Cell Research (1990), **2**, 207−215

Ultracytochemical localization of H+–adenosine triphosphatase activity in autophagic vacuoles induced by vinblastine in rat liver

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ABSTRACT

H^+–adenosine triphosphatase (H^+–ATPase) activity was demonstrated cytochemically in autophagic vacuoles (AVs) of rat hepatocytes using a modification of the method for the demonstration of neutral *p*-nitrophenyl phosphatase (*p*-NPPase) activity [1]. When an inhibitor of H^+–ATPase, N–ethylmaleimide (IqEM) or 4, 4'–diisothiocyanostilbene–2, 2'disulfonic acid, disodium salt (DIDS) was included in the incubation medium the enyzme activity was abolished indicating that *p*-NPPase demonstrated in this study represents H^+–ATPase. Autophagy was induced by a single intraperitoneal injection of vinblastine sulfate (VBL). The number of AVs increased remarkably in hepatocytes from 40 min after VBL treatment. H^+-ATPase activity was observed mainly on the membranes of lysosomes and AVs. However, early forms of AVs containing only incompletely digested material showed no H^+–ATPase activity. Most AVs revealing a positive reaction seemed to be in advanced stages of development. Acid phosphatase acticity was demonstrable in mature but not in early forms of AVs. The present investigation showed that membranes of advanced stage AVs possess an H^+–ATPase which may be derived from lysosomal membranes.

Key words: *Rat, hopatocyto, autophagy, H^+–ATPaso.*

INTRODUCTION

Increasing attention is being focused on the process of autophagy because of its importance in the intracellular catabolism of endogenous substances through the lysosomal compartment. However, the mechanism of autophagy is less understood than the mechanism of heterophagy, because autophagy has proved more difficult to manipulate

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experimentally [2]. Under physiological conditions the number of detectable autophagic vacuoles (AVs) is usually small although it varies in different cell types [3]. Therefore, morphological investigations of autophagy have been performed frequently using experimental conditions in which AVs were induced by tho administration of hormones such as glucagon [4], microtubular inhibitors such as vinblastine [5], and inhibitors of lysosomal enzymes such as leupeptin [6].

One of the major unresolved questions of the autophagic process is the origin of the limiting membrane of the AVs. On the basis of electron microscopic observation [7, 8], enzyme cytochemistry [9], osmium impregenation and freeze-fracture studies [10] it has been proposed that the membranes of AVs are derived from various existing cell membranes including lysosomal membranes and/or they are Synthesized *de novo* during the autophagic process.

Recently, we have cytochemically demonstrated p-NPPase activity in lysosomal membranes of rat liver at neutral pH and surmised that it represents H^+-ATPase activity responsible for the generation of the intralysosomal acid pH. In this study, we detected H^+–ATPase activity in membranes of AVs induced by vinblastine and discuss the relation between lysosomes and AVs from the viewpoint of H^+– ATPase activity.

MATERIALS AND METHODS

Male and female, 6－7 weeks old Wistar rats (250－300 g) were used in this experiment. The animals were fed standard chow and water *ad libitum*.

