Studies on Cellular Autophagocytosis

The Relationship of Autophagocytosis to Protein Synthesis and to Energy Metabolism in Rat Liver and Flounder Kidney Tubules In Vitro

J. D. Shelburne, MD, PhD, A. U. Arstila, MD, PhD and
B. F. Trump, MD

The purpose of the present study was to elucidate the metabolic requirements of autophagocytosis. Two model systems were used for this purpose: a) glucagon-induced autophagocytosis in the rat liver, and b) the wave of autophagocytosis which occurs when isolated flounder kidney tubules are incubated *in vitro*. In the rat liver, protein synthesis was inhibited by the administration of cycloheximide (1.5 mg/kg) to rats 2 hours prior to glucagon injection. In flounder kidney tubules, protein synthesis was inhibited at least 90% by adding cycloheximide, actinomycin D, pactamycin and puromycin to the medium. In both systems the inhibition of protein synthesis failed to inhibit the formation of autophagic vacuoles or their subsequent transformation into autolysosomes, as depicted from electron microscopic histochemical preparations. In flounder kidney tubules no differences were found in the levels of *p*-nitrophenylphosphatese, β-DL-glycerophosphatase, *N*-acetyl-β-D-glucosaminidase, arylsulphatase, β-D-galactosidase or acid proteinase when tubules were incubated up to 5 hours in the presence or absence of protein synthesis inhibitors. When ethionine was administered to rats 2 hours before glucagon injection, a decrease of approximately 75% in the ATP levels was observed. After ethionine administration, glucagon failed to induce the formation of autophagic vacuoles. The incubation of flounder kidney tubules in the presence of cyanide or in a nitrogen atmosphere decreased the ATP levels to less than 10% of controls and blocked autophagy. On the other hand, cyanide had little effect on acid hydrolase levels at 1 hour of incubation. A wide variety of other inhibitors were also shown to block autophagy. These results further support the hypothesis that, in the formation of antophagic vacuoles, preexisting enzyme and membrane pools are utilized. On the other hand, the esotropy-exotropy membrane conformational changes occurring in the formation of autophagic vacuoles seem to be energy dependent and can therefore be blocked by lowering intracellular ATP levels (Am J Pathol 73:641–670, 1973).

DURING THE PAST FEW YEARS, wide attention has been paid to the phenomenon of cellular autophagocytosis.[1,2] These studies have

From the Department of Pathology, Duke University Medical Center, Durham, NC, the Department of Cell Biology, University of Jyväskylä, Finland and the Department of Pathology, University of Maryland School of Medicine, Baltimore, Md.

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Address reprint requests to Dr. John D. Shelburne, Department of Pathology, Duke University Medical Center, Durham, NC 27710.

shown that autophagocytosis, although occurring in normal cells, is greatly augmented in a variety of conditions such as in the liver after administration of glucagon [3–8] or cyclic AMP,[9–11] in neurons after the administration of spindle inhibitors [12] and in the kidney after the administration of bacitracin.[1]

These studies have produced substantial information on the mechanisms by which autophagic vacuoles form and on their relationship to other lysosomes. On the other hand, only a few attempts have been made as yet to study the control mechanisms involved in the formation of autophagic vacuoles. However, such studies would be important to the understanding of the role of autophagocytosis both in normal and, especially, in pathologically altered cells. Although some attractive hypotheses have been presented,[1] little factual information has been gathered on this subject.

The present study was undertaken to delineate the relationship of autophagocytosis to protein synthesis and to oxidative phosphorylation. For these studies two model systems were chosen: glucagon-induced autophagocytosis in rat liver [2] and the wave of autophagocytosis which occurs when isolated flounder kidney tubules are incubated *in vitro*. These models were chosen because substantial information already exists on autophagocytosis in these two models and because autophagocytosis can be rapidly and reliably induced in both these systems.[13–16]

Materials and Methods

Rat Liver

General

Young female Sprague-Dawley rats (150 to 250 g) were used in all studies. The rats were fasted overnight (12 to 16 hours) and the experimental compounds were injected intraperitoneally the next morning (between 9:00 and 10:00 AM). Glucagon (Lilly) was injected as a single dose of 100 μg/100 g body weight, and the rats were killed 1 hour afterwards. Cycloheximide (Sigma) was injected as a single intraperitoneal dose of 1.5 mg/kg body weight, and the rats were killed 3 hours afterwards. In a number of experiments, cycloheximide administration was followed 3 hours later by a single intraperitoneal injection of glucagon. These rats were sacrificed 1 hour after glucagon administration. DL-Ethionine was injected as a single intraperitoneal dose of 1 g/kg body weight using a 0.25% solution, and the rats were killed 2 hours later. In some experiments, ethionine administration was followed by a single intraperitoneal injection of glucagon 2 hours later, and the rats were killed 1 hour after the glucagon administration. The control rats in all experiments received the same volume of physiologic saline solution intraperitoneally.

ATP Assay

Rats were lightly anesthetized with sodium pentobarbital while breathing oxygen, and ATP levels were determined using the method of Goldblatt [17] with

liquid-nitrogen-cooled brass tongs and the luciferin-luciferase assay.[18] The dosages and times postinjection of the assay are the same as noted above.

Electron Microscopy

The rats were killed by decapitation, and small pieces of liver from the left lateral lobe were rapidly removed for fixation. In some instances, perfusion fixation was accomplished by anesthetizing the rats and inserting a cannula into the portal vein. The liver was then perfused for 5 minutes with cold 4% sodium cacodylate-buffered glutaraldehyde followed by removal of small pieces of liver and rapid immersion of the pieces in the same fixative for 3 hours. After immersion or perfusion fixation in glutaraldehyde the pieces were washed overnight in sucrose-cacodylate buffer and postosmicated in 1% *s*-collidine-buffered osmium tetroxide (pH 7.4, 0 to 4 C) for 1 hour. In a number of studies, primary fixation was done by immersing small pieces of liver in 1% osmium tetroxide for 1 hour. All tissue was dehydrated in a series of ethanols, embedded in Epon, and thin sections were cut with diamond knives. The sections for routine electron microscopy were taken using Hitachi HU11E or HS8 electron microscopes.

Histochemistry

Acid phosphatase was demonstrated in control, glucagon-treated, and cycloheximide and glucagon-treated liver as follows: The livers were fixed with 4% sodium cacodylate-buffered glutaraldehyde (pH 7.4) for 3 hours as described previously.[6] After fixation the pieces were washed overnight in 0.1 M sodium cacodylate buffer (pH 7.4) containing 8% sucrose, and 5- to 50-μ-thick frozen sections were cut for light and electron microscopy. The incubation was performed according to the Gomori technic,[19] except that for electron microscopy the ammonium sulfide rinse was omitted.[20] The controls were done as described previously.[6] After incubation the tissue was postosmicated and processed as described above.

Flounder

General

These studies were done on isolated flounder (*Paralichthys lethostigma*) kidney tubules maintained at approximately 23 C in the medium described by Forster [21] which was gassed with 100% oxygen. The details of the collection of these animals, the dissection and incubation of the tubules, and the fixation for electron microscopy have been described previously.[11,22,23] Tubules were fixed by being placed in 1% osmium tetroxide buffered with *s*-collidine for 1 hour at 0 to 4 C. Dye transport was evaluated qualitatively by observing with a dissecting microscope the amount of dye concentrated in the tubular lumens.

