

Effects of Swainsonine on Rat Liver and Kidney: Biochemical and Morphological Studies

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ABSTRACT Among the reported effects of the plant toxin swainsonine in animals are a decreased level of Golgi mannosidase II activity, an increase in lysosomal α -D-mannosidase activity, oligosaccharide accumulation, vacuolization of cells, and neurological changes. We now find that, in the rat, the alkaloid rapidly induces vacuolization of both liver and kidney cells, but oligosaccharides accumulate only in the latter. We demonstrate by enzyme- and immunocytochemistry that the induced pleomorphic vacuoles are lysosomal in nature. The vacuoles do not appear to be derived from the Golgi apparatus, which retains its typical ultrastructural appearance, but are formed by autophagy. In swainsonine-fed rats, the lysosomal system is highly developed in hepatocytes, Kupffer cells, and cells of the proximal convoluted tubules. The relation of this hypertrophy of the lysosomal system to the known effects of swainsonine on glycoprotein biosynthesis and on Golgi and lysosomal α -mannosidases is not clear. In addition, in liver there occurs a marked increase in mitotic figures in the hepatocytes. This occurred in the absence of both cell death and increased liver size as estimated by gross morphology.

Plants of the genus *Swainsona* and spotted locoweed (*Astragalus lentiginosus*) have been reported to induce in animals a neurological condition mimicking the hereditary lysosomal storage disease, α -mannosidosis, which occurs in man, Angus cattle, and cats (1–5). Swainsonine, an indolizidine alkaloid, has been isolated from these plants and has been suggested to be the toxic agent that causes the neurological effects, vacuolization of cells, and accumulation of free oligosaccharide (6–8). Recent evidence, obtained in the pig, indicates that swainsonine is indeed the principal, or sole, agent in locoweed responsible for these symptoms (9).

In spite of the fact that swainsonine is a strong inhibitor of lysosomal α -D-mannosidase in vitro, both the alkaloid and locoweed fed to pigs induced increases in activities of α -D-mannosidase and several other acid glycosidases in most tissues examined. Both swainsonine and locoweed decreased liver Golgi mannosidase II levels, as expected from the potent inhibitory action of the alkaloid on this enzyme in vitro, and they both also increased plasma acid hydrolase activities and greatly increased tissue oligosaccharides, principally mannose, *N*-acetyl-glucosamine₂, in all tissues examined (9). Sim-

ilar changes in enzyme levels in swainsonine-fed rats have been reported (10).

The vacuoles induced in cells of grazing animals by ingestion of swainsonine-containing plants have been assumed to be lysosomes because of the general resemblance of the induced condition to α -mannosidosis (8). To obtain more definitive evidence on this point, we have initiated a combined biochemical and morphological study of the liver and kidney of rats administered swainsonine. This investigation has yielded unequivocal evidence that the vacuoles formed in liver and kidney are lysosomes. Moreover, although the administration of the alkaloid led to a marked vacuolization of both liver and kidney cells, only the latter showed an accumulation of free oligosaccharides. The major effects on cytological structures are (a) enlargement of the lysosomal system, including GERL and pleomorphic vacuoles, (b) induction of autophagy, and (c) mitosis in liver, which in some rats is similar to that seen in regenerating liver. There were profound morphological effects on the lysosomal system of hepatocytes, Kupffer cells, and cells of the proximal convoluted tubules of the kidney. Although swainsonine is a potent

inhibitor of Golgi mannosidase II and influences glycoprotein processing through its action on this enzyme, there seems to be no discernible morphological effect on the Golgi apparatus.

MATERIALS AND METHODS

Materials: Swainsonine isolated from *Rhizoctonia leguminicola* as previously described (11) was generously provided by Dr. H. P. Broquist of Vanderbilt University. Uniformly labeled oligosaccharides used for the calibration of the Bio-Gel P-4 column were prepared as described (12). Bio-Gel P-4 (–400 mesh) was from Bio-Rad Laboratories (Richmond, CA). Anti-mannosidase II IgG was prepared as previously reported (13). Highly specific polyclonal antibody (IgG) against lysosomal α -D-mannosidase was purified from a rabbit immunized against a homogeneous preparation of rat epididymal α -D-mannosidase. By the Ouchterlony double diffusion technique (13), this antibody was observed to react with rat liver lysosomal α -mannosidase but not with Golgi mannosidase IA, IB, or II (14) or with cytosolic α -D-mannosidase (15).

Swainsonine Administration: Male Wistar Rats (170–200 g) from Marland Breeding Farms (Hewitt, NJ) or Harlan Industries, Inc. (Madison, WI) were administered various amounts (5 μ g/ml or 50 μ g/ml) of swainsonine in their drinking water (10). In all experiments reported here, swainsonine-fed animals (experimental group) were compared to age-matched animals given tap water (control group).

Preparation of Kidney Extracts: Animals were stunned by a blow to the head and killed by decapitation. The kidneys were quickly excised, the particulate fractions prepared by homogenization and centrifugation (10), and the fractions suspended in 3 vol of homogenizing solution per gram of tissue for assay of acid hydrolase activities.

Enzyme Assays: Lysosomal acid hydrolase activities were assayed by incubation of the particulate fraction with the appropriate synthetic substrate in a total volume of 0.5 ml as described (16).

Preparation and Assay of Tissue Oligosaccharides: Crude oligosaccharide from control and experimental rat tissue was prepared according to the procedure of Dorling et al. (8), as described from our laboratory (9). In brief, the rat tissue was homogenized in 4 vol of water, the homogenate was mixed with 3 vol of ethanol, and the mixture was centrifuged at 1,000 g for 20 min. 2 vol of diethyl ether was added to the supernatant and the mixture centrifuged as above. The ethanol-ether-precipitated oligosaccharide was dried at room temperature and suspended in 0.3 ml 0.1 M acetic acid. Total hexose in this suspension was assayed by the phenol-sulfuric acid method (17) scaled down fivefold, with mannose as standard. The crude oligosaccharide was fractionated on a column of Bio-Gel P-4 as described (12, 14).

Morphology Studies: After 60 h of swainsonine administration, Wistar rats (170–200 g) were anesthetized by ether, the livers and kidneys were removed, and thin slices of the tissues were fixed by immersion in the following fixatives: (a) 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, 5% sucrose; (b) 2% formaldehyde (prepared from paraformaldehyde)/2.5% glutaraldehyde, in 0.1 M cacodylate buffer, pH 7.4, 0.025% CaCl₂, 5% sucrose; (c) 4% formaldehyde (prepared from paraformaldehyde) in 0.1 M phosphate buffer, pH 7.4, 7.5% sucrose (18). The total fixation time was 3 h. Tissues fixed in fixative *a* were used for ultrastructural studies. Tissues fixed in fixative *b* were used for cytochemical studies. Tissues fixed in fixative *c* were used for immunocytochemical studies. Cubes of liver were also fixed in cold 2% OsO₄ in 0.1 M cacodylate buffer, pH 7.4, for 1 h, followed by 48 h at 40°C (19), with renewal of OsO₄ after 16–24 h.

Cytochemical Studies: After fixation in fixative *b*, tissues were kept overnight in cold buffer before nonfrozen sections were prepared with a Vibratome (Oxford, Ted Pella, Inc., Irvine, CA). Vibratome sections ~20–30- μ m thick were collected in cold 7.5% sucrose. Sections were incubated at 37°C for thiamine pyrophosphatase (TPPase)¹ activity for 90 min or acid phosphatase (AcPase) activity for 15 min. The incubation medium for TPPase activity contained 25 mg thiamine pyrophosphate chloride (Sigma Chemical Co., St. Louis, MO), 7 ml distilled water, 10 ml 0.2 M Tris-maleate buffer, pH 7.2, 5 ml 0.025 M manganese chloride, 3 ml 1% lead nitrate in 5% sucrose. The medium was filtered before use and renewed after 30 min (18). The medium for AcPase activity consisted of 25 mg of cytidine 5'-monophosphate, disodium salt (Sigma Chemical Co.), 12 ml of distilled water, 10 ml of 0.05 M acetate buffer pH 5.0, and 3 ml of 1% lead nitrate in 5% sucrose (18). To avoid the slight precipitation that may occur during incubation, the medium was used after it developed a slight precipitation, which usually occurred after 30–60

¹ Abbreviations used in this paper: AcPase, acid phosphatase; DAB, diaminobenzidine; HRP, horseradish peroxidase; TPPase, thiamine pyrophosphatase.

min at room temperature, or 37°C. It was then filtered and used immediately. To visualize the mitotic apparatus, sections were stained by the methyl green pyronine procedure of Lillie (20). TPPase and AcPase activities were examined by light microscopy after the sections were treated with dilute ammonium sulfide, rinsed in sucrose or distilled water, and mounted in glycerol gelatin (Sigma Chemical Co.).