Vinblastine (VBL, Sigma Chemical Lo. Ltd.) dissolved in physiological saline solution was injected intraperitonealiy in doses of 20－40 mg/kg body weight. At the time intervals of 15, 30, 40 rain, 1, 2, 3 and 4 hr after VBL injection, the animals were perfused first with 0.9% NaCl containing heparin (10 I.U./ml) through the left ventricle for 5－10min under sodium pentobarbital anesthesia (Sommopentyl 0.1 ml/100g b.w., i.p.) and then perfused with a mixture of 2 % formaldehyde and 0.25 % glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, for 15 rain at room temperature. The livers were washed in 0.1 *M* cacodylate buffer, pH 7.4, containing 5 % sucrose (rinsing buffer) for 24 hr at 4℃. Non–frozen sections of 40－50 μm in thickness were prepared with a Microslicer (Dosaka EM Co. Ltd., Kyoto). The sections were incubated in the reaction medium for the detection of *p*-NPPase activity for 10－30 min at room temperature. The reaction medium consisted of 5 m*M* *p*-nitrophenyI phosphate (Mg salt), 50 m*M* KC1, 2.5 m*M* levamisole, 25% dimethyl sulfoxide, 5－10 m*M* NaF, and 10 m*M* lead citrate in 25 m*M* Tricine–KOH buffer, pH 7.4. For cytochemieal controls, an inhibitor of H^+–ATPase, 4, 4'–diisothiocyanostilbene-2, 2'–disulfonic acid (DIDS, 10－15 m*M*) or N–ethylmaleimide (NEM, 10 m*M*) was added to the incubation medium. Acid phosphatase (AcPase) activity was also demonstrated by the method of Gomori [11]. Following incubation, the sections were thoroughly washed in rinsing buffer, post–fixed with 1% OsO_4 in 0.1 *M* cacodylate buffer, pH 7.4, for 40－60 min at 4℃, dehydrated through a graded ethanol series, and embedded in Spurr's resin. Ultrathin sections were cut with an LKB Ultrotome IV 8800, stained briefly with 2% uranyl acetate, and observed under a JEOL–1200 EX electron microscope operated at 80 kV.

RESULTS

Ultrastructure of VBL –treated hepatocytes

The ultrastructures of most cell organelles were essentially normal at 15 rain after VBL injection. The first conspicuous change in VBL–reated hepatocytes was the expansion of ER cisternae at 40 min after the VBL injection (Fig. 1, arrowheads), and 60 min after VBL treatment vacuoles of various shapes ranging from 0.05 to 0.5 μm in diameter were observed in the oytoplasm. However, there were no detectable changes of mitochondrial matrix and oristae. The bile canaliouli and intercelluar junctional complexes seemed to be unaffected by VBL treatment.

In the livers of rats injected with VBL an increase in the number of antophagic vacuoles was observed 15 min after VBL injection and became evident within 40 min suggesting that VBL stimulates autophagocytosis. Although AVs were distributed throughout the cytoplasm they seemed to occur more frequently around the bile canaliculi. AVs were numerous during the entire experimental period (15 min to 4hr), and the ultrastructure of individual A Vs varied depending on the stage of degradation of the sequestered material. Newly formed A Vs had single or double limiting membranes, and contained only slightly disintegrated cytoplasmic material and/or cell organelles (for example glycogen particles, mitochondria, ER, etc.). More mature AVs were surrounded by a single limiting membrane, and contained fairly well–digested electron–dense materials.

Fig. 1 shows two AVs in a hepatocyte 40 min after injection of VBL. One of these AVs contains an incompletely digested mitochondrion and is surrounded by a double limiting membrane (small arrow). The other AV is in a more advanced stage of digestion in which the nature of the engulfed material is no longer recognizable large arrow.

Localization of p–NPPase activity in VBL–treated hepatocytes

As reported previously for normal rat hepatocytes [1], the lysosomes of hopatocytes of VBL–treated rats showed distinct *p*–NPPase activity in their membranes but not in the matrix (Fig. 2). Other organelles such as mitochondria, Golgi apparatus, peroxisome, and ER did not display any positive reaction for the enzyme. However, Shore were scattered, diffuse deposits of tho reaction product throughout the cytoplasm (Fig. 2).

In VBL–treated hepaocytes, *p*–NPPase activiSy was also observed on the AV membranes and, occasionally, in AV matrices (Figs. 3, 4). However, not all AVs revealed *p*–NPPase aetivity. In general, *p*–NPPaso activiSy was found only in the membranes of more mature AVs containing well–digested material. Neibher the membranes nor tho matrices of newly formed AVs (Fig. 4) displayed *p*–NPPase activity. Although She content of AVs usually showed very weak *p*–NPPase activity (Figs. 3, 4), small vesicles (50—300 nm in diameter) and trabecular structures occasionally revealed an intense positive reaction (Figs. 5, 6).

