologische und funktionelle Aspekte der cellulären Autophagie. - Acta Morph. Acad. Sci. Hung. 20: 247-267.

Pfeifer, U. 1976: Lysosomen und Autophagie. - Verh. Dtsch. Ges. Path. 60: 28-64.

Pfeifer, U. 1978: Inhibition by insulin of the formation of autophagic vacuoles in rat liver. A morphometric approach to the kinetics of intracellular degradation by autophagy. - J. Cell Biol. 78: 152-167.

Pfeifer, U. 1979: Inhibited autophagic degradation of cytoplasm during compensatory growth of liver cells after partial hepatectomy. - Virchows Arch. B Cell Path. 30: 313-333.

Pfeifer, U. & Guder, W. G. 1975: Stimulation of cellular autophagy by parathyroid hormone and cyclic adenosine 3', 5'-monophosphate in isolated tubular fragments from the rat's kidney cortex. - Virchows Arch. B. Cell Path. 19: 51-67.

Pfeifer, U. & Scheller, H. 1975: A morphometric study of cellular autophagy including diurnal variations in kidney tubules of normal rats. - J. Cell Biol. 64: 608-621.

de Priester, W. 1972: Lysosomes in the midgut of *Calliphora erythrocephala* Meigen. - Z. Zellforsch. 129: 430-446.

Quataker, J. R. 1971: Formation of autophagic vacuoles during human corpus luteum involution. - Z. Zellforsch. 122: 479-487.

Reaven, E. P. & Reaven, G. M. 1980: Evidence that microtubules play a premissive role in hepatocyte very low density lipoprotein secretion. - J. Cell Biol. 84: 28-39.

Reid, I. M. 1973: An ultrastructural and morphometric study of the liver of the lactating cow in starvation ketosis. - Exp. Mol. Path. 18: 316-330.

Rohr, H., Oberholzer, M., Bartsch, G. & Keller, M. 1976: Morphometry in experimental pathology: Methods, baseline data and applications. - Int. Rev. Exp. Pathol. 15: 233-325.

Saito, T. & Ogawa, K. 1974: Lysosomal changes in rat hepatic parenchymal cells after glucagon administration. - Acta Histochem. Cytochem. 7: 1-18.

Scornik, O. A. & Botbol, V. 1976: Role of changes in protein degradation in the growth of regenerating livers. - J. Biol. Chem. 251: 2891-2897.

Shelburne, J. D., Arstila, A. U. & Trump, B. F. 1973: Studies on cellular autophagocytosis: Cyclic AMP- and dibutyryl cyclic AMP-stimulated autophagy in rat liver. - Amer. J. Pathol. 72: 521-534.

Stalmans, W. & Hers, H.-G. 1975: The stimulation of liver phosphorylase b by AMP, fluoride and sulfate. - Eur. J. Biochem. 54: 341-350.

Stein, O., Slanger, L. & Stein, Y. 1974: Colchicine-induced inhibition of lipoprotein and protein secretion into the serum and lack of interference with secretion of biliary phospholipids and cholesterol by rat liver *in vivo*. - J. Cell Biol. 62: 90-103.

Stein, O. & Stein, Y. 1973: Colchicine-induced inhibition of very low density lipoprotein release by rat liver *in vivo*. - Biochim. biophys. Acta (Amst.) 306: 142-147.

Stäubli, W., Hess, R. & Weibel, E. R. 1969: Correlated morphometric and biochemical studies on the liver cell. II. Effects of phenobarbital on rat hepatocytes. - J. Cell Biol. 42: 92-112.

Sutherland, E. W. 1968: Polysaccharide phosphorylase, liver. In: Colowick, S. P. & Kaplan, N. O. (eds.), Methods in enzymology, vol. 1: 215-222 pp. Academic Press, New York.

Svoboda, G. H., Gorman, M. & Root, M. A. 1964: Alkaloids of Vinca rosea (*Catharanthus roseus*) XXVIII. A preliminary report of hypoglycemic activity. - Lloydia 27: 361-363.

Tappel, A. L. 1969: Lysosomal enzymes and other components. In: Dingle, J. T. & Fell, H. B. (eds.), Lysosomes in biology and pathology, vol. 2: 207-244 pp. North-Holland Publ. Co., Amsterdam, London.

Terry, R. D., Wisniewski, H. & Johnson, A. B. 1970: Studies on the formation of autophagic vacuoles in neurons treated with spindle inhibitors (colchicine and vinblastine). - J. Neuropathol. exp. Neurol. 29: 142-143.

Trout, J. J. & Viles, J. M. 1979: Cellular changes associated with Triton WR-1339 accumulation in rat hepatocytes. I. Autophagy. - Exp. Mol. Path. 30: 230-241.

Tyson, G. E. & Bulger, R. E. 1973: Vinblastine-induced pracrystals and unusually large microtubules (macrotubules) in rat renal cells. - Z. Zellforsch. 141: 443-458.

Venable, J. H. & Coggeshall, R. 1965: A simplified lead citrate stain for use in electron microscopy. - J. Cell Biol. 25: 407-408.

Volk, B. W., Wellmann, K. F. & Lewitan, A. 1966: The effect of irradiation on the fine structure and enzymes of the dog pancreas. I. Short-term studies. - Amer. J. Pathol. 48: 721-753.

Watson, M. L. 1958: Staining of tissue sections for electron microscopy with heavy metals. - J. biophys. biochem. Cytol. 4: 475-478.

Weibel, E. R. 1969: Stereological principles for morphometry in electron microscopic cytology. - Int. Rev. Cytol. 26: 235-302.

Weibel, E. R. & Bolender, R. P. 1973: Stereological techniques for electron microscopic morphometry. In: Hayat, M. A. (ed.), Principles and techniques of electron microscopy, vol. 3: 239-296 pp. Van Nostrand Reinhold Co., New York.

Whaley, W. G. 1975: The Golgi apparatus. Cell biology monographs, vol. II, Springer-Verlag, Wien, New York.

LEGENDS FOR FIGURES

Fig. 1. Light micrographs of mouse liver stained with hematoxylin and eosin. a) Four hours after VBL injection (10 mg/kg). Almost no changes have occurred as compared to control livers. C, central vein, P, portal area. Magnification (M): 175 x. b) Mouse liver 12 hours after VBL injection (10 mg/kg). Almost all parenchymal cells are vacuolated. Single necrotic cells are seen in the liver lobule (arrow head). M: 175 x. c) Mouse liver 4 hours after VBL injection (50 mg/kg), showing vacuolation of the hepatocytes and lipid droplet accumulation around the central vein (arrows). M: 175 x. d) Mouse liver 12 hours after VBL injection (50 mg/kg). The parenchymal cells are vacuolated and there are focal necrotic cell groups (arrow heads) diffusely scattered in the liver lobule. The central vein is dilated. M: 175 x. (From the paper of Hirsimäki, P., Trump, B. F. & Arstila, A. U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

Fig. 2. Mouse liver after VBL injection (10 mg/kg), stained with the PAS-technique. a) One hour after VBL injection. The hepatocytes contain large amounts of PAS-positive material, presumed to be glycogen, in all parts of the liver lobules. The animal starved after the injection. P, portal area, C, central vein. M: 70 x. b) Twelve hours after VBL injection. PAS-positive material is absent from both portal and central areas. The animal starved after the injection. A similar appearance is seen in overnight starved mice given only saline and in non-starved mice given VBL. M: 175 x. c) Two hours after saline injection. Little PAS-positive material is seen around the central veins, but large amounts are still left around the portal area. The animal starved after the injection. M: 70 x. d) Two hours after VBL injection. PAS-positive material has disappeared from around the central veins and diminished around the portal areas. The animal starved after the injection. M: 70 x. e) Four hours after saline injection. PAS-positive material is seen around the portal areas but very little is left around the central veins. The animal starved after the injection. M: 70 x. f) Four hours after VBL injection. Only hepatocytes around the portal areas show some PAS-positive material. The animal starved after the injection. M: 70 x.

Fig. 3. Control mouse hepatocytes after saline injection. No autophagic vacuoles are seen in the cytoplasm. N, nucleus, M, mitochondria, MB, microbody, RER, rough surfaced endoplasmic reticulum, SER, smooth surfaced endoplasmic reticulum, BC, bile canaliculus. M: 20 000 x.

Fig. 4. Control mouse hepatocyte after saline injection. Microtubules (arrows) are seen near the Golgi apparatus (Go). M: 39 000 x.

Fig. 5. Mouse hepatocytes 1 hour after VBL injection (10 mg/kg). Autophagic vacuoles containing glycogen (arrow heads), pieces of endoplasmic reticulum and cytoplasmic ground substance are seen in the cytoplasm. Glycogen is also seen in the cytoplasm (large arrows). Ly, lysosome (dense body). M: 20 000 x.

Fig. 6. Mouse hepatocyte 1 hour after VBL injection (25 mg/kg). Autophagic vacuoles (AV) at various stages of development are seen in the cytoplasm: AV_2: an apparently newly formed large AV with clearly recognizable contents limited by a double membrane, AV_1: a smaller single membrane-limited AV with still recognizable contents, RB: an AV with unrecognizable contents showing signs of transformation to a residual body. Vesicles containing VLDL particles are also seen in the cytoplasm (arrows). M: 16 000 x.