Table 1 summarizes the treatments studied and dosages used. All inhibitors were weighed and put into solution immediately prior to the incubation. Thus actinomycin D (Upjohn) was protected from exposure to light until immediately before the experiment.[24] Pactamycin (Upjohn) was first dissolved in a small volume of 0.1 N HCl which was then added to the Forster's buffer. Cycloheximide was also obtained from Upjohn. All other inhibitors were obtained from Sigma. Antimycin A and oligomycin were first dissolved in a small volume of ethanol. Relative anoxia was achieved by placing the tubules in medium bubbled continuously with 100% nitrogen. Hypothermia (0 to 4 C) was achieved by incubating the tubules on ice without bubbling oxygen. The magnesium-free medium resembled that described above [21] except for the omission of $MgCl_2$. Addition of HCl to the standard buffer was used to decrease the pH to 3.0.

Table 1—Relationship of Autophagy to Several Aspects of Cellular Metabolism: Summary of Results of Experiments on Isolated Flounder Kidney Tubules and on Rat Liver

Treatment	Concentration	Inhibition	Flounder tubule dye transport*	ATP†	Wave of autophagy	Protein synthesis†
Flounder						
Control			+	+	+	+
Mitomycin	10^{-4}	DNA synthesis	+	+§	+	+§
Actinomycin D	5, 50 μg/ml	mRNA, protein synthesis	+	+	+	0
Cycloheximide	10^{-4}, 10^{-5}	Protein	+	+	+	0
Pactamycin	10^{-4}, 10^{-5}	synthesis	+	+	+	0
Puromycin	2×10^{-4}		+	+§	+	0§
N_2	—	Electron	0	0	0	0§
CN^-	10^{-3}, 10^{-5}	transport	0	0	0	0§
Antimycin A	10^{-4}		0	0§	0	0§
Oligomycin	10^{-4}	Oxidative phosphorylation	0	0§	0	0§
2, 4 DNP	10^{-3}, 10^{-4}, 10^{-5}	Uncouple	0	0§	0	0§
Arsenate	10^{-4}	oxidative phosphorylation	0	0§	0	0§
KF	10^{-2}, 10^{-4}	Glycolysis	0	0§	0	0§
IAA	10^{-3}, 10^{-4}		0	0§	0	0§

Ouabain	$10^{-3}, 10^{-4}$	Na-K ATPase	0	0§	0	0§
PCMBS	$10^{-3}, 10^{-5}$	Binds sulfhydryl	0	0§	0	0§
$HgCl_2$	$10^{-3}, 10^{-5}$	groups	0	0§	0	0§
0 to 4 C	—	Nonspecific	0	0§	0	0§
decreased pH	3.0	metabolic	0	0§	0	0§
Mg^{++}-free	—	effects	0	0§	0	0§
Rat						
Glucagon				+	+	+§
Glucagon + Cycloheximide		Protein synthesis		+	+	0§
Glucagon + Ethionine		Traps adenine		0	0	+§

* + = ability to concentrate chlorphenol red dye, 0 = markely impaired ability to concentrate dye

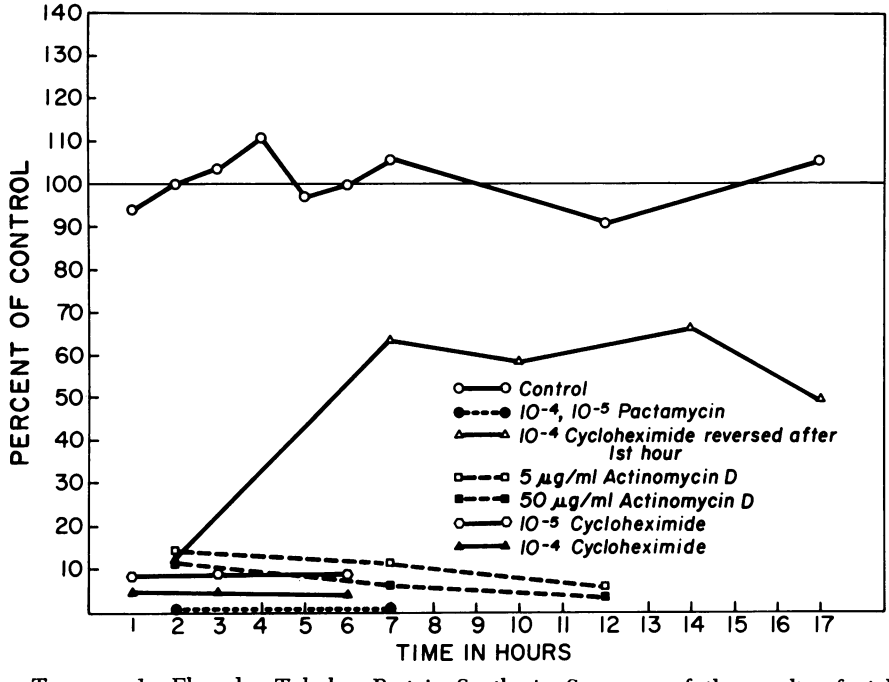
† + = maintainance of normal or near normal ATP levels or protein synthesis, 0 = a significant drop occurred early in the experiment

‡ + = presence of wave of autophagy as determined by electron microscopy, 0 = absence of autophagy

§ not documented by experiments reported in this paper; presumed correct information based on the available literature and other experiments has been shown.

Note that the wave of autophagy occurs only when ATP values are maintained. In those cases in which ATP was not actually measured, lack of dye transport is assumed to indicate an ATP drop. The wave of autophagy occurs both when protein synthesis is inhibited or not inhibited. (In those cases in which protein synthesis was not actually measured it is assumed that lack of ATP will block protein synthesis. The only exception may be the case of ethionine administration as reported by Villa-Trevino, Shull and Farber[3]- due to the time course of these experiments.)

All inhibitors were present during the entire incubation period with the exception of one experiment with cycloheximide in which the reversibility of inhibition was studied. In this experiment, after 1 hour of incubation in 10^{-4} M cycloheximide, the remaining tubules were rapidly washed three times and then allowed to continue incubating in control medium without cycloheximide. All tubules were incubated in these experiments as described above, except for the addition of 1.6 mM unlabeled leucine to control and experimental dishes. Two different assay technics were employed. In one, aliquots of the tubules in various control or experimental dishes were incubated with 2.2 μM labeled leucine (L-leucine 4,5-^{3}H, New England Nuclear Corporation, 5 Ci/mmole) during 1-hour intervals immediately prior to homogenization. This technic was used in the experiments in which the reversibility of cycloheximide inhibition was studied and in the antinomycin D and pactamycin experiments. In the other, labeled leucine was present during the entire experimental period. This technic was employed in the experiments with cycloheximide. In all experiments, control tubules were assayed for protein synthesis using the same technics at the same times as treated tubules. At the various times noted in Text-figure 1, tubules were removed from the dishes and homogenized in a ground glass homogenizer in deionized water. Aliquots of the homogenate were analyzed for protein by the method of Lowry [25] *et al.* Other aliquots of the homogenate were pipetted onto borosilicate filter discs and prepared by