Immunocytochemistry: Vibratome sections (~20 μ m) of tissues fixed in fixative *c* were exposed to rabbit mannosidase II IgG or to rabbit lysosomal mannosidase IgG at 4°C for 18–22 h, rinsed in cold 0.1 M phosphate buffer (pH 7.4)/sucrose several times and treated with protein A-horseradish peroxidase (HRP) for 1 h or anti-rabbit F(ab)₂ fragments labeled with HRP for 1–3 h at room temperature. After several rinses in 0.1 M phosphate buffer/sucrose, the sections were incubated in diaminobenzidine (DAB) medium at pH 7.6 (21) at room temperature for 10 min in the dark. The incubating medium was prepared as follows: 10 mg of DAB was dissolved in 10 ml of 0.1 M Tris-HCl buffer containing 7.5% sucrose. The medium was adjusted to pH 7.6 before adding 0.2 ml of 1% H₂O₂ (freshly prepared) and was filtered before use. The following controls were performed: (i) exposure of sections to non-immune rabbit antibody (Cappel Laboratories, Cochranville, PA) followed by treatment with protein A-HRP and incubated in DAB, as described above; (ii) treatment with protein A-HRP and incubation in DAB, as above, but without prior exposure to immune or nonimmune antibody; (iii) treatment with unlabeled protein A and incubation in DAB, as above; and (iv) incubation with DAB, as above, but no treatment with antibody, protein A-HRP, or protein A (22).

Electron Microscopy: For ultrastructural studies, small pieces of liver and kidney (~2 mm³) were initially fixed in fixative *a* for 2 h, postfixed in 1% osmium, and buffered with 0.1 M cacodylate buffer, pH 7.4, containing 5% sucrose. For enzyme cytochemical studies, Vibratome sections, with and without treatment in dilute ammonium sulfide (7.5% sucrose used to dilute the ammonium sulfide), were fixed in cold 1% OsO₄/0.1 M cacodylate buffer for 1 h. Vibratome sections used for immunoelectron microscopy studies were fixed in cold 2.5% glutaraldehyde in phosphate buffer, pH 7.4, and postfixed in cold 1% OsO₄/0.1 M phosphate buffer, pH 7.4. Tissue cubes and sections were then stained en bloc in uranyl acetate, dehydrated in alcohols, and embedded in Epon (18). Ultrathin sections (50–70 nm) were prepared and examined with or without lead staining in a Philips 300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ).

RESULTS

Effect of Swainsonine Administration on Oligosaccharides of Rat Liver and Kidney

A dose of 5 μ g/ml swainsonine does not cause any accumulation of free oligosaccharide in the liver of rats examined after 1, 2, 4, 7, 10, and 28 wk of treatment. This result is in contrast to those obtained in the pig, in which all tissues tested showed marked oligosaccharide accumulation (9). Even with a swainsonine dose of 50 μ g/ml, no oligosaccharide accumulation was observed in rat liver. On the other hand, there was a rapid and massive accumulation of oligosaccharide in rat kidney, as shown in Table I. With only 2 d of swainsonine administration, the kidney oligosaccharide had more than tripled. The accumulated oligosaccharide in kidney (Fig. 1) was isolated in pure form and shown to consist primarily of mannose₃-N-acetylglucosamine, mannose₄-N-acetylglucosa-

TABLE I. Oligosaccharide in the Liver and Kidney of Control and Swainsonine-Fed Rats

Swainsonine μ g/ml H ₂ O	Liver		Kidney	
	2 d	5 d	2 d	5 d
	μ g hexose/g tissue			
0	12,790	9,960	308	200
5	11,200	10,770	963	1,814
20	12,400	11,480	1,490	3,543
50	13,464	11,400	1,493	3,143

See Materials and Methods for details. This Table records the results of a typical experiment on a single animal. Similar results were obtained in several additional experiments.

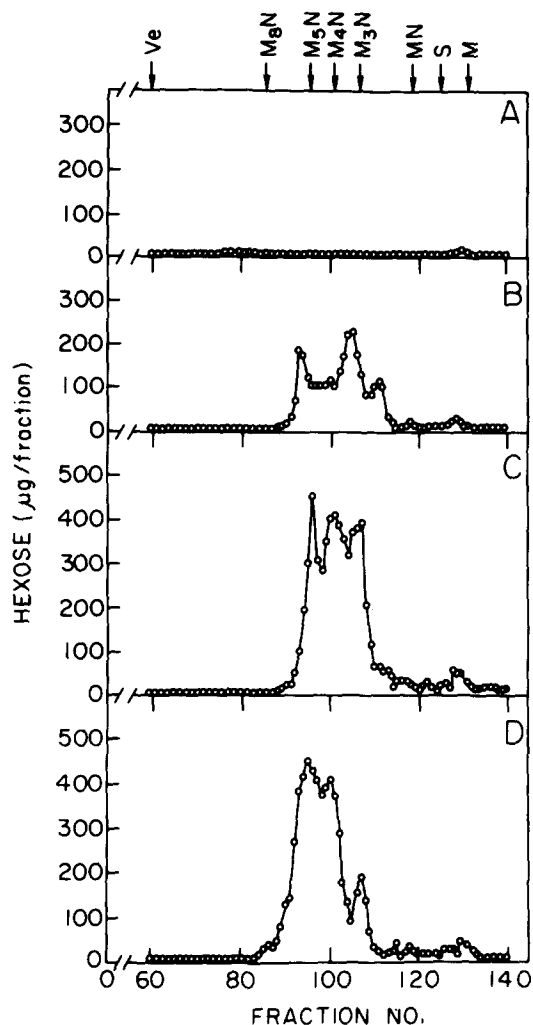


FIGURE 1 Fractionation of oligosaccharide from the kidney of control and swainsonine-fed rats. The ethanol-ether-precipitated oligosaccharide was prepared from 1.5 g of kidney from control (A) and swainsonine-fed (B-D) animals. Male Wistar rats (200 g) were fed various concentrations of swainsonine in drinking water (B, 5 $\mu\text{g}/\text{ml}$; C, 20 $\mu\text{g}/\text{ml}$; and D, 50 $\mu\text{g}/\text{ml}$). The precipitated oligosaccharide, suspended in 0.3 ml of 0.1 M acetic acid, was applied to a Bio-Gel P-4 column (0.8 \times 136 cm, -400 mesh) equilibrated with 0.1 M acetic acid. Fractions (0.5 ml) were collected at a flow rate of 2.5 ml/h. Aliquots from each fraction were assayed for hexose by the phenol-sulfuric acid method as described in Materials and Methods. The column exclusion volume (V_e) was determined using bovine serum albumin. The standards are M_6N , (Man) $_6$ GlcNAc; M_5N , (Man) $_5$ GlcNAc; M_4N , (Man) $_4$ GlcNAc; M_3N , (Man) $_3$ GlcNAc; MN , ManGlcNAc; S, sucrose; M, mannose.

mine, and mannose $_3$ -N-acetylglucosamine (Tulsiani, D. R. P., and O. Touster, manuscript in preparation). These oligosaccharides appear between fractions 90 and 110 in Bio-Gel P-4 gel filtration (Fig. 1). Fig. 2 shows that swainsonine has little effect on the oligosaccharide content of liver.

The increased level of oligosaccharide in kidney was unrelated to observed changes in lysosomal α -D-mannosidase levels. We have previously reported that 3 d of swainsonine administration (5 $\mu\text{g}/\text{ml}$ drinking water) resulted in an increased level of activity of this enzyme in liver (176% of normal), with much higher values after 1 or 2 wk (10). In kidney, we find 140% of normal levels at 3 d and 170% at 5 d. These similar, gradual increases in enzyme activities are in