Fig. 7. Mouse hepatocyte 4 hours after VBL injection (50 mg/kg). Numerous autophagic vacuoles (1-14) are seen in the cytoplasm. Most of these are limited by a double membrane with clearly recognizable contents such as mitochondria (6 and 13) or cytoplasmic ground substance containing pieces of endoplasmic reticulum (1-5, 7, 12 and 14). Some of the autophagic vacuoles contain digested material (8-11) and are limited by a single membrane (8). Rough surfaced endoplasmic reticulum (RER) is vesiculated and partly degranulated. M: 16 000 x. (From the paper of Hirsimäki, P., Trump, B. F. & Arstila, A. U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

Fig. 8. Autophagic vacuoles (AV) with various contents. a) An AV containing fragments of endoplasmic reticulum and cytoplasmic ground substance (ERGS). M: 30 000 x. b) An AV containing a mitochondrion. M: 30 000 x. c) An AV containing a microbody and ERGS. M: 30 000 x. d) An AV containing fat and ERGS. M: 30 000 x. e) An AV (arrow head) containing a vacuole with VLDL particles and ERGS. M: 30 000 x. f) An AV (arrow head) containing glycogen. M: 20 000 x.

Fig. 9. Mouse hepatocyte 12 hours after VBL injection (10 mg/kg). No autophagic vacuoles are seen in the cytoplasm. M: 26 000 x. (From the paper of Hirsimäki, P., Trump, B. F. & Arstila, A. U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

Fig. 10. Mouse hepatocyte 12 hours after VBL injection (50 mg/kg). Autophagic vacuoles (large arrows) are present in the cytoplasm. They are mostly limited by a single membrane, showing transformation to residual bodies. Note secretory vesicles containing VLDL particles (arrow heads) and lipid droplet (L) accumulation in the cells. The Golgi cisternae (Go) are swollen, containing VLDL particles. Ly, lysosome. M: 8 000 x. (From the paper of Hirsimäki, P., Trump, B. F. & Arstila, A. U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

Fig. 11. Mouse hepatocytes and a Kupffer cell (K) 12 hours after VBL injection (50 mg/kg). Degraded material (large arrow) resembling that found in hepatocyte residual bodies (RB) is seen in the sinusoidal lumen (L). Phagocytized material is seen in the Kupffer cell phagosomes (arrow heads).

M: 16 000 x. (From the paper of Hirsimäki, P., Trump, B. F. & Arstila, A. U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

Fig. 12. Mouse hepatocyte 12 hours after VBL injection (50 mg/kg). Secretory vesicles containing VLDL particles (arrows) are accumulated near the space of Disse (D). M: 16 000 x. (From the paper of Hirsimäki, P., Trump, B. F. & Arstila, A. U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

Fig. 13. Mouse hepatocyte 12 hours after VBL injection (50 mg/kg). The Golgi cisternae (Go) are swollen and contain VLDL particles. The rough surfaced endoplasmic reticulum (RER) is vesiculated and partly degranulated. M: 20 000 x. (From the paper of Hirsimäki, P., Trump, B. F. & Arstila, A. U. 1976: Studies on vinblastine-induced autophagocytosis in the mouse liver. I. The relation of lysosomal changes to general injurious effects. - Virchows Arch. B Cell Path. 22: 89-109. By permission of Springer-Verlag)

Fig. 14. A control mouse hepatocyte stained with unbuffered OsO_4. The outermost cisternae and the adjacent vesicles on the forming face of the Golgi apparatus (Go) are densely stained. The degree of unbuffered OsO_4 staining is graded across the stack. The cisterna enclosing an autophagic vacuole (AV), and some vesicles within the AV are also stained. M: 20 000 x. (From the paper of Hirsimäki, P. & Reunanen, H. 1980: Studies on vinblastine-induced autophagocytosis in mouse liver. II. Origin of membranes and acquisition of acid phosphatase. - Histochemistry 67: 139-153. By permission of Springer-Verlag)

Fig. 15. Mouse hepatocyte 2 hours after VBL injection (50 mg/kg) stained with unbuffered OsO_4. The vesicles within multivesicular bodies (MVB) and unidentified cytoplasmic vesicles (arrow heads) are stained. The dilated outer cisternae of the Golgi apparatus (Go) are also stained. The cisternae enclosing autophagic vacuoles (AV) are not stained but an older AV has osmium deposits in its matrix (arrow). M: 16 000 x. (From the paper of Hirsimäki, P. & Reunanen, H. 1980: Studies on vinblastine-induced autophagocytosis in mouse liver. II. Origin of membranes and acquisition of acid phosphatase. - Histochemistry 67: 139-153. By permission of Springer-Verlag)

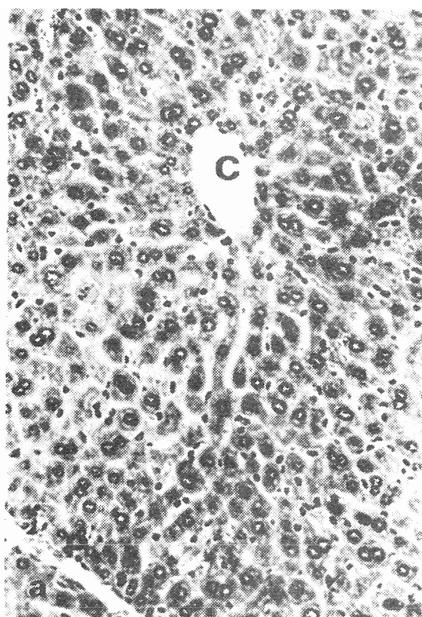
Fig. 16. Mouse hepatocytes 15 min after VBL injection (50 mg/kg) stained with unbuffered OsO_4. a) An early stage of autophagic vacuole formation. A stained cisterna partially encloses a mitochondrion. M: 20 000 x. b) Later stage of autophagic vacuole formation. Portions of two hepatocytes around a bile canaliculus (BC). A part of the cytoplasm is surrounded by double membranes and the space between these is stained with unbuffered OsO_4. Note the dilation of the cisternae of the Golgi apparatus (Go) and the staining of cytoplasmic vesicles (arrow heads). M: 20 000 x. (From the

paper of Hirsimäki, P. & Reunanen, H. 1980: Studies on vinblastine-induced autophagocytosis in mouse liver. II. Origin of membranes and acquisition of acid phosphatase. - Histochemistry 67: 139-153. By permission of Springer-Verlag)

Fig. 17. Two mouse hepatocytes from the same section 15 min after VBL injection (50 mg/kg) stained with unbuffered OsO_4. The degree of staining of the endoplasmic reticulum (ER) varies in different cells. a) The ER shows a light granular staining pattern. The ER cisternae and the mitochondria are lightly swollen. AV, autophagic vacuole, BC, bile canaliculus. M: 16 000 x. b) The ER is heavily stained and the mitochondria are swollen. M: 16 000 x. (From the paper of Hirsimäki, P. & Reunanen, H. 1980: Studies on vinblastine-induced autophagocytosis in mouse liver. II. Origin of membranes and acquisition of acid phosphatase. - Histochemistry 67: 139-153. By permission of Springer-Verlag)

Fig. 18. Mouse hepatocytes 30 min after VBL injection (50 mg/kg) stained for inosine diphosphatase activity. Enzyme activity is seen in the cisternae of the rough surfaced endoplasmic reticulum and in the vesicles of the smooth surfaced endoplasmic reticulum (arrows). a) An early stage of autophagic vacuole formation. The membranes partly surrounding a mitochondrion (arrow heads) have no enzyme activity, but the cisternae of endoplasmic reticulum probably being engulfed have. M: 17 000 x. b) No enzyme activity can be seen between the limiting membranes surrounding an autophagic vacuole but the engulfed material is stained (arrow heads). M: 20 000 x. c) No enzyme activity can be seen between the double membranes (2) or in the single membrane (1) surrounding autophagic vacuoles. Go, Golgi apparatus. M: 16 000 x. (From the paper of Hirsimäki, P. & Reunanen, H. 1980: Studies on vinblastine-induced autophagocytosis in mouse liver. II. Origin of membranes and acquisition of acid phosphatase. - Histochemistry 67: 139-153. By permission of Springer-Verlag)