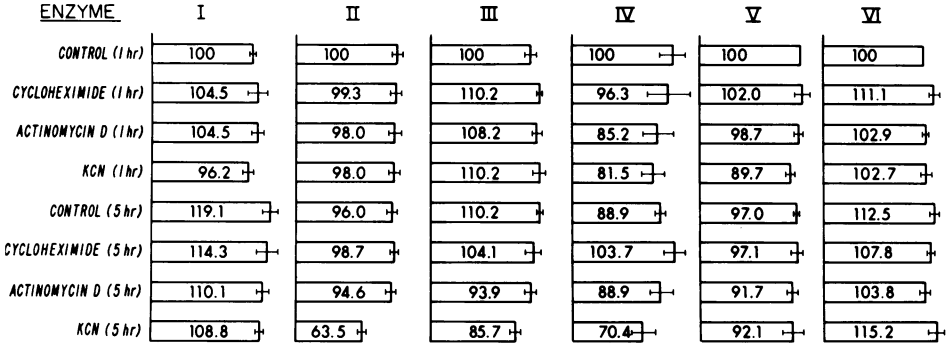
TEXT-FIG 1—Flounder Tubules, Protein Synthesis. Summary of the results of eight separate experiments. Each point represents the mean value of leucine incorporation per milligram protein per hour of incubation under the various conditions. In the case of control data, the pooled mean values are plotted as a percent of the mean 2-hour value to show the variability of the controls with time. In controls the mean incorporation of leucine into protein over the interval from 1 to 2 hours of incubation was 1400 ± 220 (mean ± SE, N = 6) μμmoles/mg protein/hr. In the case of values from inhibited tubules, they are given as percents of the 2-hour control values obtained in the same experiment.

the method of Mans and Novelli,[26] modified as follows: the discs were immediately plunged into 5% trichloracetic acid (TCA) at 4 C, washed in two changes of 5% TCA at room temperature, washed in absolute ethanol for 1 minute at room temperature, washed in ethanol-cholorform-ether (2:2:1) for 1 minute at room temperature, and washed in two changes of ether at room temperature. Samples were air dried and placed in low-potassium glass scintillation vials. Liquid scintillation solution containing 5 g of 2,5-diphenyloxazole and 0.5 g of 2,2-*p*-phenylenebis(5-phenyloxazole)/liter in anhydrous toluene was added to each vial. Samples were then counted in a liquid scintillation counter at an efficiency of 25%.

Enzyme Assays

Aliquots of approximately 50 mg of flounder tubules treated as indicated in Text-figure 2 were removed from the incubation medium and placed on small squares of aluminum foil in a slight depression. Excess medium was rapidly removed with a Pasteur pipette connected to a vacuum line. The tip of the pipette was placed near the edge of the depression, and the medium was rapidly decanted. Immediately, the aluminum foil and tissue were plunged into liquid nitrogen. The frozen tissue was subsequently removed from the foil into 5 ml of deionized water in a ground glass homogenizer and homogenized with twenty strokes. The homogenate was then decanted into plastic vials, which were immediately frozen until assayed.

For determination of total activities, homogenates were treated with 0.2% Triton X-100 for 30 minutes and suitable dilutions were then made for analyses. Acid proteinase activity was assayed at pH 4.8 by the method described by Riekkinen and Clausen [27] using denatured human hemoglobin as a substrate. Re-

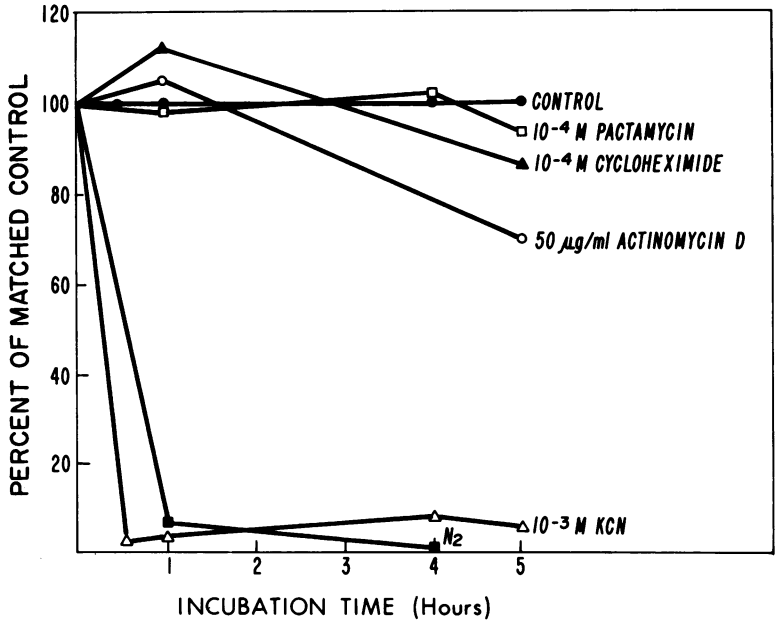


TEXT-FIG 2—Flounder Tubules, Enzymes. The figure shows the levels of six different flounder kidney tubules enzymes at 1 and 5 hours of incubation. Three different treatments were studied in addition to the controls: 10^{-4} M cycloheximide, 50 μg/ml antinomycin D and 10^{-3} M KCN. In each case, the mean of three separate experiments done at different times is shown by the length of the bar, plus or minus the standard errors. The mean values are expressed as a percent of the mean 1-hour control values, except for those of V and VI, where, because of the marked variations in the controls between the three different experiments, each separate experiment was compared with its matched 1-hour control value. The graph shows the mean of the three percents ± SE. *I* = *p*-nitrophenylphosphatase (100% control = 39.8); *II* = β-DL-glycerophosphatase (100% control = 14.8); *III* = acid proteinase (100% control = 4.9); *IV* = aryl sulfatase (100% control = 2.7); *V* = β-galactosidase (100% = 13.1) *VI* = β-glucosaminidase (100% control = 111.0). The absolute units are described in Material and Methods.

sults are expressed as nanomoles tyrosine liberated per milligram protein per minute. Acid phosphatase (EC No 3.1.3.2) activity was assayed at pH 4.8 using both *p*-nitrophenylphosphate and β-DL-glycerophosphate as substrates, as described by Riekkinen and Clausen [27] and Sellinger and Hiatt.[28] Results for *p*-nitrophenylphosphatase are expressed as nanomoles liberated *p*-nitrophenyl per milligram protein per minute and those for β-DL-glycerophosphatase as nanomoles liberated phosphate per milligram protein per minute. Aryl sulphatase (EC No. 3.1.6.1) activity was assayed at pH 5.0 using *p*-nitrocatechol sulfate as substrate, as described by Sellinger and Hiatt.[28] The results are expressed as nanomoles liberated *p*-nitrocatechol per milligram protein per minute. β-Glucosaminidase (EC No. 3.2.3.39) activity was assayed at pH 4.8 using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as substrate, as described by Sellinger and Hiatt.[28] The results are expressed as nanomoles liberated *p*-nitrophenyl per milligram protein per minute. β-Galactosidase (EC No. 3.2.1.23) activity was assayed at pH 4.2 using *p*-nitrophenyl-β-D-glactopyranoside as a substrate, as described by Beck and Tappel.[29] The results are expressed as nanomoles liberated *p*-nitrophenyl per milligram protein per minute.