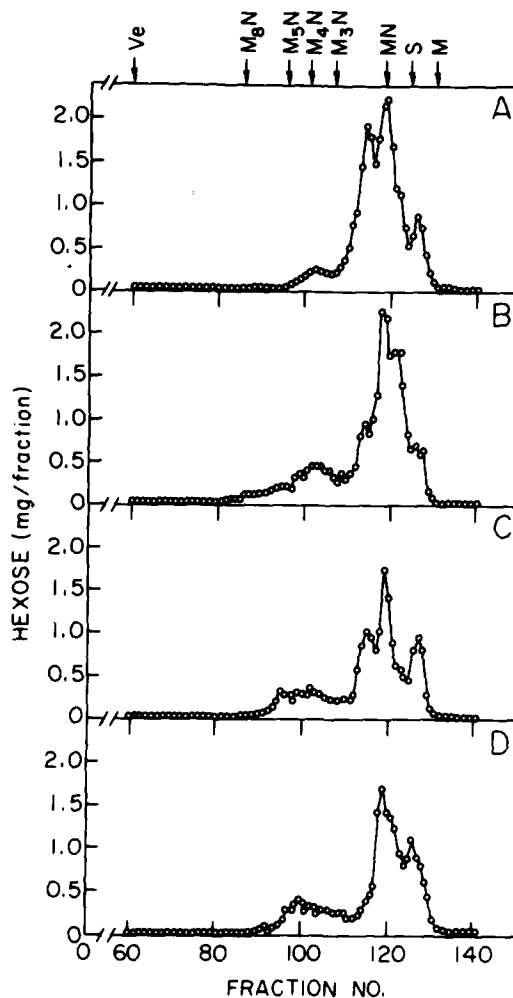


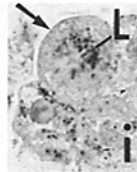
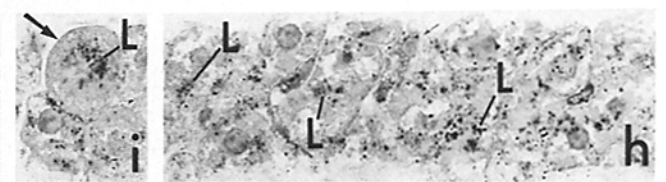
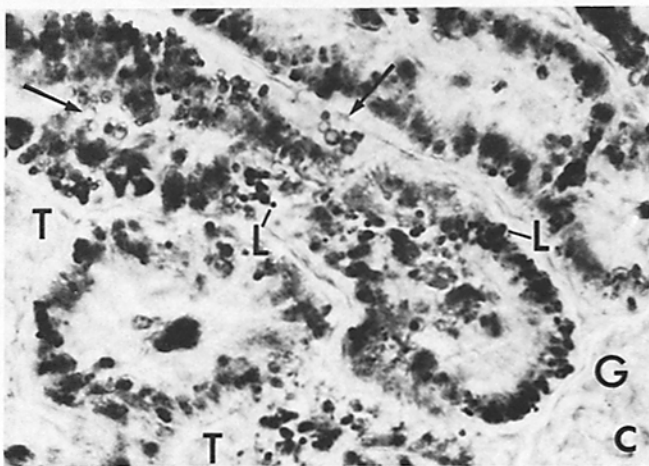
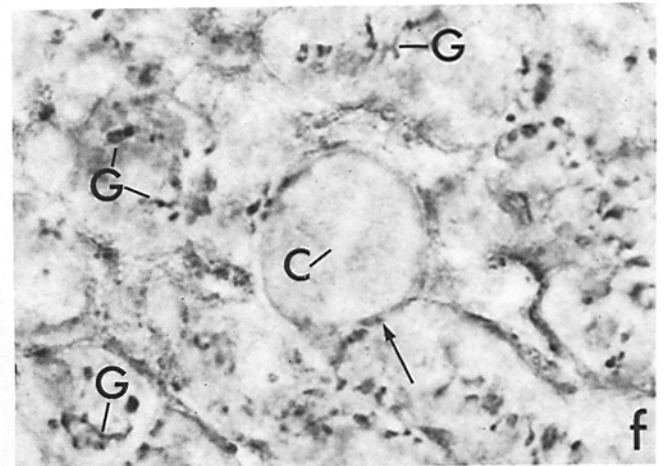
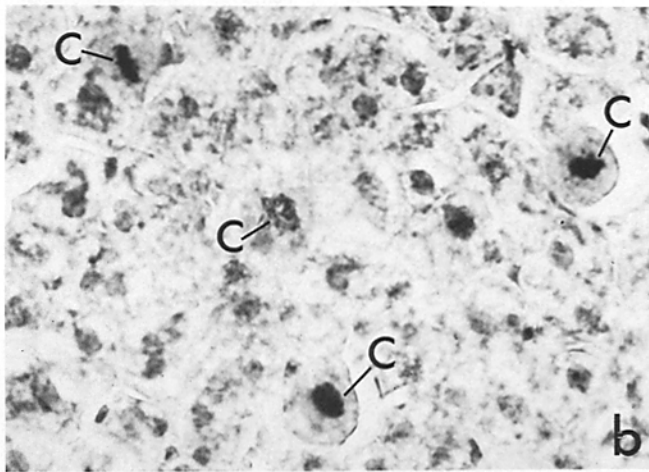
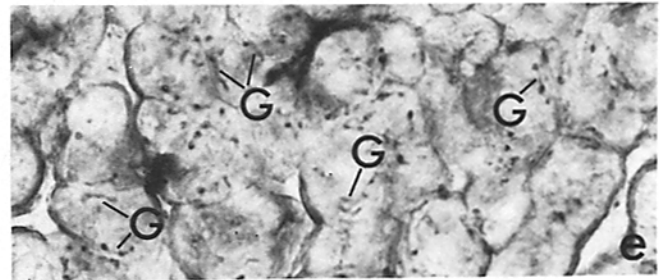
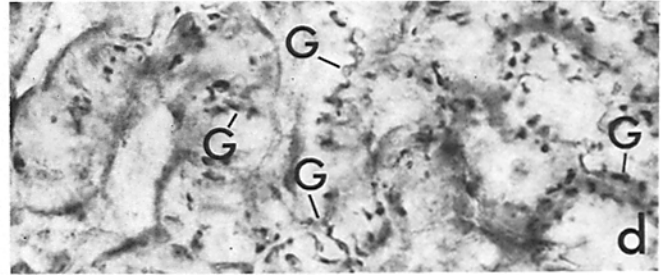
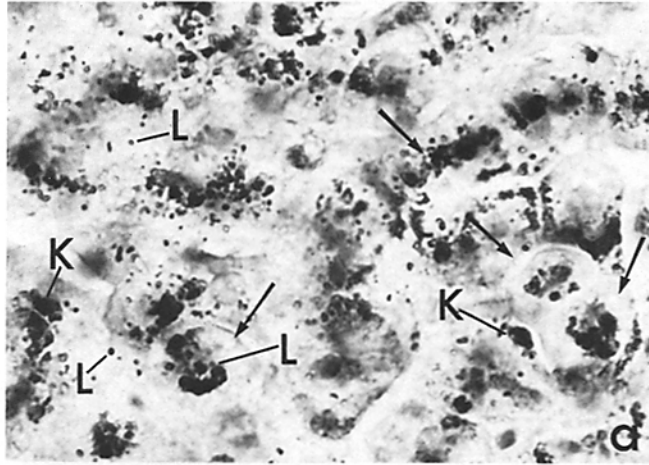
FIGURE 2 Fractionation of oligosaccharide from the liver of control and swainsonine-fed rats. The ethanol-ether-precipitated oligosaccharide was prepared from 2 g of liver from control (A) and swainsonine-fed (B-D) animals. Other details are the same as in Fig. 1.

marked contrast to the different findings on oligosaccharide levels in the two tissues. In these experiments, α -L-fucosidase levels increased slowly, and other acid glycosidases remained essentially normal.

Light Microscopic Observations

In frozen sections of liver from rats administered swainsonine, all hepatocytes exhibited a marked increase in the size and number of lysosomes, as revealed by AcPase enzyme cytochemistry (Fig. 3a). Many of the enlarged lysosomes showed a clear central area surrounded by dense reaction product; these proved to be autophagic vacuoles when examined by electron microscopy (Figs. 4 and 5a). In addition to their usual pericanalicular location, lysosomes also were found more widely distributed in the cytoplasm (Fig. 3a). Kupffer cells and their lysosomes were also found to be enlarged (Fig. 3a). Hepatocytes in division were encountered frequently (Fig. 3, a and b). In the dividing hepatocytes, lysosomes were clustered around the mitotic apparatus.

In similar sections of rat kidney after swainsonine was administered, AcPase cytochemistry showed enlarged lysosomes to be present only in proximal convoluted tubules and not in distal convoluted tubules or glomeruli (Fig. 3c). The