Fig. 19. Mouse hepatocytes after VBL injection (50 mg/kg) stained for acid phosphatase activity. a) 30 min after injection. A number of newly formed autophagic vacuoles with clearly recognizable contents show no or little (arrow) acid phosphatase activity. A lysosome (Ly) has enzyme activity. M: 20 000 x. b) 2 hours after injection. A double membrane-limited autophagic vacuole with lead deposits between the membranes. M: 20 000 x. c) 2 hours after VBL injection. An older autophagic vacuole with lead deposits in the matrix. Go, Golgi apparatus. M: 20 000 x. (From the paper of Hirsimäki, P. & Reunanen, H. 1980: Studies on vinblastine-induced autophagocytosis in mouse liver. II. Origin of membranes and acquisition of acid phosphatase. - Histochemistry 67: 139-153. By permission of Springer-Verlag)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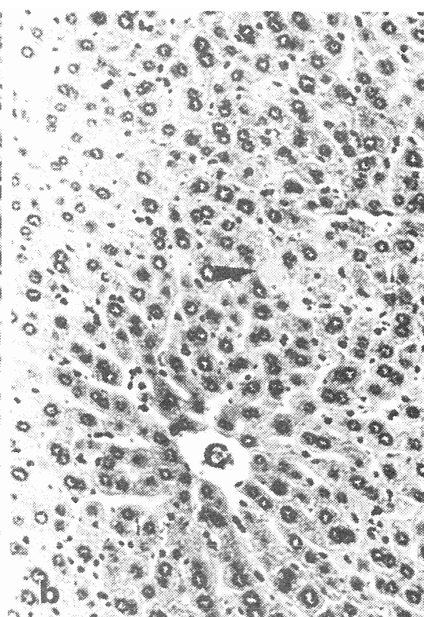
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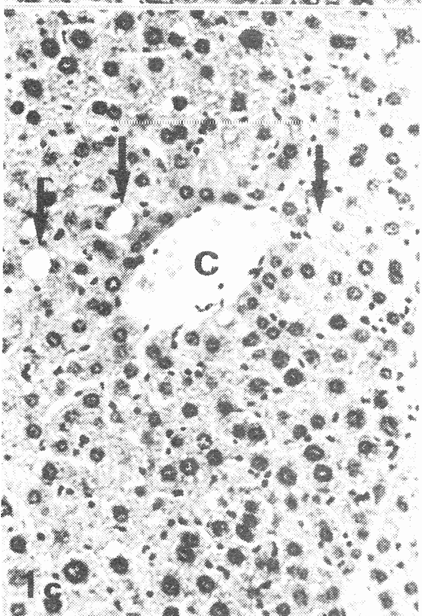
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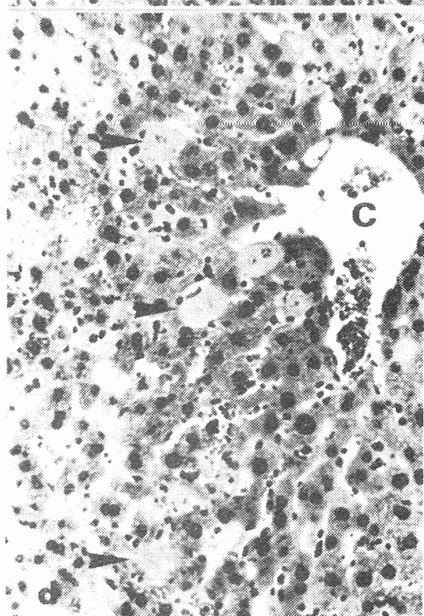
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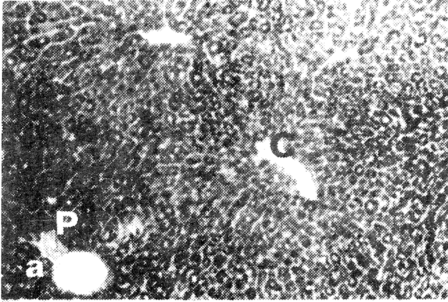
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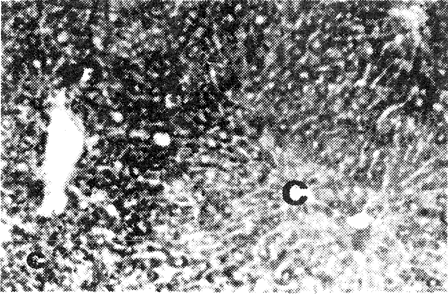
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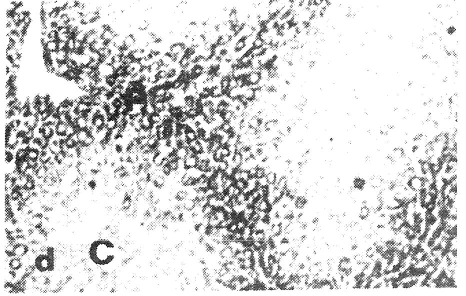