ATP Assay

Aliquots of approximately 200 mg of flounder tubules treated as shown in Text-figure 3 were removed from the incubation medium and placed on small squares



TEXT-FIG 3—Flounder Tubules, ATP Levels. The graph shows the level of ATP in flounder tubules under the following conditions: incubation in control medium alone, incubation in 10^{-4} M pactamycin, in 10^{-4} M cycloheximide, in 50 µg/ml actinomycin D, 10^{-3} M KCN or bubbled in N_2. Each value is expressed as the percent of its matched control value, *ie,* tissue maintained in control medium for the same time as the treated tissue. Each point represents, in most cases, the mean of two or three determinations of the ATP levels in the seven separate experiments which were done to accumulate this data. The mean of the control ATP level at 1 hour was 0.30 ± 0.11 (mean ± SE, N = 4) and at 5 hours, 0.37 ± 0.11 (mean ± SE, N = 4) µmoles/g wet weight of tissue.

of aluminum foil in a slight depression. Excess medium was rapidly removed with a Pasteur pipette connected to a vacuum line. The tip of the pipette was placed near the edge of the depression and the medium was rapidly decanted. Immediately, the aluminum foil and tissue were plunged into liquid nitrogen. The frozen tissue was subsequently weighed and triturated in 9 ml of frozen 3.14% perchloric acid. ATP was analyzed by the luciferin-luciferase assay.[18]

Results

Rat Liver

ATP Values

As shown in Table 2, ethionine administration dropped the ATP levels in 2 hours to 25% of the control values.[30,31] This low level was maintained also after subsequent injection of glucagon to these ethionine-treated rats. Glucagon administration alone had little effect on the ATP level.

Table 2—Rat Liver ATP Values as Percent of Matched Control Values

Treatment	No. of pairs	Value (% of matched control value)	Significance (treated vs matched control)
Glucagon	11	97	$P \leq 0.1$
Ethionine	10	25	$P \leq 0.001$
Ethionine and glucagon	5	11	$P \leq 0.03$

The pairs of rats represent 1 treated and 1 control. For the various treatments, significance tests (Wilcoxon matched pair signed rank test [75]) were conducted comparing the pairs of control vs treated rats, using for each rat the mean of at least two determinations of ATP. For a given treatment, the mean of all the treated values was divided by the mean of the matched control values to obtain the percent level of ATP. The mean of the control ATP level at 1 hour is 2.17 ± 0.28 (SEM, N = 11) μmoles/g wet wt.

Electron Microscopy

Controls. Autophagic vacuoles* were only rarely observed in the liver cells of fasted animals. Approximately one autophagic vacuole was seen per three liver cells. The distribution of acid phosphatase reaction product was similar to that described previously.[6]

Glucagon Administration. One hour after glucagon injection a marked increase in the number of autophagic vacuoles was readily noticeable, mainly in areas around the bile canaliculus. The fine structure of the vacuoles was similar to that described previously [6] and fell

* Autophagic vacuoles in both model systems [6,13] are defined as membrane-bounded spaces containing recognizable organelles or bits of cytoplasm in various stages of degradation. Autophagic vacuoles are thus one type of cytosegresome as defined by Ericsson.[1] The reasons for regarding the cytosegresomes described here as autophagic and not heterophagic vacuoles have been reviewed by Ericsson.[1]

into two categories: a) vacuoles limited by a double membrane and b) vacuoles limited by a single membrane only (Figure 1). In preparations incubated for the demonstration of acid phosphatase activity, it was seen that newly formed autophagic vacuoles, devoid of acid phosphatase activity, acquired lead phosphate reaction product by fusion with primary or secondary lysosomes.[6,16]

Cycloheximide Administration. Three hours after cycloheximide administration, morphologic changes were limited to the endoplasmic reticulum and the Golgi apparatus, as described by Verbin, Goldblatt and Farber.[32] Autophagic vacuoles were only infrequently seen in cells, and in this respect the cells resembled the control cells. The distribution of acid phosphatase reaction product was similar to that seen in controls and has been described previously.

Cycloheximide and Glucagon Administration. Pretreatment with cycloheximide did not seem to have any effect on the formation of glucagon-induced autophagic vacuoles. There appeared to be as many autophagic vacuoles around the bile canaliculus as in cells treated with glucagon alone. The distribution of acid phosphatase reaction product (Figure 2) was similar to that seen after glucagon administration alone.[6]

Ethionine Administration. Two hours after ethionine injection, marked changes in the fine structure of the liver cells were readily noticeable. Since these changes have been well described in previous studies,[32–34] only alterations in lysosomes will be described in this connection. The secondary lysosomes were larger than in controls, and it also appeared that their inner contents were more electron lucent than those of secondary lysosomes in controls. The pale appearance of lysosomes was especially striking in osmium-fixed and double-stained preparations. Also, the shape of the lysosomes was more irregular, and thin tail-like processes were often seen to protrude from the lysosomes. Double-membrane-limited autophagic vacuoles were only rarely seen in these preparations, but the number of single-membrane-limited autolysosomes appeared to be somewhat greater than in controls.[35]

Ethionine and Glucagon Administration. Fine structural changes in these cells (Figure 3) closely resembled those seen after ethionine administration alone. Double-membrane-limited autophagic vacuoles were only rarely seen. The number of single-membrane-limited autophagic vacuoles was considerably less than after glucagon treatment only.

Flounder Kidney Tubules

Inhibition of Protein Synthesis

The results of the protein synthesis assays are shown in Text-figure 1.

Protein synthesis was most markedly inhibited in tubules treated with pactamycin and was less than 10% of control levels in tubules treated with cycloheximide. Actinomycin D was slightly less effective initially but increased in effectiveness with time. The inhibition produced by cycloheximide could be partially reversed by changing the medium.

ATP Values

The results of the ATP assays are shown in Text-figure 3 and in Table 1. Tubules incubated anoxically by bubbling with N_2 or in 10^{-3} M KCN showed a prompt drop in ATP levels to less than 10% of the control values by 1 hour, whereas tubules incubated with cycloheximide, pactamycin or actinomycin D showed no significant changes early in the incubation. By 5 hours, tubules treated with actinomycin D did show a decrease to 70% of the control value.

Enzyme Determinations

The results of the enzyme determinations are shown in Text-figure 2. It is seen that the activities of the six acid hydrolases were quite stable during the experiment, except in the experiments in which tubules were incubated for 5 hours in the presence of cyanide. It is seen that by this time some enzyme activities were noticeably decreased. No significant increases in enzyme activities were noted.

Dye Transport

Dye transport was not affected over the 5-hour period in control tubules or in tubules treated with cycloheximide, puromycin or mitomycin. Tubules treated with actinomycin D and pactamycin also resembled controls; however, a very slight decrease in dye transport was noted in some experiments by 5 hours. As is shown in Table 1, all other treatments resulted in a marked loss of dye transport.

Electron Microscopic Findings

The results are summarized in Table 1, which shows whether or not a wave of autophagy occurred in the various treatments. In tubules fixed immediately after removal of the kidney, very few autophagic vacuoles were observed. After 1 hour of incubation in control medium, however, an increased number of autophagic vacuoles was seen as described previously. The majority of these newly formed autophagic vacuoles were localized in the apical cytoplasm in the area between the Golgi apparatus and the apical microvilli. These autophagic vacuoles were often limited by a double membrane and contained well-preserved organelles such as mitochondria. After 5 hours of incubation, the number of autophagic vacuoles was approximately the same as

after 1 hour. However, at this time the majority of the autophagic vacuoles were limited by a single membrane only, and the organelles within the autophagic vacuoles were considerably deteriorated (Figure 4).

Treatment with cycloheximide, pactamycin, actinomycin D, puromycin [36] and mitomycin [37] failed to affect the wave of autophagy. After 1 hour of incubation there was a conspicuous increase in the number of autophagic vacuoles which was of the same magnitude as that in the tubules incubated in the control medium. As in the control experiments, most of these autophagic vacuoles were limited by a double membrane and contained easily recognizable cytoplasmic organelles. At 5 hours the number of autophagic vacuoles was approximately the same as after 1 hour, but the majority of the vacuoles were now limited by a single membrane only (Figure 5).