In control experiments, when sections were incubated in a medium containing an inhibitor of H^+–ATPase, NEM or DIDS, no deposits of the reaction product were observed in AV membranes (Fig. 7).

AcPase activity in VBL–treated hepatocytes

AcPase activity of VBL–treated hepatecytes was localized in the matrices of lysosomes and AVs showing an advanced–stage of digestion of content (Fig. 8). These AVs seem to correspond morphologically to those AVs which also showed *p*–NPPase activity (Figs. 3, 4). Interestingly, no AcPase activity was observed on either the membranes or in the matrices of AVs at early stages of contenft–digestion (Fig. 9), just as such AVs failed to show any *p*–NPPase activity. Deposits of the reaction product of AcPase activity were mostly concentrated in AV matrices and the AV membranes revealed only sparse deposits of the reaction product in their inner surface. However, the distribution pattern of AcPase activity on the AV membranes did not seem to be identical with that of *p*–PPase activity; the reaction prouduots of *p*–NPPase activity were found on the membranes on their outer (cytoplasmic) surface which accentuated the electrolucent space (hallow) beneath the membrane of AVs or lysosomes (Figs. 2, 3).

DISCUSSION

The ATP–driven vacuolar proton pump (H^+–ATPase) which is responsible for maintaining the acidity in organelles and vacuolar systems has been studied biochemically in a variety of intracellular organelles and acidic vacuolar systems such as lysosomes [12], Golgi apparatus [13], endoplasmic reticulum [14], mitochondria [15], endosomes [16], secretory granules [17], clathrin–coated vesicles [18J, plasmalemmal vesicles in urinary epithelial cells [19], and acrosomes [20]. However, the ultracytochemical demonstration of H^+–ATPase activity in these cellular structures has not been reported, with the exception of our recent data on the demonstration of H^+–ATPase activity in rat liver lysosomes [1] and in Golgi complex [21]. In the present study, H^+–ATPase activity was detected in AVs and lysosomes in VBL–treated rat hepatocytes.

Neutral *p*-NPPase activity was mainly observed on the cytoplasmic surface of lysosomal membranes in VBL–treated hepatocytes. This localization is similar to that seen in hepatocytes of untreated rats. The VBL treatment seemed to have little effect on H^+–ATPase activity, although several ultrastructural changes of organelles were observed. Since the incubation medium contained NaF in a concentration sufficient to completely inhibit acid phosphatase activity, the *p*–NPPase activity observed on lysosomal and AV membranes was not derived from that of lysosomal acid phosphatase. In addition, the *p*–NPPase activity on the lysosomal and AV membranes was inhibited by NEM and DIDS. These results suggest that the neutral *p*–NPPase activity most likely represents H^+–ATPase activity on lysosomal and AV membranes in VBL–treated hepatoceytes. Both the AVs and lysosomal membranes revealed the *p*–NPPase activity sensitive to the H^+–ATPase inhibitors, but not all AV membranes showed enzyme activity. AV membranes in advanced stages of content. digestion revealed an intense H^+–ATPase activity, but the membranes of early AVs containing incompletely digested material did not. Vacuoles that have newly sequestered materials but do not yet contain hydrolase within their matrices are called autophagosomes. It follows that the H^+–ATPase activity is present in the membranes of autophagolysosomes, but not in the membranes of autophago seines. Autophagosomes having no H^+–ATPase

activity on the membranes revealed no AcPase activity in either the membranes or matrix. The H^+–ATPase of AV membranes may be derived from lysosomal membranes, possibly by fusion or *de novo* synthesis. Although clear fusion of AVs with lysosomes showing H^+–ATPase has not been observed in the present study, an arrow in Fig. 5 suggests possible fusion of AV with H^+–ATPase positive lysosome, and it is likely that H^+–ATPase of AV membranes is derived from the lysosomal membranes.