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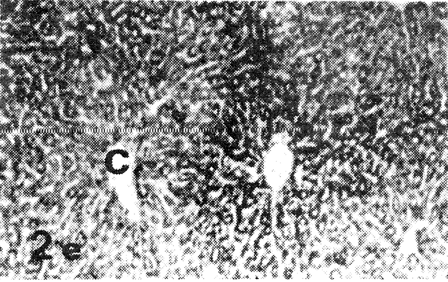
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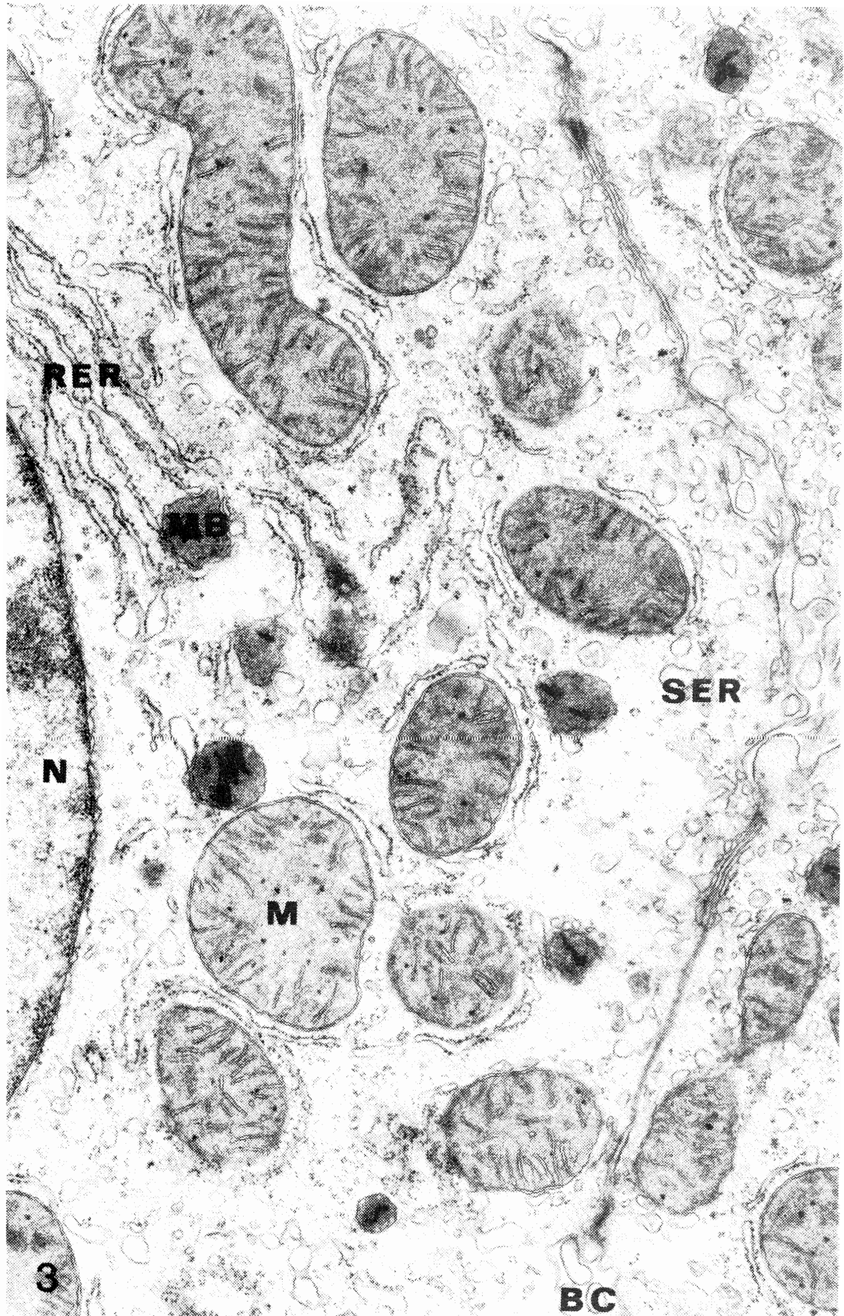
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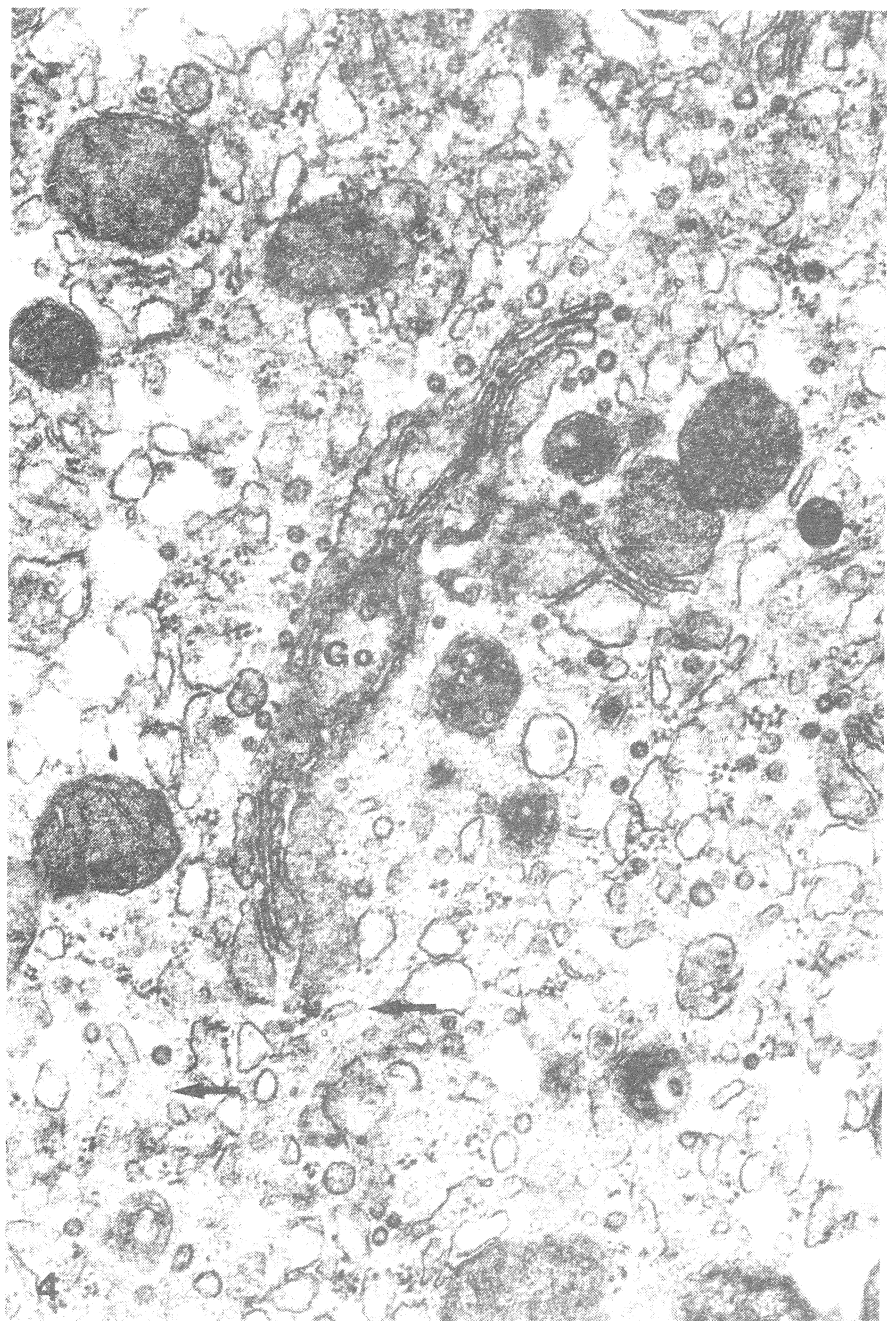
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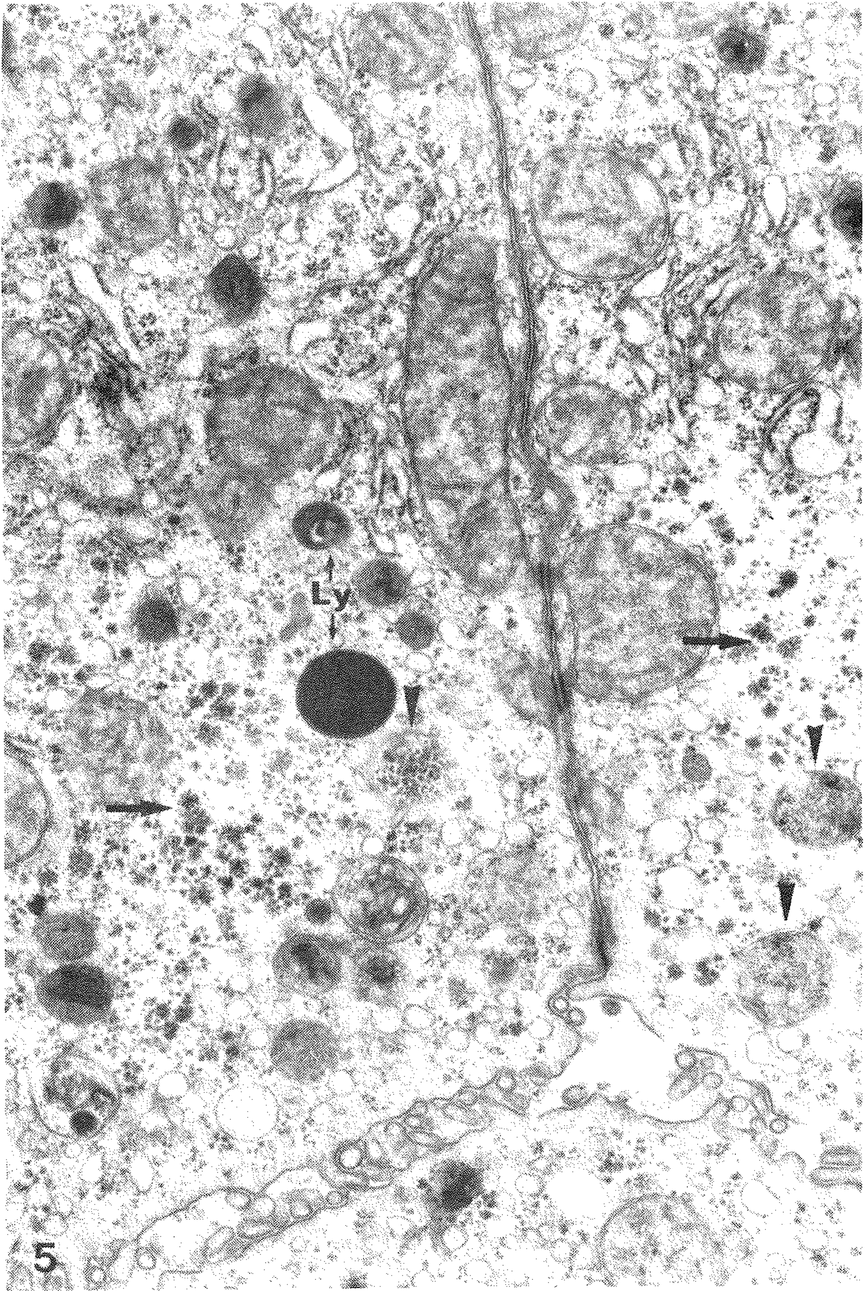
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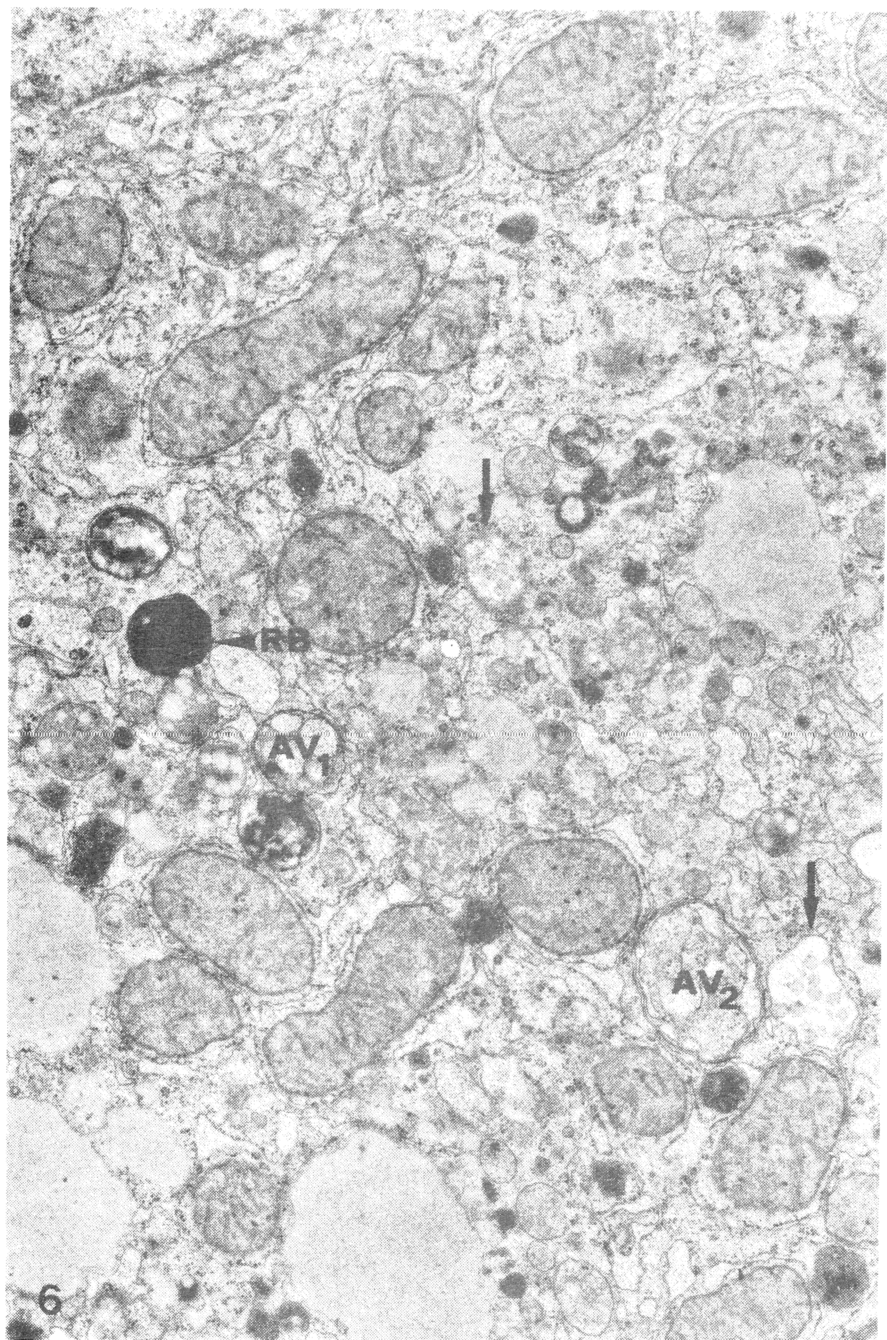
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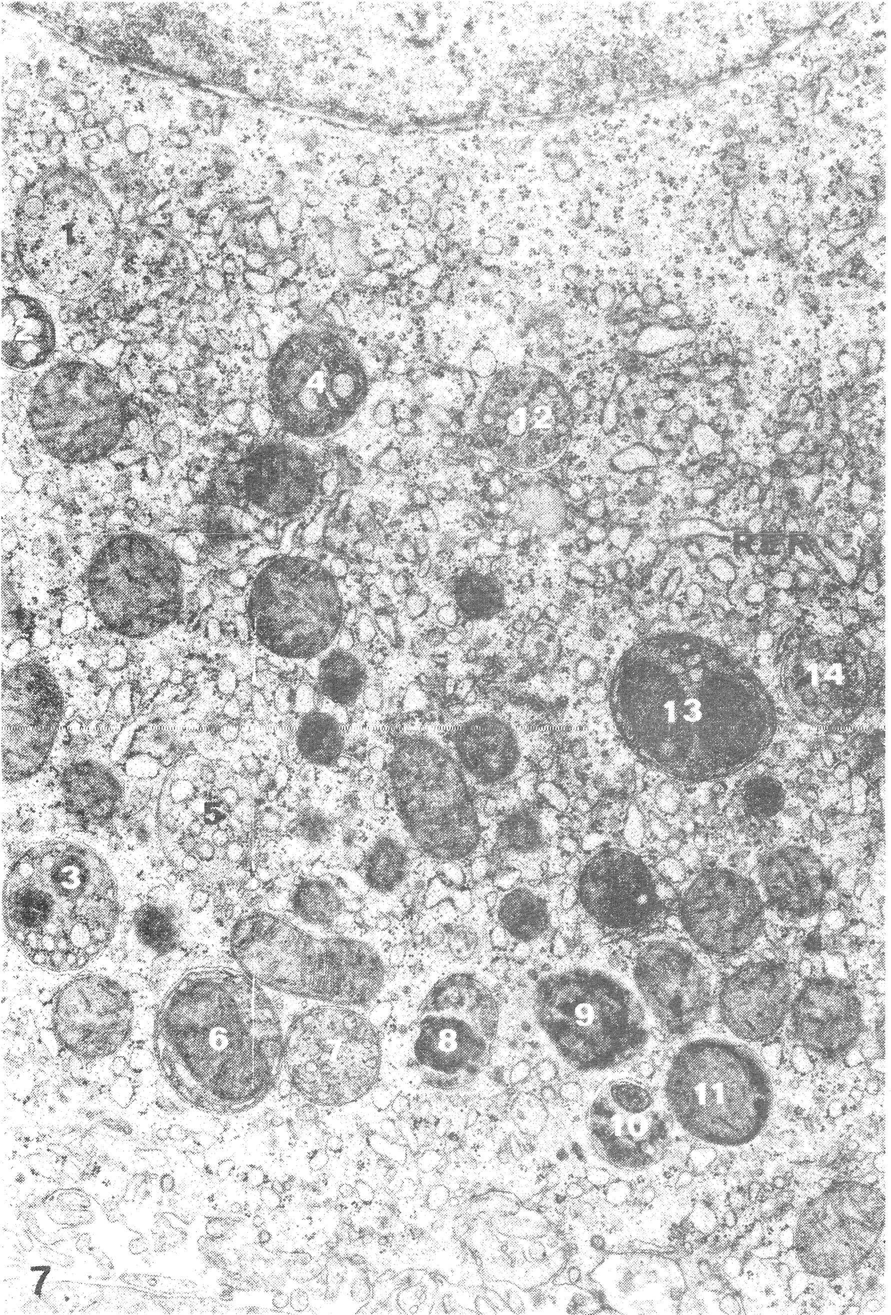
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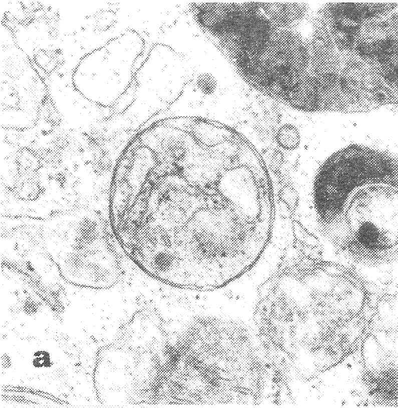
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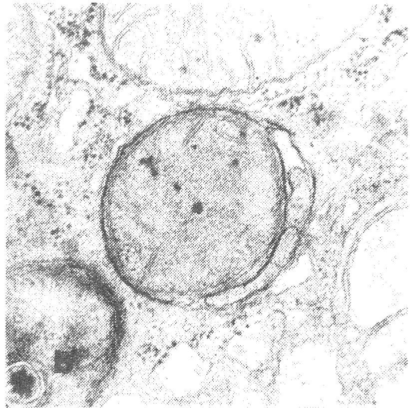