It was furthermore noted in the tissue incubated with both doses of cycloheximide that no ultrastructural effects of the inhibition of protein synthesis could be observed at 1 or 5 hours (Figure 5). Polysome patterns were retained, and no signs of chromatin clumping were noted. Mitochondria were intact. The tubules resembled the control tubules in every respect. Tubules treated with 50 μg/ml of actinomycin D, however, did show marked loss of polysomal arrays, chromatin clumping with margination around the periphery of the nucleus and nucleolar stratification by 5 hours of incubation (Figure 7A). Tubules incubated with the lower dose of antinomycin D (5μg/ml) also showed this nucleolar stratification (Figure 7B) and loss of polysomal arrays but did not exhibit chromatin clumping. Tubules incubated with pactamycin showed only a loss of polysomal arrays by 5 hours but otherwise resembled controls. Tubules incubated with puromycin (Figure 6) and mitomycin exhibited few changes from normal.[38,39]

As noted in Table 1, all other treatments not only blocked dye transport but also blocked autophagy (Figures 8–11). Interestingly, all of these treatments, with the exception of hypothermia, also eventually (by 5 hours) resulted in high amplitude mitochondrial swelling (Figure 8). Tubules incubated at 0 to 4 C showed little dye transport or autophagy (Figure 9) but appeared capable of retaining zero time morphology for up to 24 hours.[39] Details of the ultrastructure of tubules treated with ouabain or *p*-chloromercuribenzenesulfonate (PCMBS) have been presented previously.[22,23] Tubules incubated anoxically by being continuously bubbled with nitrogen (Figure 8) resembled essentially those incubated with cyanide and described previously.[40] Details

of other aspects of the ultrastructure of these and other treatments will be described elsewhere.

Discussion

In order to discuss the metabolic requirements of the membrane movements in autophagy, a brief description of the terminology used is necessary. Recently, Arstila and Trump introduced the term *cytocavitary network*[41] to refer to certain functionally interconnected, membrane bounded organelles in the cytoplasm other than mitochondria: the endoplasmic reticulum, microbodies, the Golgi apparatus, various types of transport, storage and secretory vacuoles, digestive vacuoles (lysosomes) and phagosomes. Accordingly, the cell sap can be regarded as the true intracellular space, separated from the extracellular space and from the space of the cytocavitary network by a membrane, which includes both the membrane of the cytocavitary network and the plasma membrane. Although both structural and functional differences have been described in various parts of the membrane, it appears from many studies that the conformational changes which occur in this membrane during membrane movements and during the flow of materials in the network are basically similar in all parts. To further define these conformational changes with respect to the intracellular space as they are seen under the electron microscope, the following new terms were introduced.[42]

Esotropy. This term refers to a membrane protrusion towards the cell sap and the total fission of a portion of the invaginated membrane to form a new membrane-bounded cavity. Also we include in this term the reverse process, in which a protrusion occurs when a membrane-bounded cavity fuses with the membrane.

Exotropy. This term refers to the protrusion of the membrane into the extracellular space or into the cytocavitary network. Similar to esotropy, this can either be associated with fission and the formation of a new membrane-bounded cavity or it may be preceded by fusion of the membrane-bounded cavity to form protrusions.

It should be pointed out that these phenomena are opposite, both in respect to the cell sap and in the sense that in exotropy the membrane is arranged similarly to the cell membrane so that the outer lamina and associated glycoprotein coat is outwards, whereas in esotropy the membrane is inverted so that the glycoprotein coat faces the lumen of the cavity.

As discussed in the previous paper[41] the formation of autophagic

vacuoles presents the most common type of exotropy in the normal cell. However, it is not the only type of exotropy. The extrusion of uncoated myxoviruses from cells appears to occur through exotropy into the extracellular space so that during this process the virus becomes coated with a small portion of plasma membrane.[43]

Inhibition of Protein Synthesis

In their classic paper on actinomycin D toxicity and ultrastructural effects, Jézéquel and Bernhard [44] noted both nucleolar stratification and large numbers of autophagic vacuoles in pancreatic exocrine cells. They speculated that deranged protein synthesis due to the actinomycin D might have caused the formation of these autophagic vacuoles. The finding in this study, and in that of Verbin, Goldblatt and Farber,[32] that injections of cycloheximide do not produce autophagic vacuoles argues against this theory of formation, since cycloheximide injections inhibit protein synthesis over 90% within 2 hours in rat liver.[45,46]

In order to form a new autophagic vacuole, proteins will be needed for two purposes: a) for the formation of the membrane, which eventually will enclose a part of cytoplasm and transform into the membrane of an autophagic vacuole, and b) for the synthesis of acid hydrolases. Thus, for autophagy to occur, either new membranes and enzymes must be synthesized or preexisting membranes and enzymes must be utilized.

Arstila and Trump [6] proposed the latter theory—that, in the rat liver, autophagic vacuoles form in acute experiments by enclosure of a part of cytoplasm by the membranes of endoplasmic reticulum, and that the acid hydrolases are transported into newly formed autophagic vacuoles from preexisting sources, mainly from the Golgi apparatus and from secondary lysosomes.

The independence of autophagocytosis from new protein synthesis has been further confirmed by this study. Despite its marked effect on protein synthesis, cycloheximide seemed to have no effect on the formation of autophagic vacuoles by glucagon nor on their subsequent transformation into autolysosomes. The same was true in the flounder kidney tubules. The almost total inhibition of protein synthesis by protein synthesis inhibitors did not block the usual autophagic wave and had little effect on the levels of acid hydrolase activities. Note that these levels are expressed as the ratio of enzyme to cellular protein. In control incubations no increase in this ratio was seen with time (5 hours compared to 1 hour). From this it can be concluded that a) no significant synthesis of enzyme occurred during autophagy unless b) synthesis of other proteins also occurred to balance the increased amount of

enzymes and keep the ratio of enzyme to general protein constant. The results of the incubations with protein synthesis inhibitors rule out this second possibility, since protein synthesis was inhibited but autophagy still occurred. Thus synthesis of new enzyme is not necessary for autophagy in this model.

It was also of interest to note that cycloheximide, an inhibitor of peptide bond formation,[47–49] had no detectable pathologic effects on the fine structure of the kidney tubules, even though protein synthesis was inhibited. These findings support those of Verbin, Goldblatt and Farber [32] in rat liver. The inhibition of protein synthesis in stable nondividing cells is apparently not lethal to the cell for several hours at least. Actinomycin D treatment did result in several changes. The formation of nucleolar caps has been noted by several other authors [22,24] and provides further evidence that this agent did indeed penetrate the cells. The ribosomal scattering noted was probably due to direct toxic effects of the actinomycin D to polyribosomes and ribosomes.[50,51] These effects, rather than inhibition of messenger RNA synthesis, are also thought to be primarily responsible for the inhibition of protein synthesis observed, since messenger RNA synthesis inhibition should not result in such a rapid inhibition of protein synthesis (Text-figure 1).[50] Furthermore, Laszlo *et al* [52] have shown that actinomycin D has other effects on mammalian cellular glycolysis and oxidative phosphorylation, and these may be responsible for the drop in ATP observed by 5 hours. The slight loss of polysomal arrays noted with pactamycin [53,54] probably reflects the fact that this agent interferes with the association of messenger RNA, ribosomes and transfer RNA.[51,57]

Since autophagocytosis can be envisioned as a secretory process in which lysosomal hydrolases are secreted into the lumen of the cytocavitary network instead of the extracellular space, it is of interest to compare these results to the metabolic requirements of secretion. In pancreatic exocrine cells, Jamieson and Palade [58] have shown that after the secretory proteins have been synthesized the transport and discharge of the secretory proteins [59] does not require new protein synthesis during the secretory process. The independence of autophagocytosis of protein synthesis is, however, even greater, since the cells seemingly have a large enough pool of preexisting acid hydrolases which after the induction of autophagocytosis can be transported into the newly formed autophagic vacuoles. Whether the preexisting membrane and/or enzyme pool is large enough to allow the formation of repeating cycles of autophagocytosis is not known. Both in the liver and in the kidney, it seems as if the formation of autophagic vacuoles was limited to the

beginning of the experiment and that new autophagic vacuoles did not form after this initial period.