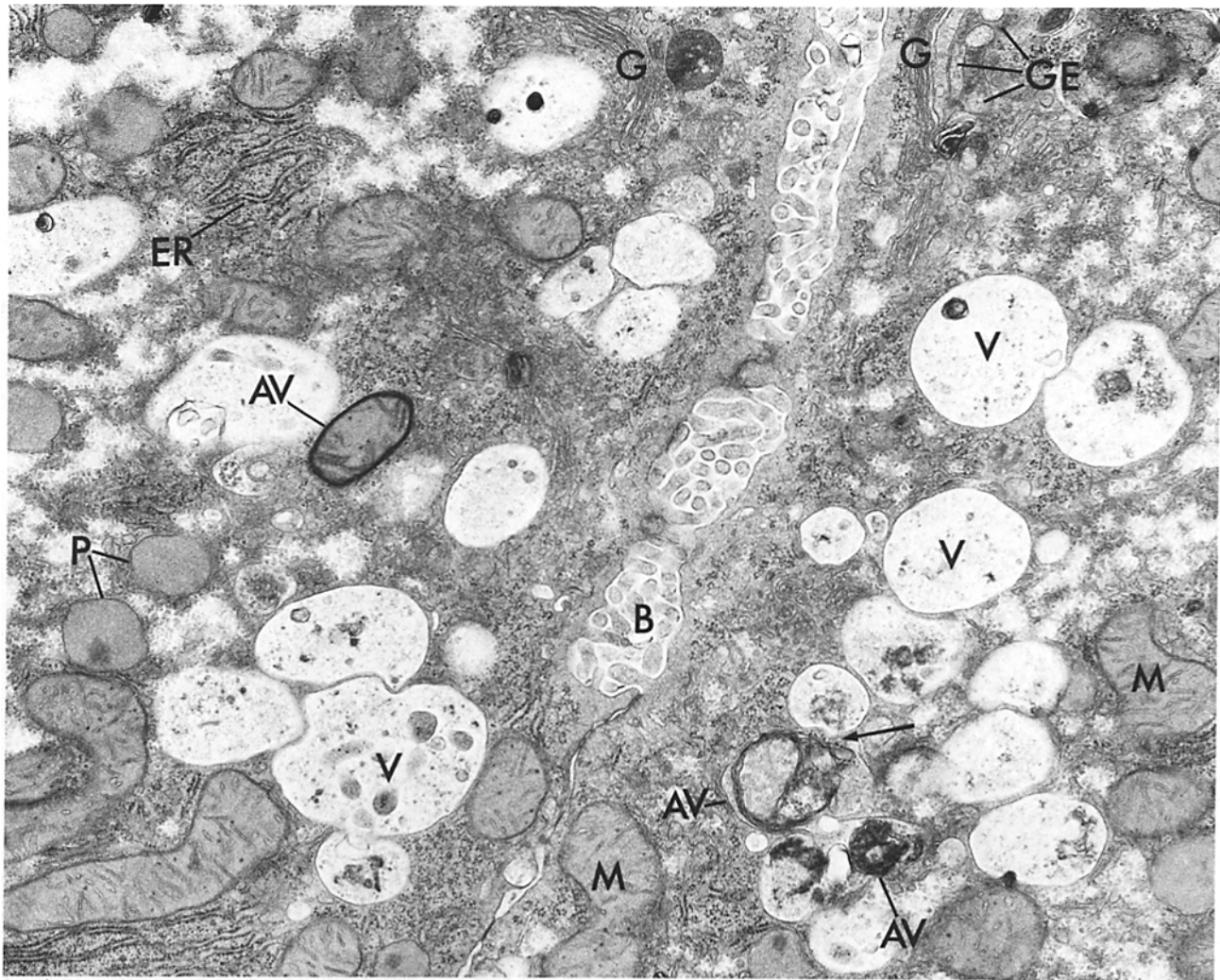


FIGURE 4 Sections of liver from rat fed swainsonine, unincubated. Note the numerous pleomorphic vacuoles (V) and autophagic vacuoles (AV). Many of the vacuoles contain heterogeneous material. The early autophagic vacuoles contain recognizable cellular constituents (mitochondria, M). GERL cisternae are evident at the upper right (GE), and contain electron-opaque material. Also labeled are mitochondria (M), peroxisomes (P), Golgi apparatus (G), bile canaliculus (B), and endoplasmic reticulum (ER). The elements of the Golgi apparatus seem to be flattened, and very low density lipoprotein particles are absent. $\times 20,000$.

FIGURE 3 (a) A frozen section ($10\ \mu\text{m}$) of rat liver after swainsonine ingestion, incubated for AcPase activity (10 min), and then stained with methyl green pyronine. The lysosomes (black dot-like structures) are large, numerous, and found widespread in the cytoplasm. Some lysosomes appear with a clear central zone (L). In untreated rat livers, most lysosomes are found near the bile canaliculi. Three hepatocytes in various stages of mitosis are evident (arrows). The lysosomes in these cells are clustered at the mitotic poles. The chromosomes are evident in these cells. The lysosomes of the Kupffer cells are also enlarged (K). $\times 800$. (b) A frozen section of rat liver after swainsonine ingestion stained with methyl green pyronine. Four hepatocytes in various stages of mitosis are seen; the chromosomes (C) are evident in these cells. $\times 600$. (c) A frozen section of rat kidney after swainsonine ingestion, incubated for AcPase activity (5 min). Only proximal convoluted tubules show numerous, enlarged lysosomes (L), many with clear central zones (arrow). The distal convoluted tubule (T) and glomerulus (G) barely show small lysosomes. $\times 400$. (d and e). Nonfrozen sections ($20\text{--}50\ \mu\text{m}$) from control rat liver showing the immunocytochemical localization of mannosidase II. The Golgi apparatus is visualized in all hepatocytes (G). Some portions of the Golgi apparatus are found near the bile canaliculus, and others extend to the nucleus. d employs the protein A-HRP procedure, and e the second antibody procedure (HRP-labeled anti-F[ab]₂ fragments-rabbit IgG). $\times 500$. (f) Nonfrozen section of liver from rat fed swainsonine, incubated for mannosidase II activity. The Golgi apparatus (G) is visualized in all nondividing hepatocytes. The Golgi apparatus is not seen in the dividing hepatocyte (arrow). The chromosomes (C) are unstained and appear as a clear region in the center of the hepatocytes. $\times 800$. (g-i) Epon sections ($2\text{--}3\ \mu\text{m}$), cut through the depth of nonfrozen liver section ($\sim 20\ \mu\text{m}$), incubated for lysosomal mannosidase activity using the protein A-HRP immunocytochemical procedure. (g) A control liver; (h and i) a swainsonine-treated liver, show this enzyme localized to lysosomes (L). These organelles are larger and more numerous in hepatocytes from swainsonine-fed rats (h) than in control (g). A dividing cell is seen at the arrow (i); note that the lysosomes are clustered near the mitotic apparatus (Fig. 8a). $\times 400$.