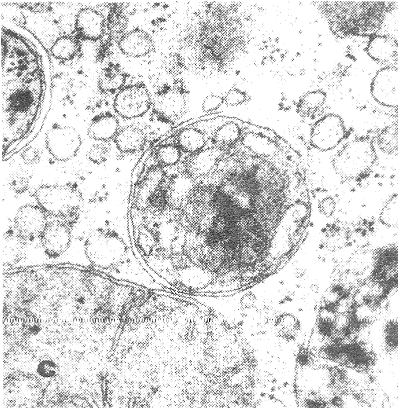
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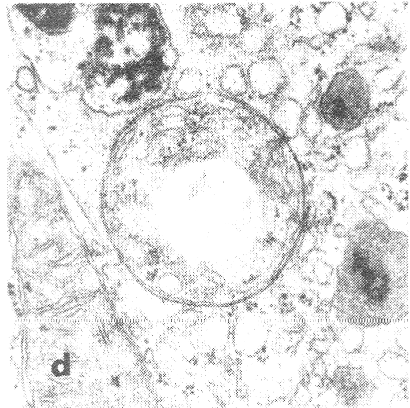
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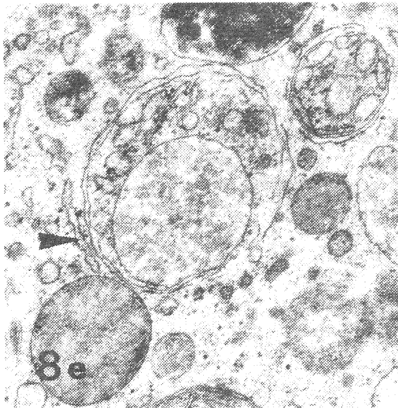
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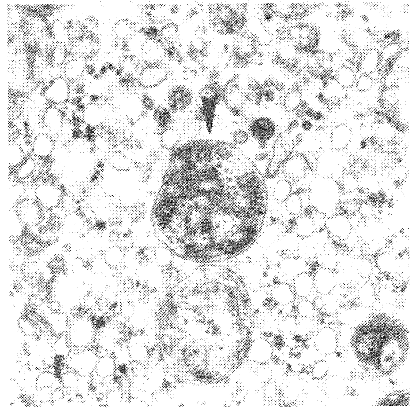
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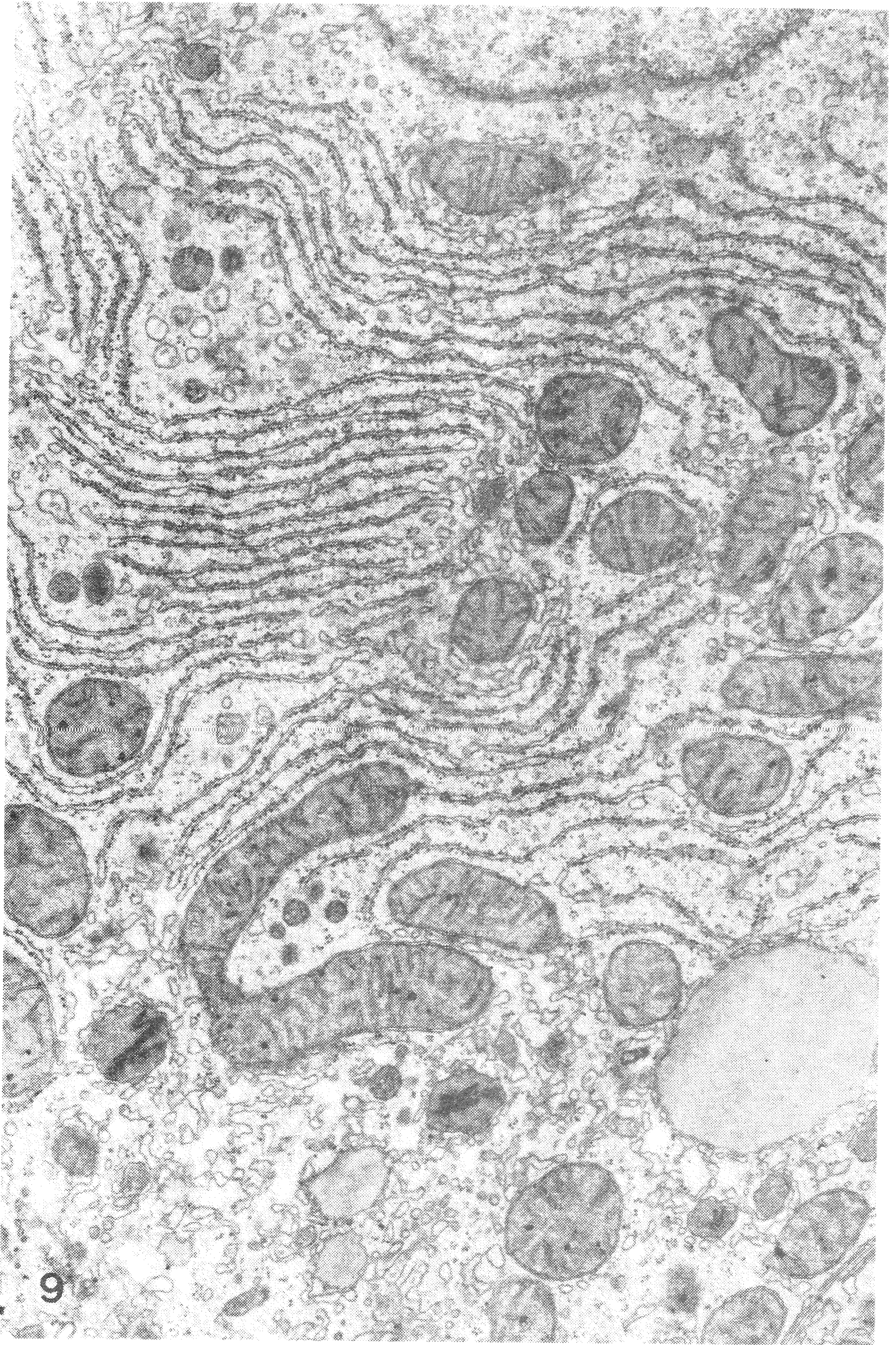
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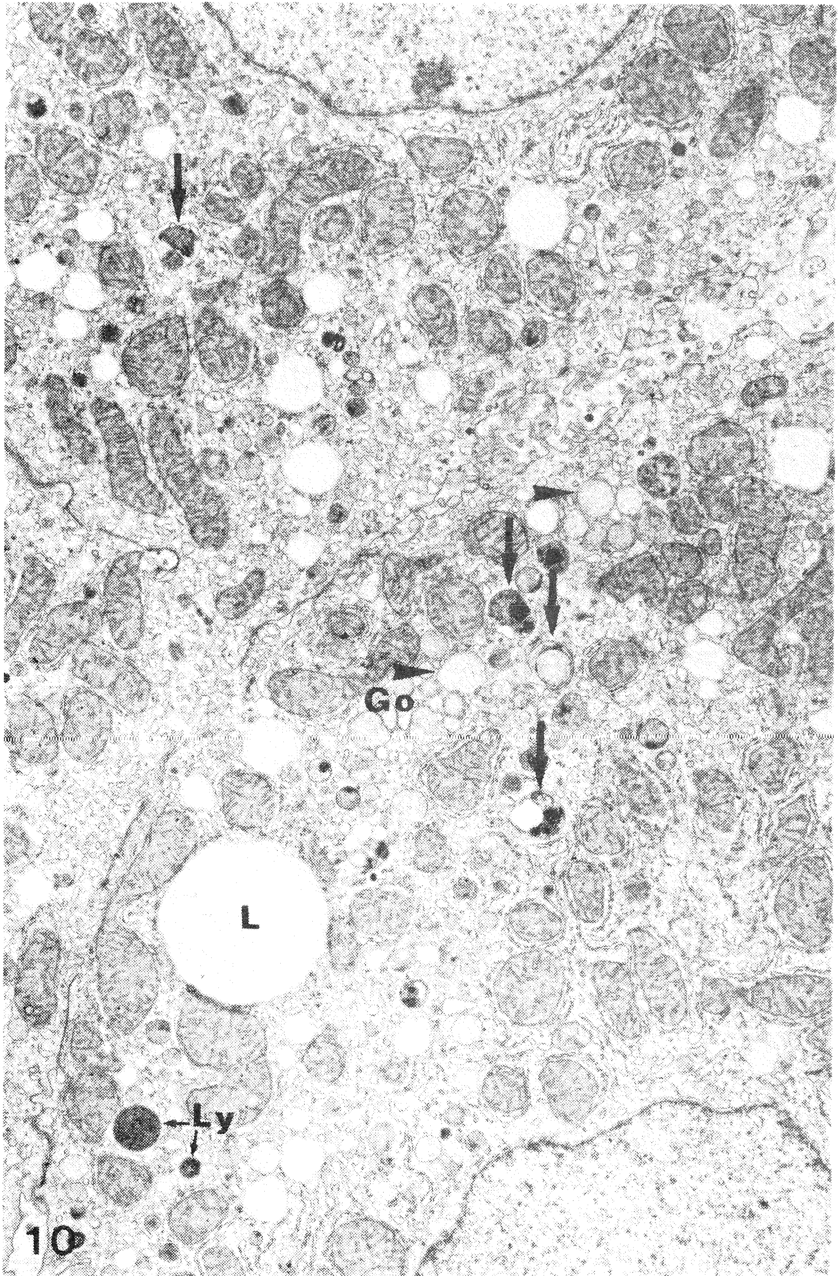
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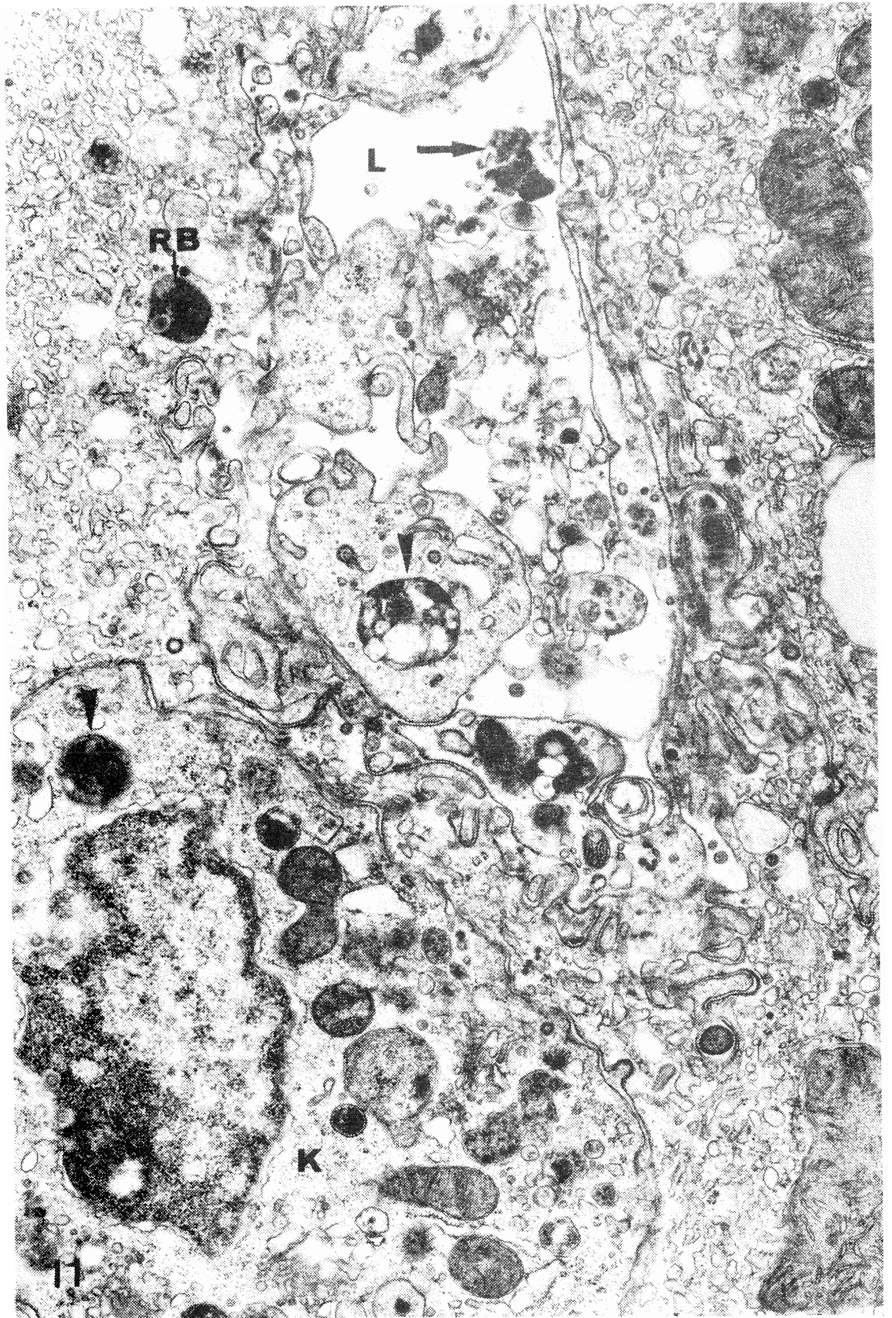