Relationship to Energy Metabolism

The present study showed that glucagon failed to induce autophagocytosis when ethionine was injected 2 hours before glucagon administration. Since the intracellular effects of ethionine at 2 hours are mainly associated with a decrease in ATP levels,[31] it may be assumed that the lowered energy metabolism was directly associated with the inhibition of autophagocytosis. Although ethionine also inhibits protein synthesis,[60] especially at longer time intervals, this probably cannot contribute to the inhibition of autophagocytosis for the reasons stated above. The dependence of autophagocytosis on energy metabolism was even more clearly demonstrated in flounder kidney tubules incubated anoxicly or in the presence of cyanide (Table 1, Text-figures 2 and 3). These kidney tubules failed to show any autophagocytosis. Dye transport was blocked, and the other fine structural effects of these inhibitors were readily visible. That the inhibition of energy metabolism by cyanide primarily affected the esotropy-exotropy conformational changes involved in autophagocytosis was apparent on the basis that the cyanide had little effect on the levels of acid hydrolases up to 1 hour. The decreased levels of enzymes noted at 5 hours was probably the result of leakage to the medium from the markedly necrotic cells.[40]

The inhibition of autophagy by antimycin A, oligomycin, 2,4-dinitrophenol, arsenate, fluoride and iodoacetic acid is assumed to be due to decreased ATP levels, but whether these decreases are due to inhibition of specific sites in the electron transport chain and glycolysis [61–64] or rather to nonspecific toxic effects must await further study.

Inhibition of autophagy by PCMBS, $HgCl_2$ and ouabain presumably resulted from decreased ATP levels secondary to the widespread membrane damage caused by these agents,[22,23,64] Hypothermia, low pH and magnesium deficiency presumably blocked autophagy by nonspecific metabolic effects, the chief consequence of which was probably low ATP levels.

Clearly, treatment of tubules with some of these agents is so harsh as to cause marked structural damage as well as ATP deficiency, and it is quite possible that such structural damage might in itself prevent autophagy. For example, it is difficult to see how the dilated cisternae of the endoplasmic reticulum seen in anoxia (Figure 8) could envelope mitochondria. Nevertheless, several of these treatments which blocked autophagy did not cause marked structural changes. Tubules incubated

at 0 to 4 C showed essentially normal ultrastructure (Figure 9). The endoplasmic reticulum was not dilated. Tubules treated with 2,4-dinitrophenol (Figure 11) were normal early in the incubation, although at later times they did show high amplitude mitochondrial swelling. Also, ethionine treatment did not produce marked alterations of mitochondria or endoplasmic reticulum in the rat liver experiments.

Thus for a wave of autophagy to occur in the glucagon model or in the flounder model ATP must be available. As is shown in Table 1, in every case in which dye transport was blocked autophagy was also blocked. Generally, by 5 hours these same treatments also produced high amplitude mitochondrial swelling which is known to be associated with failure of mitochondria to synthesize ATP.[65,66] This decrease in ATP levels was measured in the case of cyanide and anoxia (Textfigure 3). Also measured (Table 2) was the decreased ATP produced by ethionine which also blocked autophagy.

The energy requirement of esotropy–exotropy conformational changes has also been well documented in other systems. The studies of Jamieson and Palade [58] have shown that the transport of secretory proteins from the site of synthesis at the rough surfaced endoplasmic reticulum into acinar lumens requires energy at two points: a) in the transport of protein from rough surfaced endoplasmic reticulum to condensing vacuoles and b) in the secretion of mature zymogen granules into acinar lumens. That the release of secretory granules into the extracellular space is an energy-requiring process has also been shown by Hokin and Hokin [67] and by Schramm.[68] Previous studies have also demonstrated that esotropy–exotropy transformation can be induced *in vitro* by adding high energy compounds into the incubation medium. Thus Penniston and Green [69] have reported the ATP-stimulated esotropy of red cell ghosts.

Finally, our recent finding that the effects of glucagon can be mimicked by cyclic AMP [10] support the possibility of a relationship of this cyclic AMP stimulation to the ATP requirement. Cyclic AMP is synthesized from ATP. Recently Kuo and Greengard [70] in collaboration with Sutherland proposed a unifying theory for the mechanism of action of cyclic AMP. These authors were able to demonstrate cyclic-AMP-dependent protein kinases in *all* of the thirty sources of many mammalian tissues examined, including rat liver, and suggested that cyclic AMP always exerts its effects through activation of tissue-specific protein kinases.[71] Furthermore, Knodell *et al* [72] have shown that cyclic AMP stimulates

pancreatic exocrine secretion. In light of these and other discoveries, Rasmussen [73] has proposed a model of secretion in which microtubular contractile systems are activated by cyclic AMP. Since the addition of acid hydrolases during the autophagic wave is a form of intracellular secretion and since autophagy involves many membrane movements, it would seem possible that similar microtubular or microfilaments contractile systems may be of great importance in stimulating autophagy. Thus the recent discovery that colchicine and vinblastine will stimulate autophagy [74] may be of great significance.

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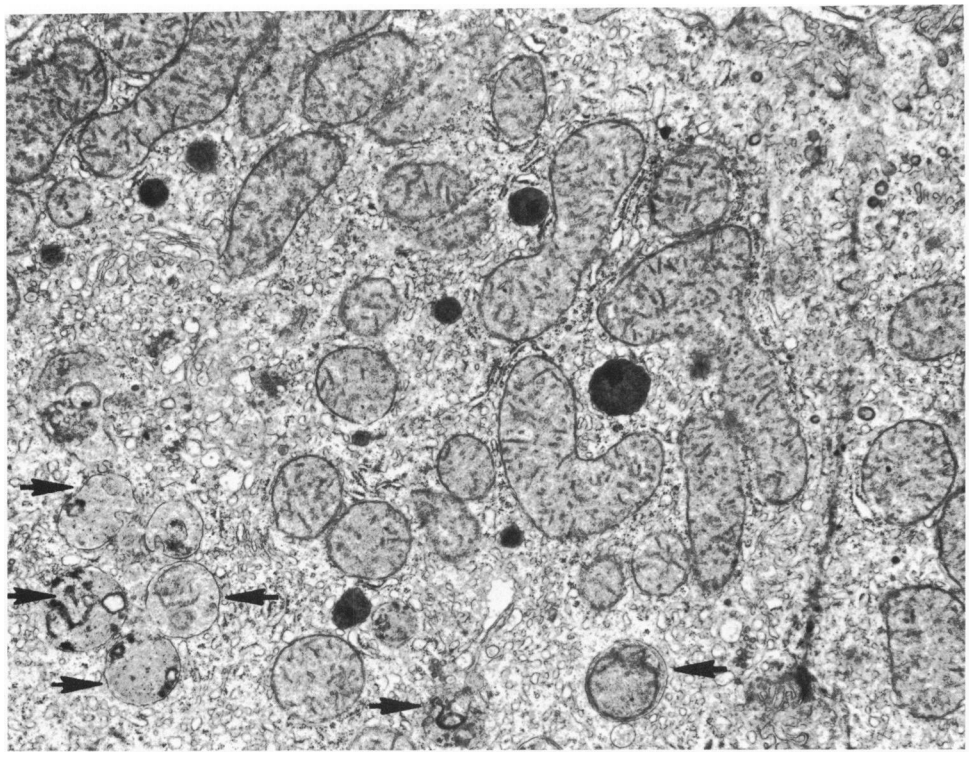
Legends for Figures