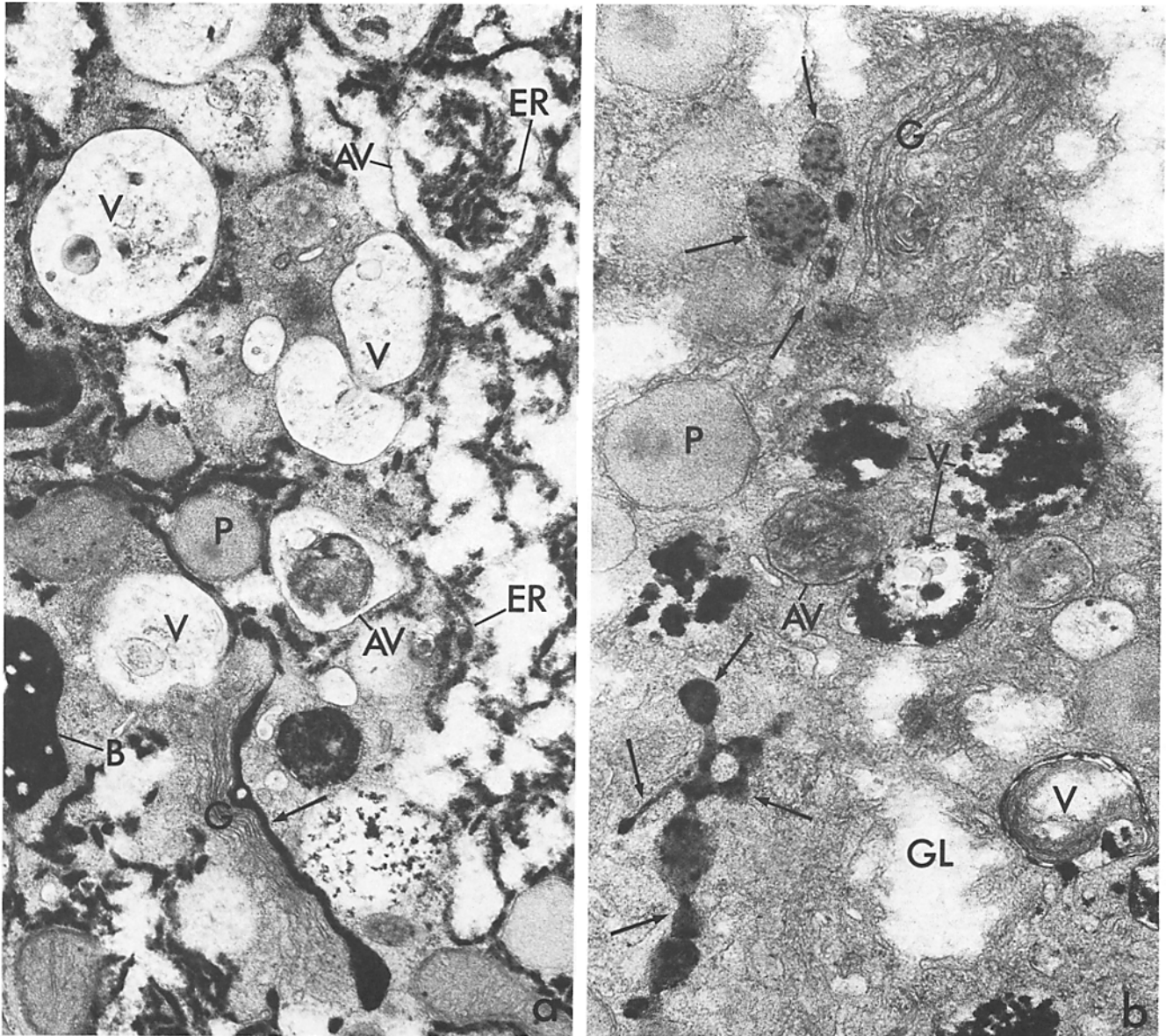


FIGURE 5 (a) Section of liver of rat fed swainsonine, incubated for nucleoside diphosphatase activity. Reaction product is present in the endoplasmic reticulum (ER), and the *trans* element of the Golgi apparatus (G) (arrow). Pleomorphic vacuoles (V) are negative. Note within one autophagic vacuole (AV), the presence of ER with reaction product, and another with a mitochondrion lacking reaction product. $\times 32,000$. (b) Section of liver of rat fed swainsonine, incubated for AcPase activity. Reaction product present in GERL (arrows) and pleomorphic vacuoles (V). Note that the Golgi apparatus (G), peroxisomes (P), and glycogen (GL) are free of reaction product. $\times 43,000$.

lysosomes in these cells were much larger than in hepatocytes. Many more lysosomes with clear central areas surrounded by dense reaction product were found. As in liver, these proved to be autophagic vacuoles (Fig. 10, *a* and *b*).

Immunocytochemical studies included the localizations of Golgi mannosidase II and lysosomal α -mannosidase in the livers from untreated and swainsonine-treated rats. In untreated rats, mannosidase II was localized exclusively to the Golgi apparatus of all hepatocytes (Fig. 3, *d* and *e*). The extensive nature of the apparatus is evident. It appears as a darkly stained tortuous structure distributed throughout the hepatocyte cytoplasm. Portions of the apparatus are seen near the bile canaliculi and close to the nucleus. Antigenic sites of this enzyme showed a similar distribution in nonfrozen sections when either HRP-labeled protein A (Fig. 3*d*) or HRP-

labeled $F(ab)_2$ fragments of anti-rabbit IgG (Fig. 3*e*) were used. The appearance of the Golgi apparatus was found to be similar in all hepatocytes with both indirect immunocytochemical procedures. Some reaction product is evident at the sinusoidal aspect of hepatocytes, which indicates possible localization of the enzyme to the apical plasma membrane. However, until the possibilities of adsorption of hemoglobin from lysed erythrocytes and the release of endogenous peroxidase from disrupted Kupffer cells can be eliminated, the specificity of this localization seems uncertain. To ascertain the specificity of this reaction, we are studying the immunolocalization of this enzyme in cultured rat hepatocytes.

Fig. 3*f* shows mannosidase II activity in hepatocytes from liver of a rat given swainsonine. As in the untreated hepatocytes (Fig. 3, *d* and *e*), the Golgi apparatus is intensely stained

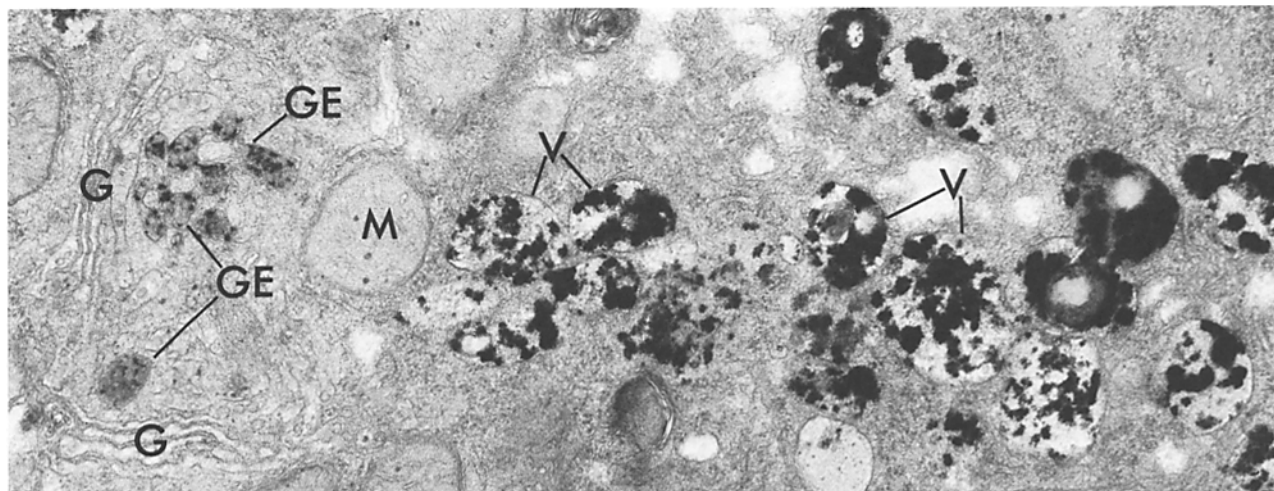


FIGURE 6 Nonfrozen sections of liver from rat administered swainsonine, incubated for AcPase activity. Reaction product is evident in the cisternae of GERL (GE) and in the numerous vacuoles (V) many of which are clustered. GERL has a variable ultrastructural appearance and shows an anastomosing tubular structure in this section. The ultrastructure of the Golgi apparatus is organized into a number of cisternae or elements parallel to each other and with an equal distance between each cisterna or element; GERL differs from the Golgi apparatus in its ultrastructure. $\times 33,000$.

and widespread in distribution. In hepatocytes during division, the Golgi apparatus is not evident at the level of the light microscope (Fig. 3*f*, arrow; cf. Fig. 8*b*).

Epon sections of nonfrozen material (Fig. 3, *g-i*) show lysosomal α -mannosidase localization in spherical dot-like structures. These structures are similar in size and distribution to those seen in AcPase cytochemistry (Fig. 3*a*). This identifies these structures as lysosomes. The lysosomes in the hepatocytes from swainsonine-treated rat livers (Figs. 3, *h* and *i*) are more numerous, larger, and more widespread than those found in the untreated hepatocytes (Fig. 3*g*). In Fig. 3*i*, a hepatocyte in division shows numerous immunoreactive lysosomes clustered around the mitotic apparatus. Parts *g-i* of Fig. 3, which are cut through the depth of an Epon-embedded nonfrozen section, illustrate that all the immunoreagents penetrate the section.

The distribution of lysosomal α -mannosidase activity in hepatocytes from both untreated and swainsonine-treated rat livers differs from that of mannosidase II activity. The former enzyme was found in lysosomes, and the latter in the Golgi apparatus.

Electron Microscope Observations

The most striking ultrastructural effect of swainsonine on rat liver and kidney is the induction of numerous membrane-delimited pleomorphic vacuoles. In the liver the only cell types that accumulate these vacuoles are the hepatocytes and Kupffer cells (Fig. 9). In the kidney, only the epithelial cells of the proximal convoluted tubules show enlarged membrane-delimited vacuoles (Fig. 10). These vacuoles contain electron-opaque material of a heterogeneous nature, presumably resulting from degradation of cytoplasmic constituents. Some vacuoles contain recognizable portions of cell organelles; these vacuoles are presumably early stages of autophagic vacuoles (Figs. 4; 5*a*; 11). Many of the vacuoles are interconnected. Use of enzyme and immunocytochemistry demonstrated that these vacuoles are lysosomes that form part of an extensive lysosomal system. In rat hepatocytes, two lysosomal enzymes, AcPase (Figs. 5*b*; 6, *a* and *b*) and acid mannosidase (Fig. 7*b*),

were localized within these vacuoles.

AcPase activity is also seen in cisternae of GERL. The appearance of the cisternae varies. In Fig. 5*b*, the cisternae show multiple distended regions, and in Fig. 6*a*, the cisternae are anastomosed. These figures are sections at different levels of an extensive continuous lysosomal system. The extensive lysosomal system is not only dramatically revealed by the generally used enzyme cytochemical marker (AcPase) but also by another lysosomal enzyme, namely, acid mannosidase (Fig. 7*b*). This mannosidase is localized in hepatocytes in both untreated (Fig. 7*a*) and swainsonine-fed (Fig. 7*b*) rats in the same structures that exhibit AcPase (see Figs. 5*b* and 6). The occurrence of another lysosomal enzyme, β -galactosidase, in lysosomes, GERL, and endoplasmic reticulum, but not in the Golgi apparatus, was previously reported (21). The structure of GERL is distinct from the Golgi apparatus which is composed of several cisternae or elements, each parallel to each other and separated from each other by a fairly constant distance (Figs. 4; 5, *a* and *b*; 6; 7, *a* and *b*). In Kupffer cells (Fig. 9*b*) and in rat kidney (Fig. 10*b*), AcPase is localized in the vacuoles. These vacuoles do not possess the enzymatic properties characterizing the Golgi apparatus; that is, the vacuoles have neither TPPase activity (Fig. 5*a*) nor mannosidase II activity (not illustrated). In the cell types in which the vacuoles accumulate, the Golgi apparatus retains its usual configuration with the elements arranged in stack-like fashion (Figs. 4 and 10). However, in hepatocytes from rats ingesting swainsonine, the Golgi elements that comprise the stacks appear to be flatter than in untreated hepatocytes, with fewer observable very low density lipoprotein particles.