In addition to the H^+–ATPase activity on AV membranes, enzyme activity was frequently observed in small vesicles and trabecular structures in advanced stages of degradation of their content. Since the relatively larger AVe frequently contained H^+–ATPase positive vesicles and trabeculae with frequent connections with AV membranes, these structures may play an important role in recycling the AV membranes.

Although our observations did not directly resolve the problem of the origin of the AV membrane, they suggest that the early forms of AV membranes in hepatocytes of VBL–treated rate probably do not originate from lysosomal membranes. However, evidence has been proposed that in various tissues A Vs are formed by the transformation of lysosomal membranes, and different terns such as lysosomal wrapping mechanism LWM [22, 23, 24], microautophagy [25, 26], and lysosomophagy [27, 28] have been proposed to describe this process. This discrepancy may be resolved by assuming the existence of two mechanisms of formation of autophagic vacuoles. One of these may involve lysosomal wrapping, as described in glucagon– or cyclic AMP–perfused rat hepatocytes [29, 30], and the other postulate is that the lysosomes, which fuse with AVs later, do not contribute to the original formation of A Vs. Further experiments are necessary to define the relationship between H^+–ATPase activity on lysosomal membranes and the lysosomal wrapping mechanism.

ACKNOWLEDGEMENT

We wish to express thanks to Prof. T. Barka for his critical review of this manuscript

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Received 5–6–1990. Revised 26–6–1990. Accelpted 16–7–1990.