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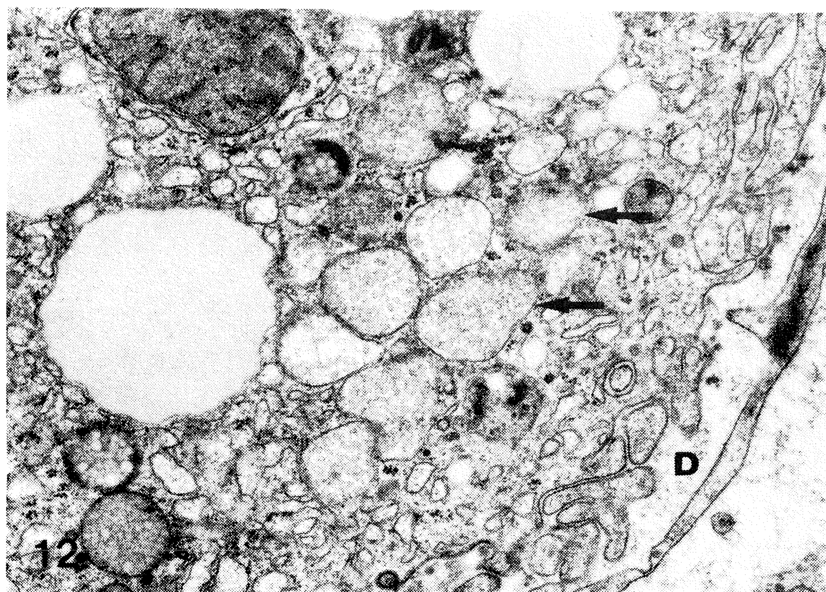
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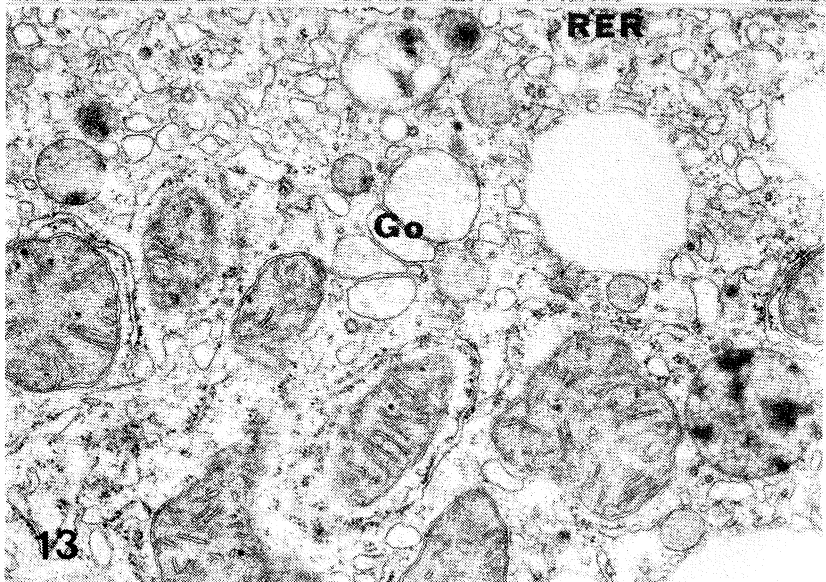
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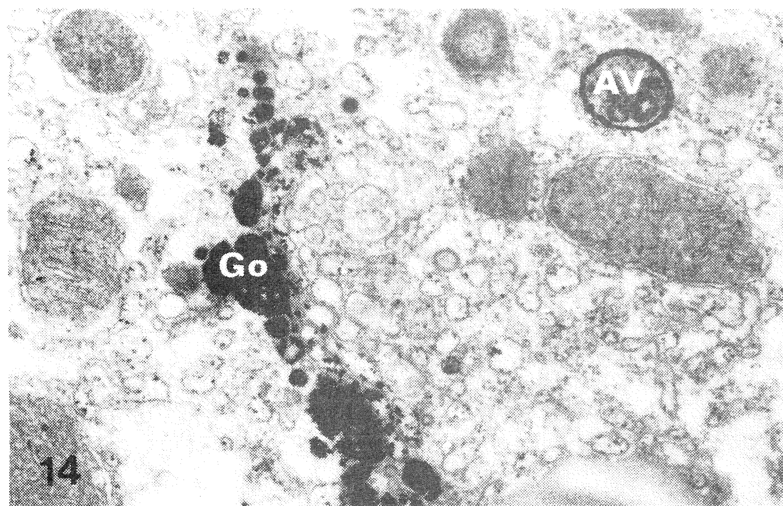
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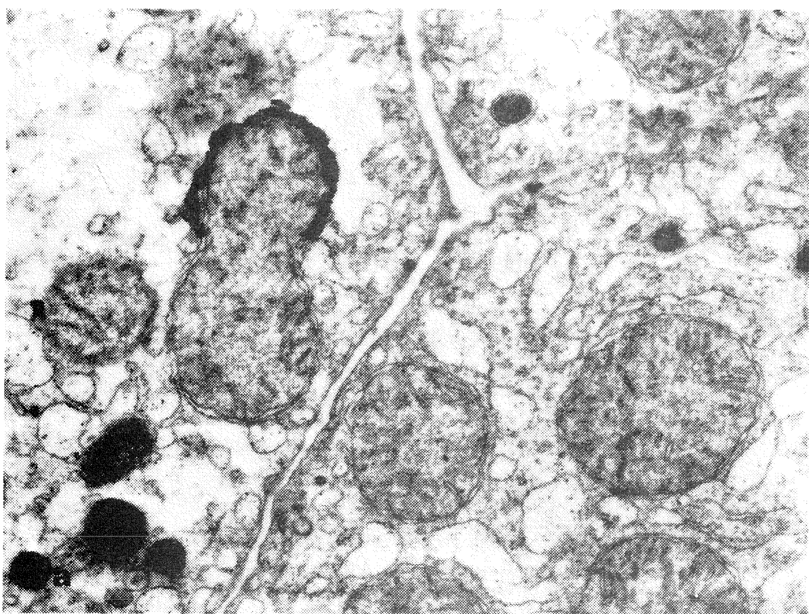
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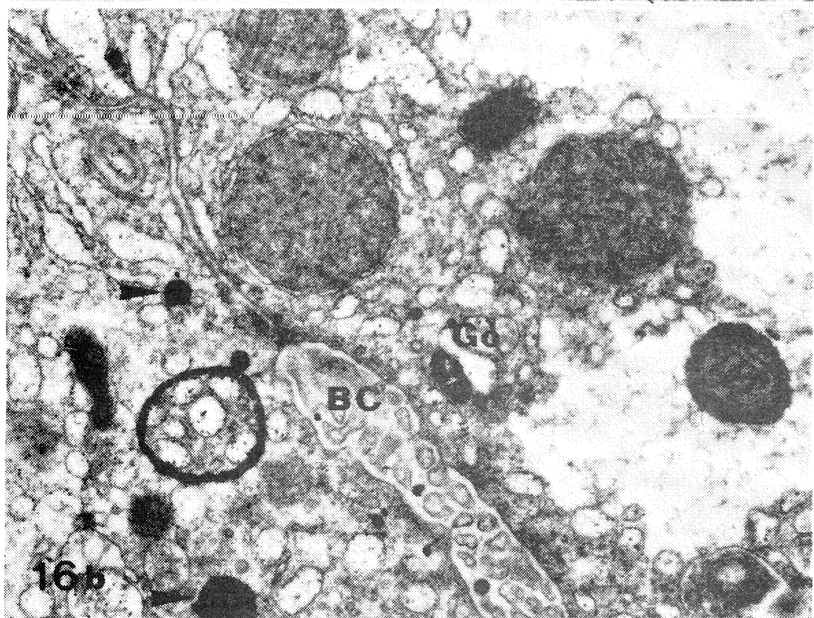
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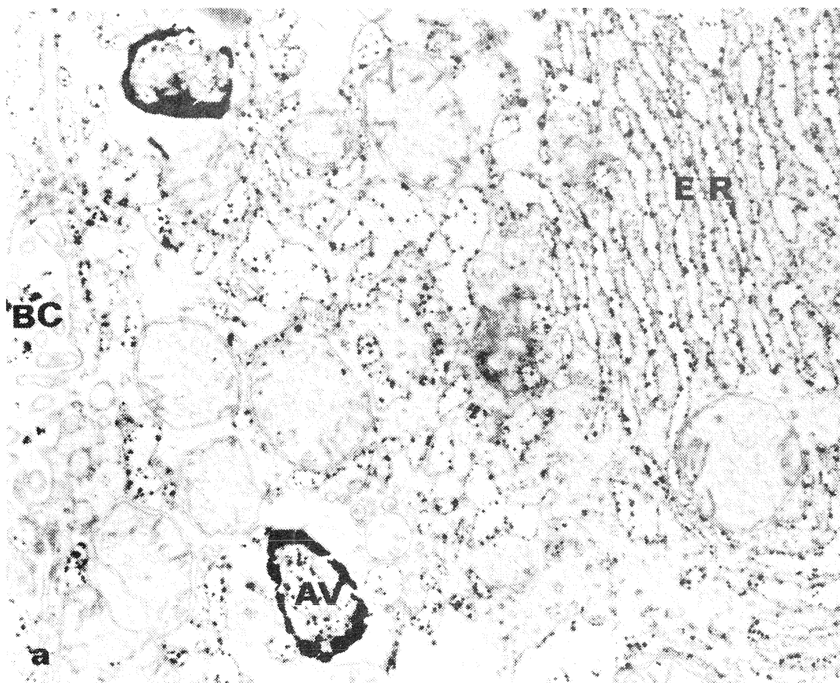
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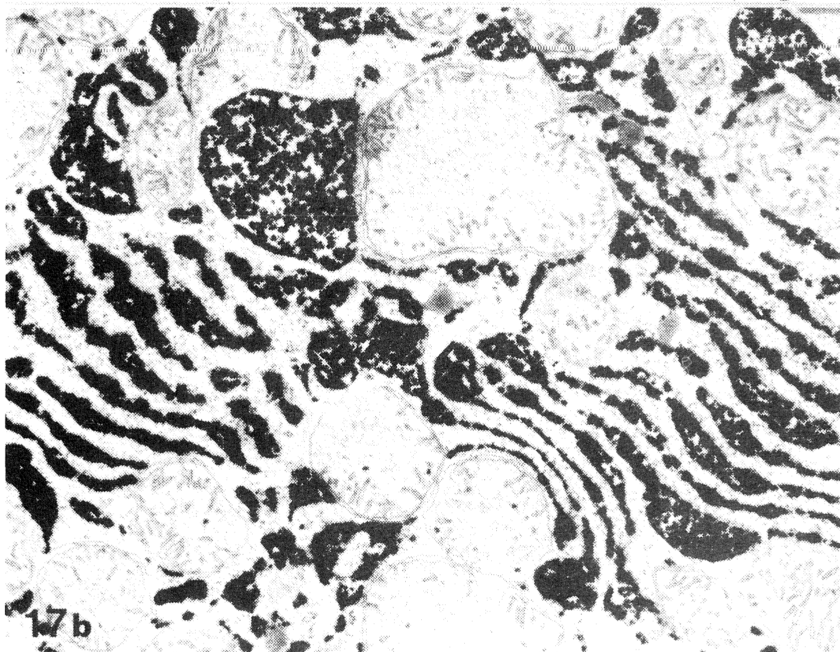