Fig 1—Glucagon-stimulated autophagy, acid phosphatase. Several peribiliary autophagic vacuoles (*arrows*) in a rat given glucagon 1 hour prior to sacrifice. The autophagic vacuole (*right*) probably contains a mitochondrion. Compare with Figure 2 (Osmium alone, × 14,900).

Fig 2—Low power view of acid phosphatase staining of glucagon-stimulated autophagic vacuoles (*arrows*). This animal was pretreated with cycloheximide. Heavy concentrations of reaction product are seen in the numerous autophagic vacuoles (× 8600).



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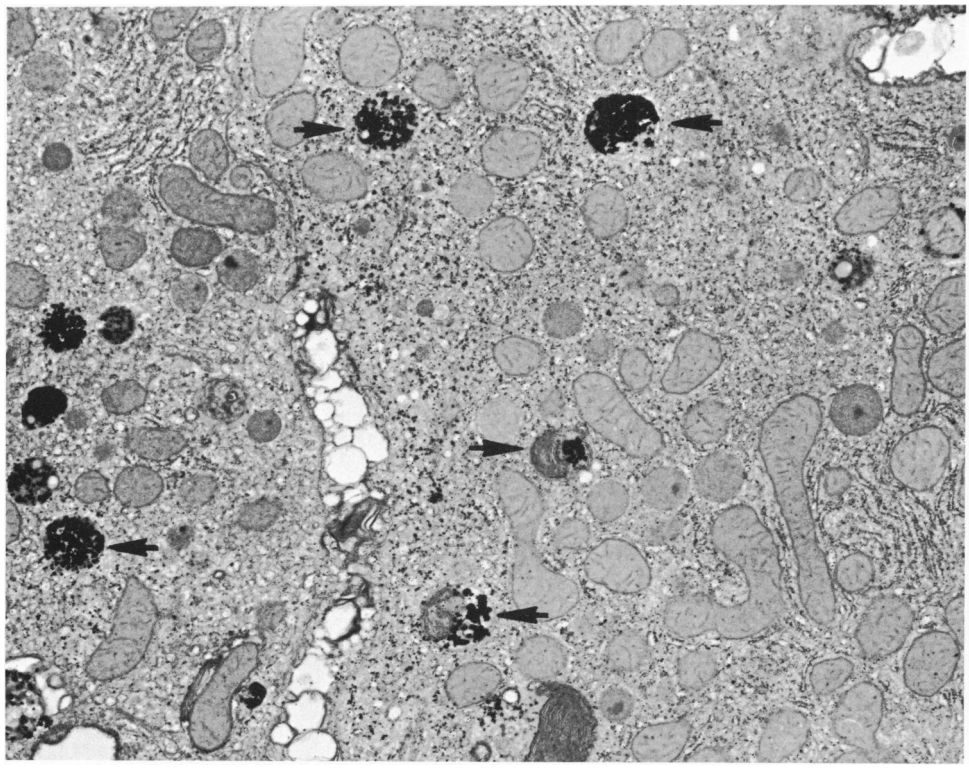

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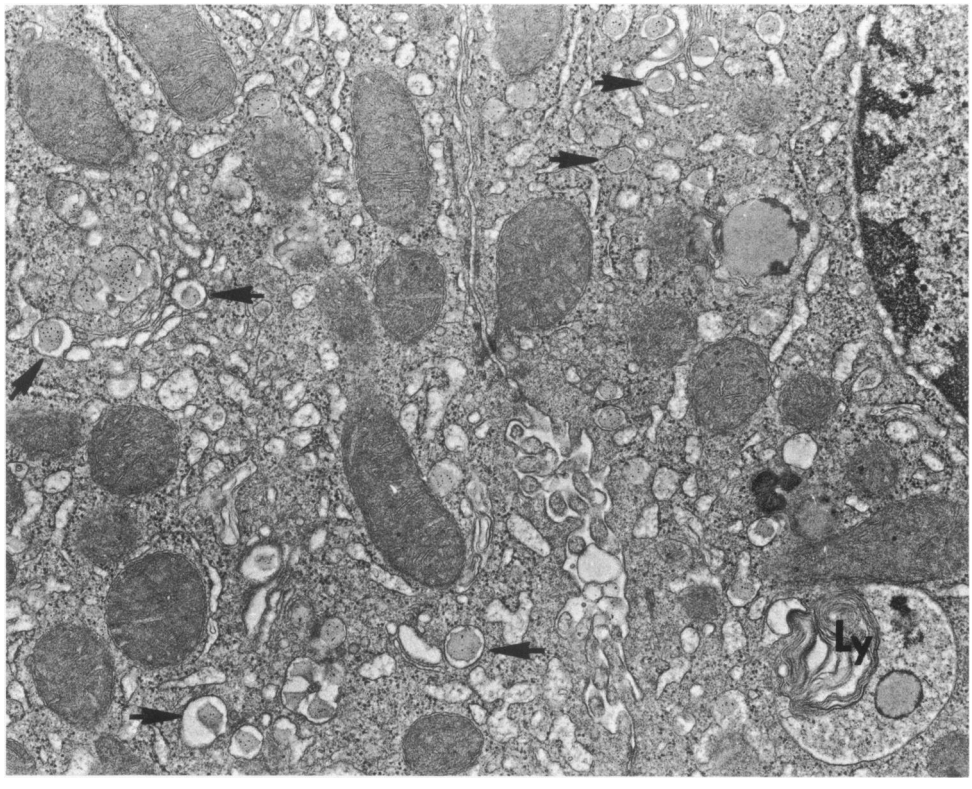
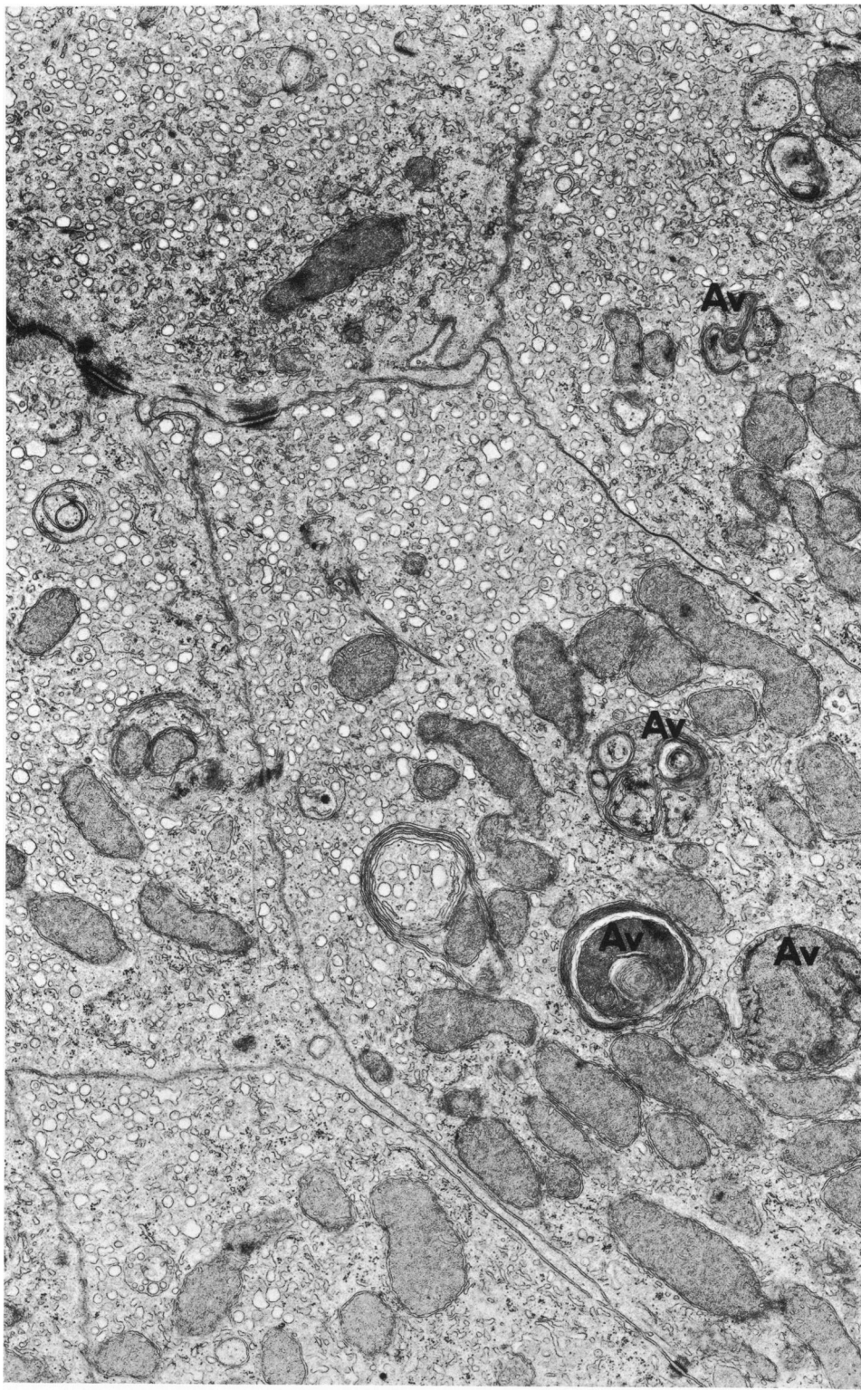