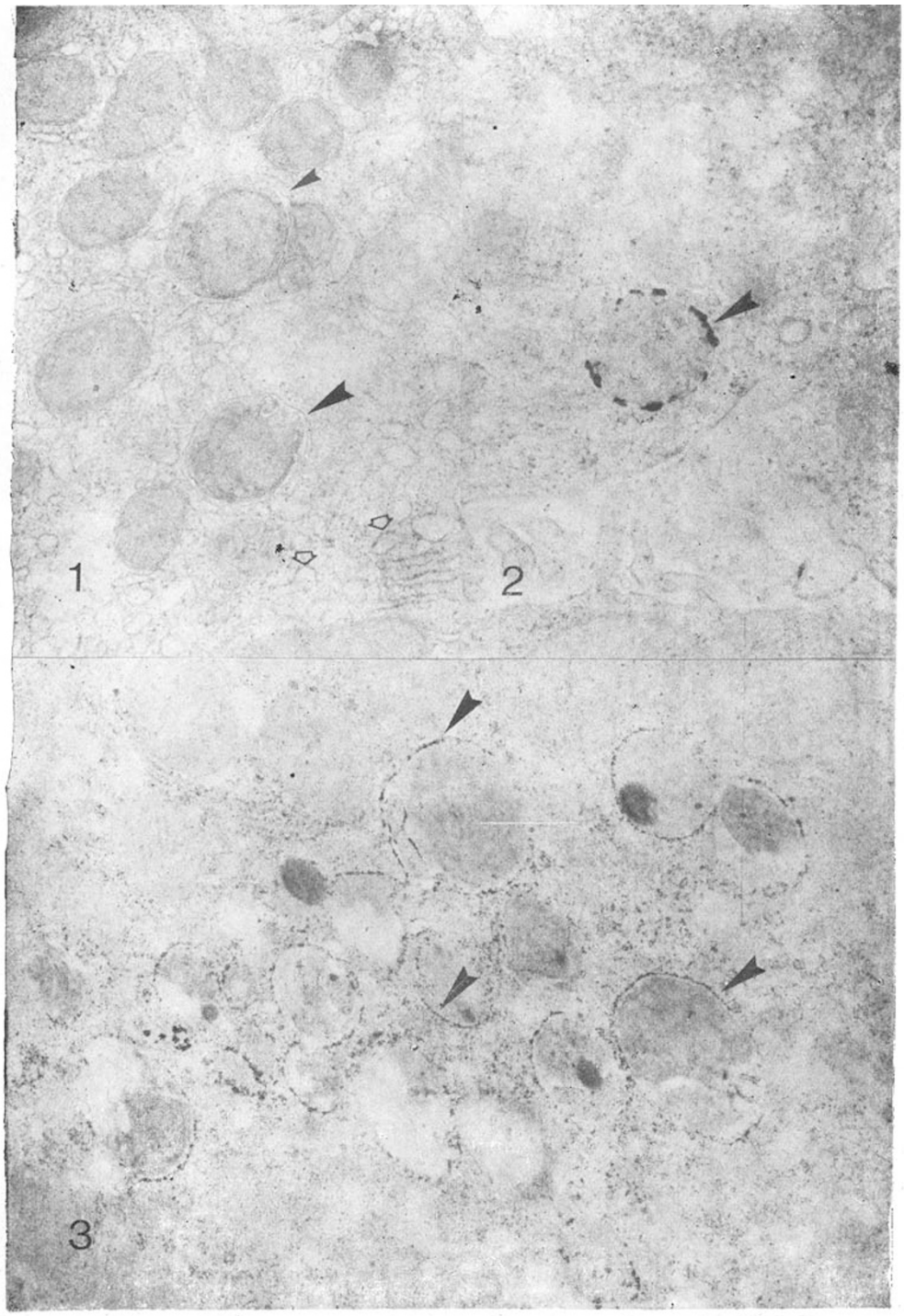

Fig. 1 Ultrastructure of rat hepatocyte 40 min after the injection of VBL. An AV containing a recognizable mitochondrion (small artery) and an AV containing undefined digested material (large arrow) are shown. The expansion of ER cisternae is seen (arrow heads). × 22, 500

Fig. 2 *p*–NPPase activity of lysosomes in a hepatocyte 2 hr after treatment with VBL. *p*–NPPase activity is observed on the membrane but not in the matrix of the lysosome (arrow). × 24,000

Fig. 3 Same hepatocyte shown in Fig. 2. Many A Vs showing membranes *p*–NPPase activity (arrows) are Seen. × 25, 000