The enzymatic profile of the Golgi apparatus is the same in Kupffer cells and kidney proximal convoluted cells of control and swainsonine-fed rats; namely, it shows TPPase and mannosidase II activities. Even though swainsonine causes the lysosomal system to be hypertrophied with a concomitant increase in acid mannosidase levels (10), neither AcPase nor acid mannosidase was observed in the Golgi apparatus (Figs. 5*a*; 6; 7*b*).

Fig. 8*a* shows one of the hepatocytes in mitosis, which increases upon administration of swainsonine. The lysosomal

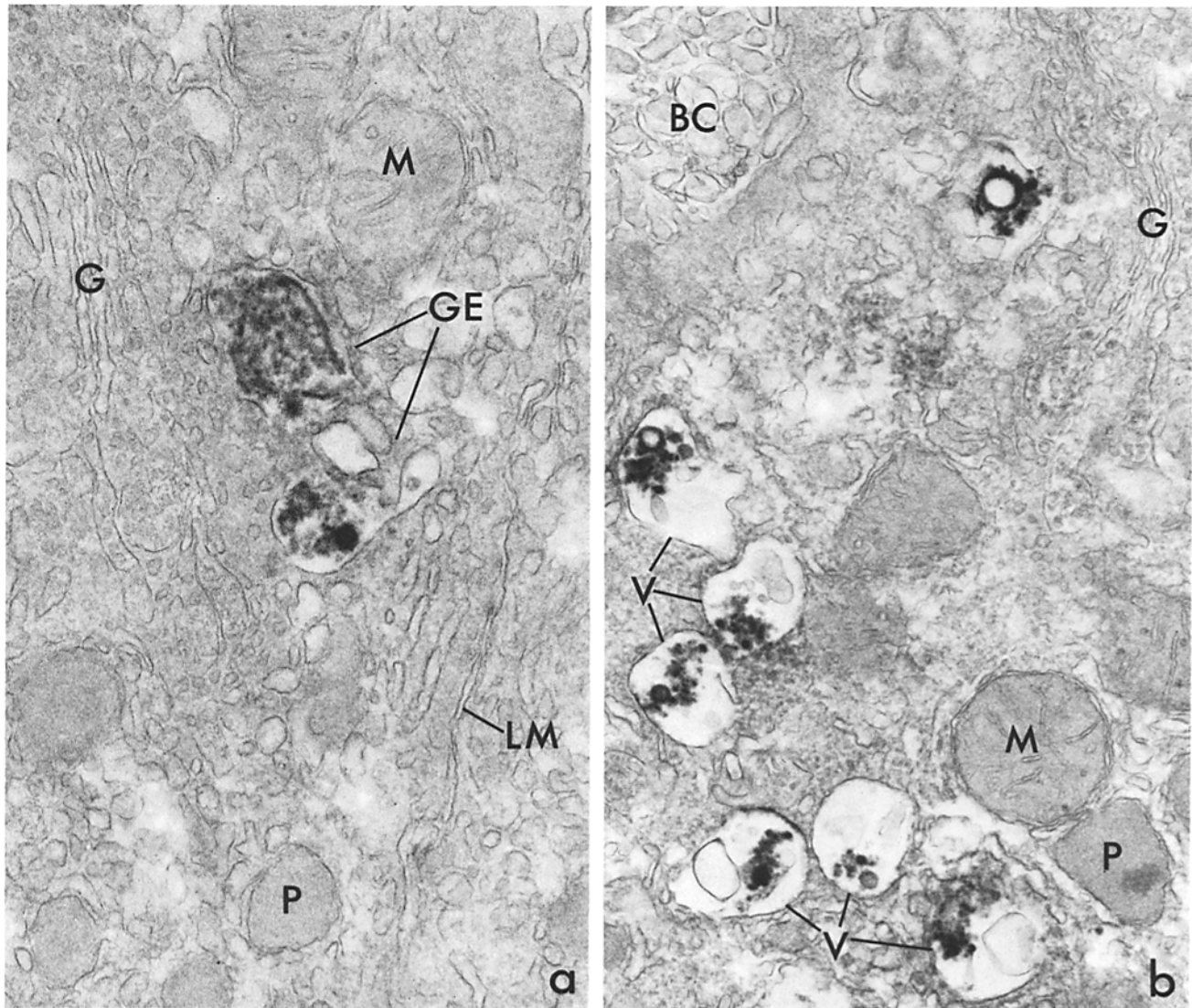


FIGURE 7 Nonfrozen sections of rat liver showing the localization of acid mannosidase activity by the protein A-HRP immunocytochemical method. (a) Control rat; (b) rat administered swainsonine. The pleomorphic vacuoles and portions of GERL (GE) show immunoreactivity. All the elements of the Golgi apparatus (G) are without acid mannosidase activity. Also without reaction product are mitochondria (M), peroxisomes (P), and lateral plasma membrane (LM). a, $\times 33,000$; b, $\times 33,000$.

system, which develops in the nondividing hepatocytes, is also found to be extensive even in dividing hepatocytes (Fig. 8a). The lysosomal system also retains its high acid hydrolase activity. AcPase is present in all pleomorphic vacuoles, many of which are closely apposed to each other and are possibly interconnected. The characteristic Golgi stack of cisternae is not evident in the dividing hepatocytes. The Golgi apparatus is reduced to what appears as a single cisterna, or element, spatially related to the chromosomes. The Golgi elements are difficult to identify except when specific cytochemical markers are used. TPPase, a marker for the Golgi apparatus (*trans* element) in nondividing hepatocytes, is localized to one of the dispersed Golgi elements that is found in the dividing cells (Fig. 8b).

DISCUSSION

We report evidence that the pleomorphic vacuoles induced by swainsonine administration in rat liver and kidney are lysosomes. These lysosomes, along with the cisternae of

GERL, form part of a lysosomal system. This system, which is acid hydrolase-rich, was found to be hypertrophied in Kupffer cells, hepatocytes, and proximal convoluted tubules in rats after swainsonine ingestion. It had already been reported, on the basis of light microscopic analysis, that *Swainsona canescens* ingestion by sheep causes a marked increase in the number of lysosomes in Kupffer cells (8). Clearly, hepatocytes, Kupffer cells, and proximal convoluted tubule cells show proliferation of lysosomes, as demonstrated by the extensive electron microscopic studies reported in the present paper. With *in situ* procedures, the vacuoles gave positive reactions for lysosomal enzymes, AcPase and acid mannosidase, but not the Golgi enzymes, nucleoside diphosphatase, and mannosidase II. Moreover, the ultrastructural appearance and enzymatic properties of the Golgi apparatus were not affected. By analogy with the effect of monensin in causing dilation of the Golgi apparatus in mouse plasma cells (23), the fact that swainsonine has a potent effect on the level of Golgi mannosidase II and modifies glycoprotein processing suggested the possibility that the swainsonine-induced vacu-