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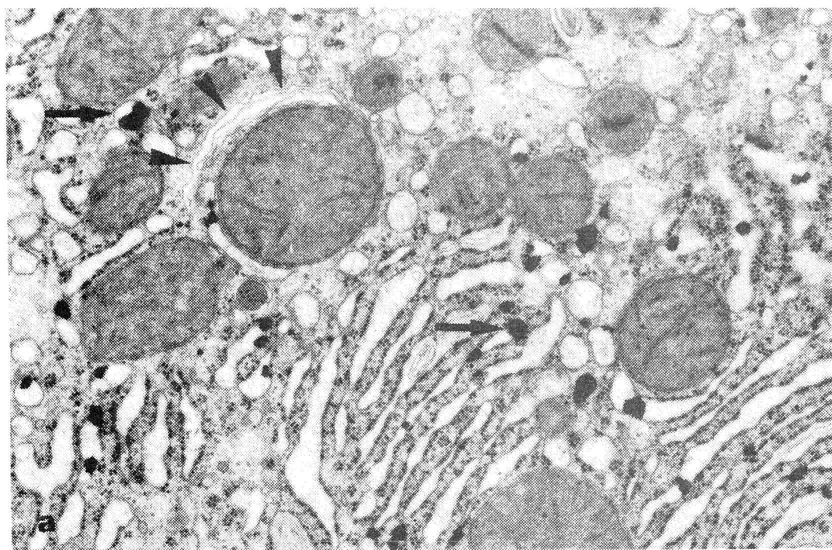
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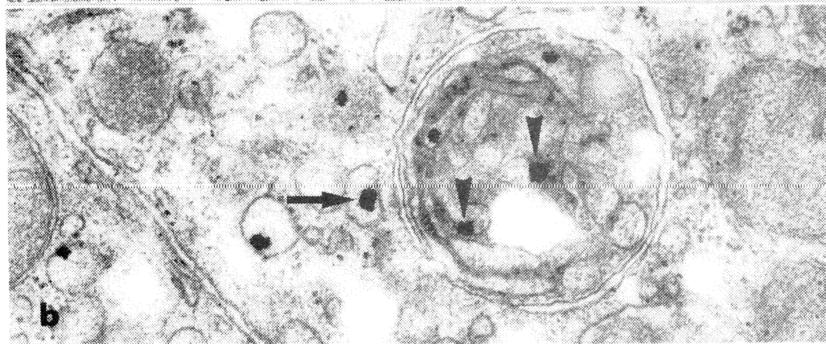
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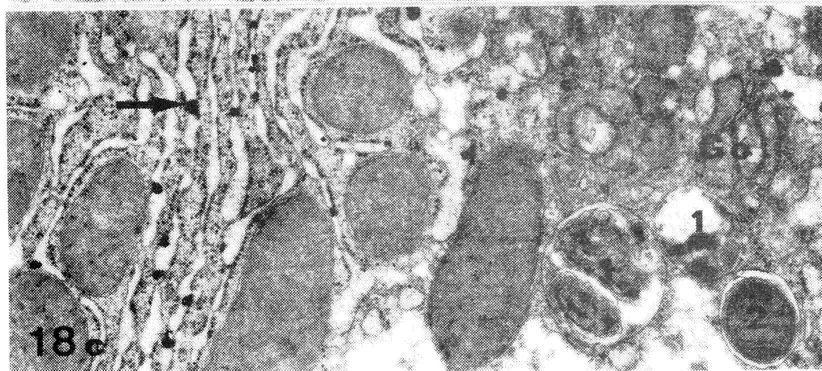
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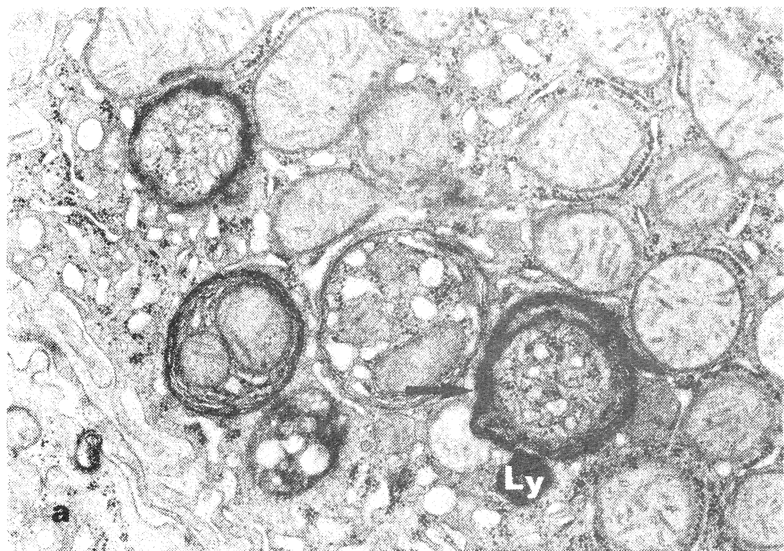
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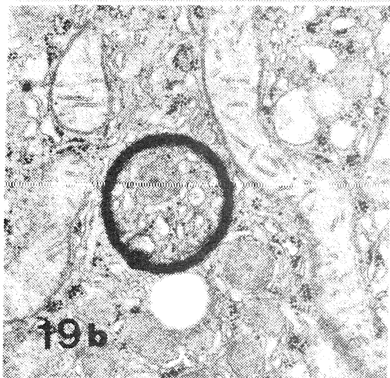
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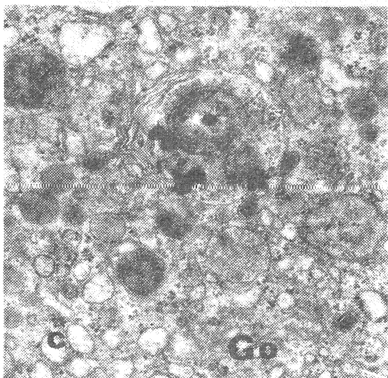
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