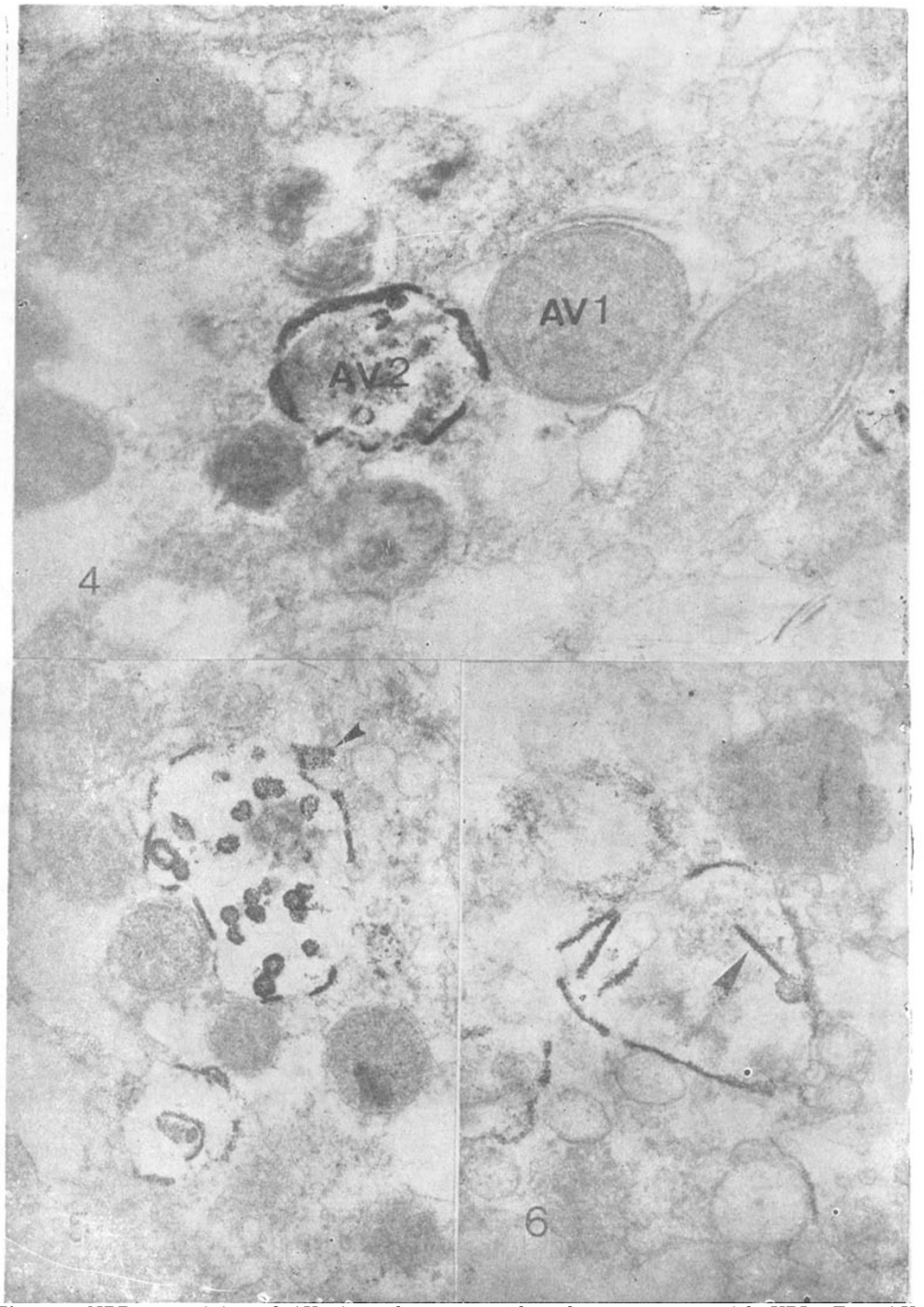

Fig. 4 *p*–NPPase activity of AVs in a hepatocyte 1 hr after treatment with VBL. Two AVs (AV1, AV2) are seen. The content of AV1 containing a clearly re ognizable mitochondrion shows no *p*-NPPase activity. Deposit reaction products are seen on the membranes of AV 2 which has an electron–dense content. ×31, 000

Fig. 5 *p*–NPPase activity of an A V in a hepatocyte 3 hr after tre tment with VBL. A large AV showing *p*–NPPase activity on the membrane is illustrated. In the matrix, there are many vesicles showing p–NPPase activity in their membranes. An arrow indicates the possible site of fussion of AV with a lysosome having H+– ATPase activity. ×28, 000

Fig. 6 A hepatocyte 3 hr after the injection of VBL. There are trabeculae in the matrix of an AV showing *p*–NPPaze positive membrane reaction (arrow). × 32, 000