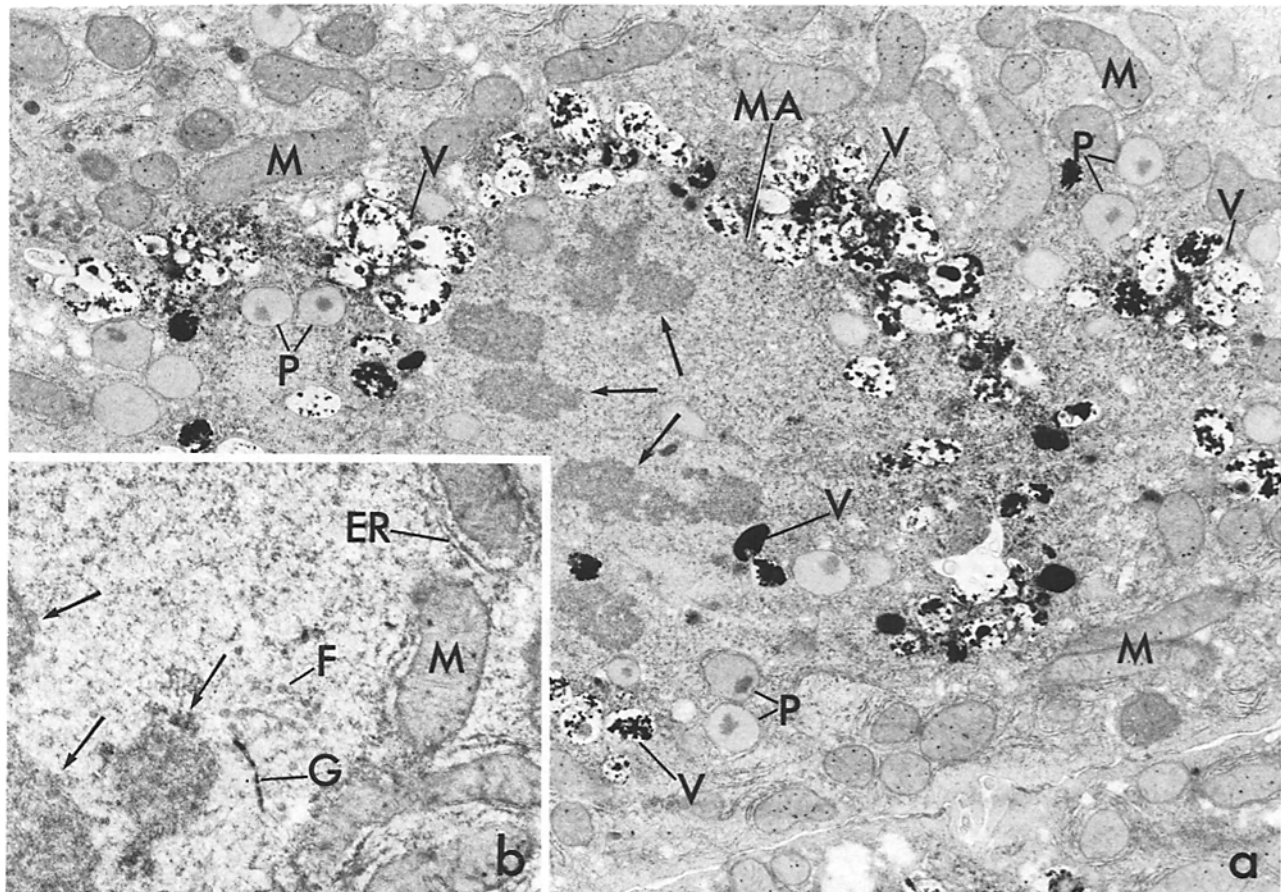


FIGURE 8 (a) Section of liver of rat fed swainsonine, incubated for AcPase activity. Most of the field is occupied by a hepatocyte in division. Reaction product is seen in the pleomorphic vacuoles (V) that are clustered near the mitotic apparatus. The chromosomes, seen at the arrows, are unreactive as are the mitochondria (M), peroxisomes (P), and other organelles. The peroxisomes are also grouped near the chromosomes. $\times 11,000$. (b) Section of liver of rat fed swainsonine, incubated for TPPase activity. The Golgi apparatus (G) is very much reduced; a portion of the TPPase-positive element is evident. Also seen are mitotic fibers (F), chromosomes (arrows), mitochondria (M), and endoplasmic reticulum (ER). $\times 15,000$.

olization might reflect a change in the Golgi apparatus. Our results clearly rule out this possibility. In fact, swainsonine appears to cause the Golgi elements of hepatocytes to be flatter and to have fewer discernible lipoprotein particles than in controls.

Since swainsonine is a potent inhibitor of both lysosomal α -D-mannosidase and Golgi mannosidase II (24), the severe toxic effects of the alkaloid may be due to actions on lysosomal function or on Golgi function, or both. Dorling et al. (8) have stated that (a) oligosaccharide accumulation is due to the inhibition of lysosomal α -mannosidase in the lysosomes, and (b) oligosaccharide accumulates within the lysosomes. With regard to the first point, it should be stated that although the increase in lysosomal α -mannosidase levels observed in rat liver and kidney extracts do not support this suggestion, it is still not known whether swainsonine in intact tissues is depressing mannosidase activity. Moreover, it has not been demonstrated that the oligosaccharide is in the same vacuoles that contain the α -mannosidase. Experiments on these questions are in progress. In this regard, it is noteworthy that the effects of swainsonine on liver indicates that vacuolization and oligosaccharide accumulation are not coupled.

The nature of the endogenous glycoproteins to be catabolized undoubtedly is of considerable importance in determining whether oligosaccharide accumulation occurs, as well as

the structures of the oligosaccharides that accumulate. Also pertinent is the fact that the substrate specificity of lysosomal α -D-mannosidase has not as yet been reported, and that the oligosaccharides that accumulate in hereditary α -mannosidosis are smaller (2-4) than those found in swainsonine poisoning. Since swainsonine induces the formation of hybrid glycoproteins in place of complex glycoproteins (12), the substrates for lysosomal α -D-mannosidase are different in swainsonine-fed animals from those in control animals or those with α -mannosidosis of genetic origin.

It is also of interest that pig tissues accumulate diacetylchitobiose derivatives (i.e., $\text{Man}_2\text{GlcNAc}_2$) (9), whereas rat kidney accumulates mono-GlcNAc derivatives (i.e., $\text{Man}_n\text{-GlcNAc}$). These differences are evidently due to a variation in endoglycosidase and/or oligosaccharide-asparagine hydrolase activities.

The pentamannosyl oligosaccharide ($\text{Man}_5\text{GlcNAc}$) found in the kidneys of rats administered swainsonine is probably derived from the hybrid type glycoproteins following the action of endo-N-acetyl- β -D-glucosaminidase and cleavage of distal sugars, N-acetylneuraminic acid, galactose, and N-acetylglucosamine, by exoenzymes. The tetra- and trimannosyl derivatives are likely formed as a result of partial cleavage of $\text{Man}_5\text{GlcNAc}$ by residual mannosidase activities. This explanation is supported by the data in Fig. 1, which show that a

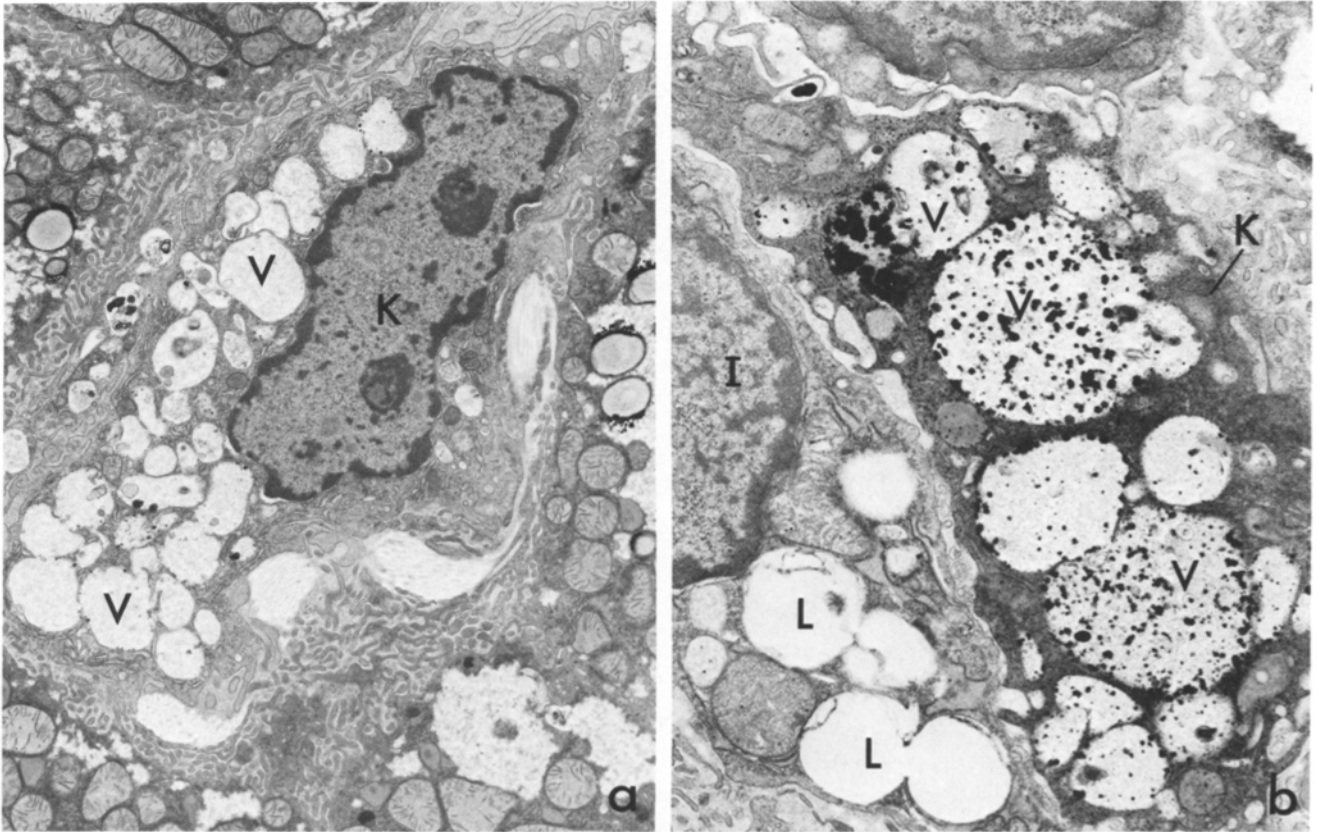


FIGURE 9 (a) Section of liver of rat fed swainsonine, unincubated. The Kupfer cell (K) occupies most of the field. Note the pleomorphic vacuoles (V). $\times 7,200$. (b) Section of liver of rat fed swainsonine, incubated for AcPase activity. The Kupfer cell (K) occupies a large part of the field. Reaction product is present in the pleomorphic vacuoles (V). In the Ito cell (I), there is no reaction product. The lipid droplets (L) are unreactive. $\times 13,000$.