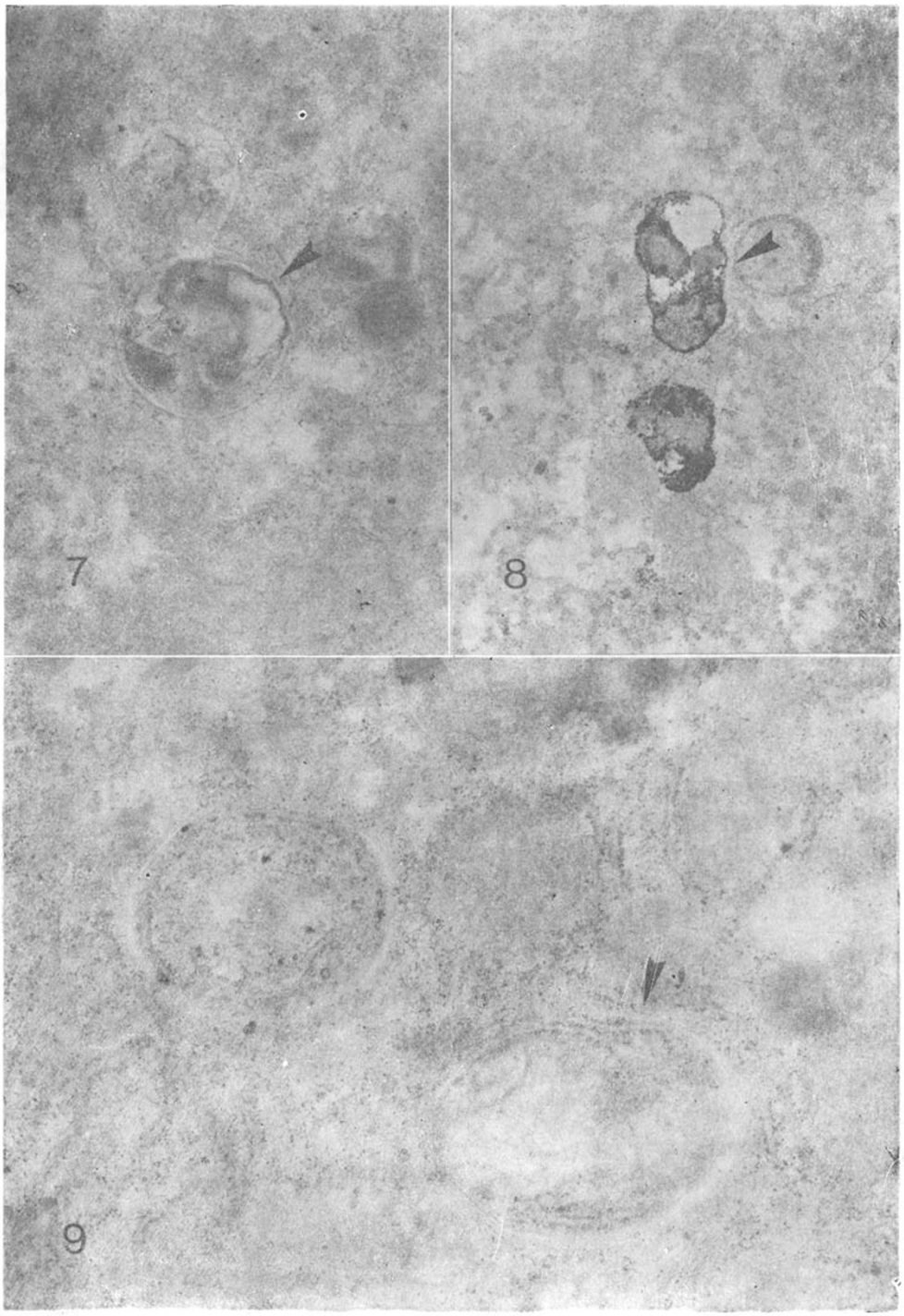

Fig. 7 Control *p*–NPPase reaction of an AV in rat hepatocyte 2 hr after treatment with VBL. No p–NPPase activity was found on membrane of AV with eleotron–dense content When the section was incubated in the presence of NEM (arrow). × 32,000

Fig. 8 AcPase activity of A Vs in rat hepatocyte 1 hr after treatment with VBL. AVs with electron-dense content show AcPase positive reaction in both membranes and matrices (arrow). × 25, 500

Fig. 9 A cPase activity o2 AV in rat hepatocyte 2 hr after treatment with VBL. There is no AcPase activity in the AV containing a recognizable mitochondrion (arrow). × 34, 500