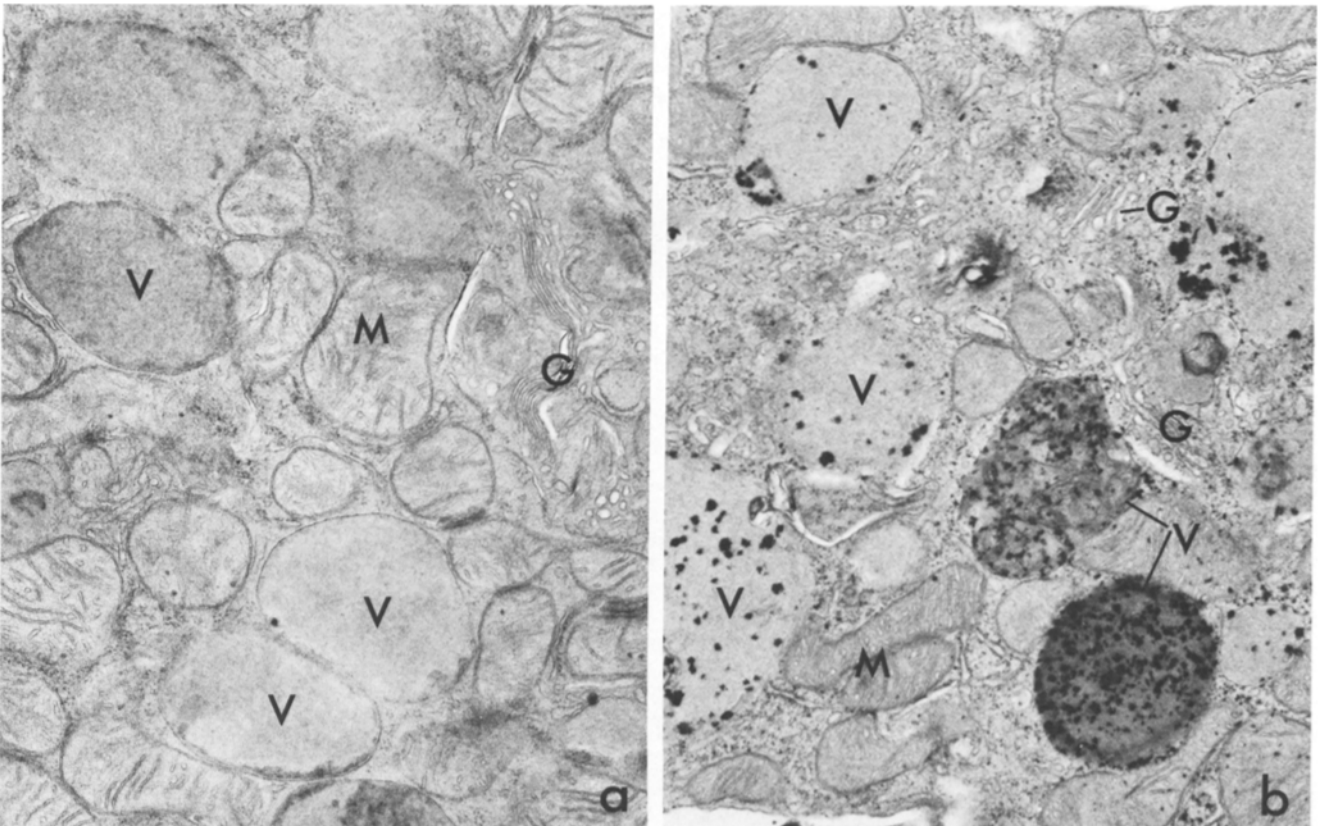


FIGURE 10 (a) Section of kidney of rat fed swainsonine, unincubated. A portion of the flattened Golgi apparatus is seen at G. V indicates the pleomorphic vacuoles. $\times 15,000$. (b) Section of kidney of rat fed swainsonine, incubated for AcPase activity. Reaction product is seen in the pleomorphic vacuoles (V). The Golgi apparatus (G) and other organelles are unreactive. Note the flattened elements of the Golgi apparatus. $\times 13,000$.

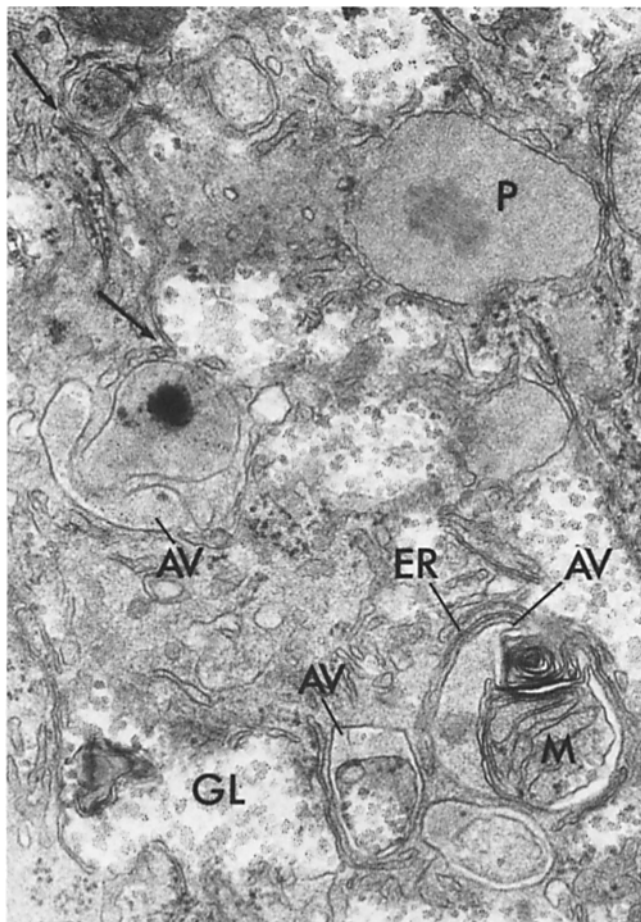


FIGURE 11 Sections of rat liver from rat fed swainsonine, unincubated. The early stage of vacuole formation is seen at AV. Note the proximity of rough endoplasmic reticulum to autophagic vacuoles (arrows). Also labeled are peroxisome (P) and glycogen (GL). A mitochondrion that is being enveloped by smooth endoplasmic reticulum (ER) to form an autophagic vacuole is seen at M. $\times 54,000$.

low dose of swainsonine ($5 \mu\text{g}/\text{ml}$ water) leads to a relatively high accumulation of trimannosyl derivatives (Fig. 1B). A high dose of the alkaloid, on the other hand, leads principally to the accumulation of penta- and tetramannosyl derivatives (Fig. 1D). It is reasonable to assume that animals administered higher levels of swainsonine will accumulate more of the alkaloid in their kidneys, thereby leading to greater inhibition of α -D-mannosidases.

Regardless of the eventual resolution of the above questions, the situation in rat liver is striking in that lysosomal hypertrophy occurs in the absence of oligosaccharide accumulation. Moreover, the marked increase in mitotic activity observed in the liver, occurring in the apparent absence of cell death or increased liver size, has no ready explanation. Our observations indicate that the proportion of mitotic figures in some rats is higher than that in regenerating liver.

It therefore appears that hypertrophy of the lysosomal system, the stimulation of autophagy, and the increase in mitotic figures in liver are results of hitherto unreported swainsonine-induced cellular events. An increased rate of autophagy may be related to rapid structural remodeling or metabolic reorientation. Since the toxin has marked effects

on the synthesis and structure of glycoproteins, which are key constituents of cell membranes, the changes in cell morphology may be consequences of altered membrane composition, turnover, fusion, and/or recycling. Additional studies with swainsonine may contribute to our understanding of membrane dynamics in liver and kidney.

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