Fig 3—Portions of two hepatocytes from an animal pretreated with ethionine and then given glucagon. A bile canaliculus is present in right center. Typical effects of ethionine administration are seen: accumulations of liposomes in the endoplasmic reticulum (*arrows*), swelling of lysosomes (*Ly*) and some ribosomal scattering. Autophagic vacuoles are not seen (Glutaraldehyde and osmium, × 24,100).

Fig 4—Apical region of control flounder kidney tubule after 6 hours of incubation. Note the numerous autophagic vacuoles (*AV*). The ultrastructure appears otherwise normal. Mitochondria are intact (× 30,000).



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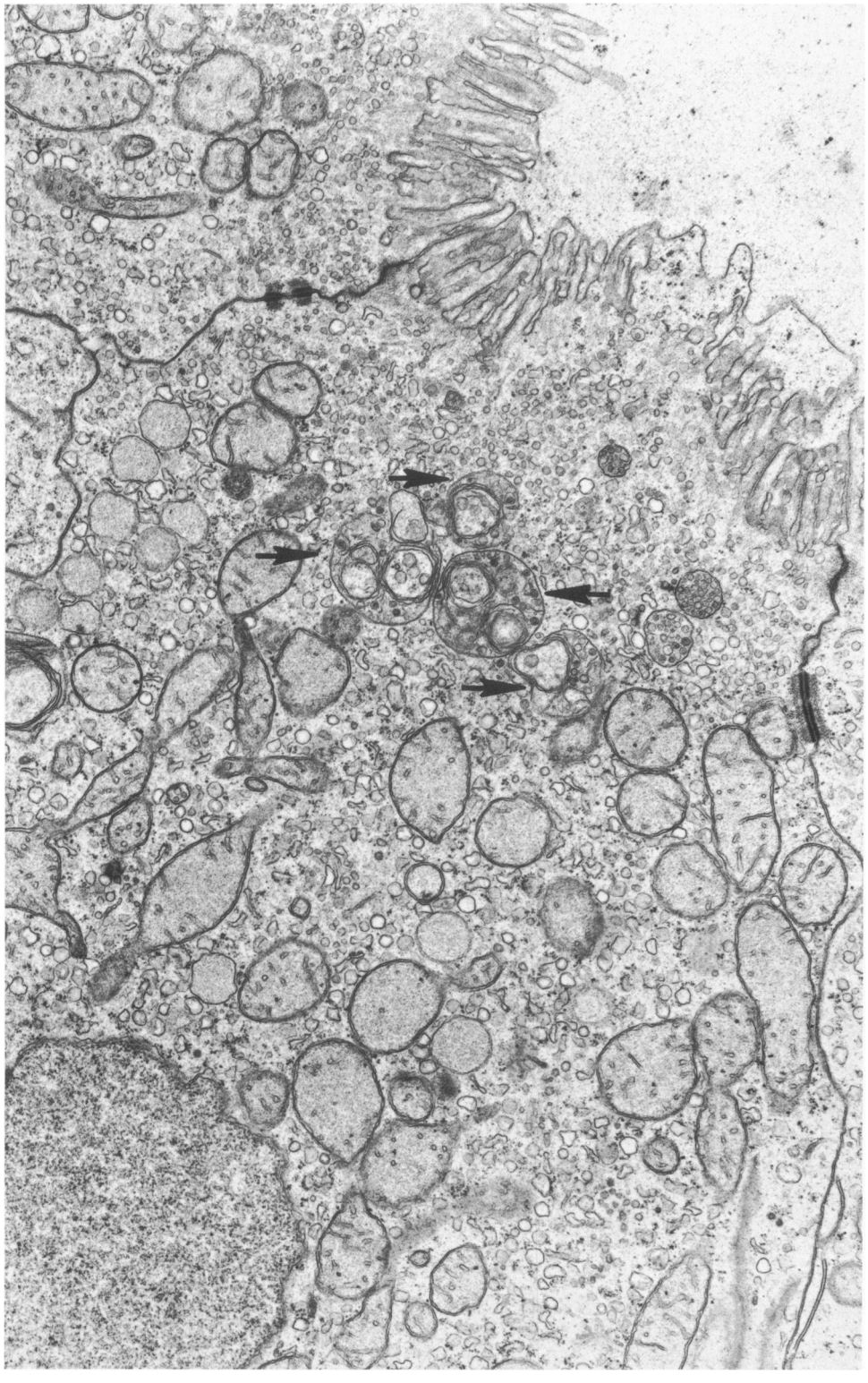
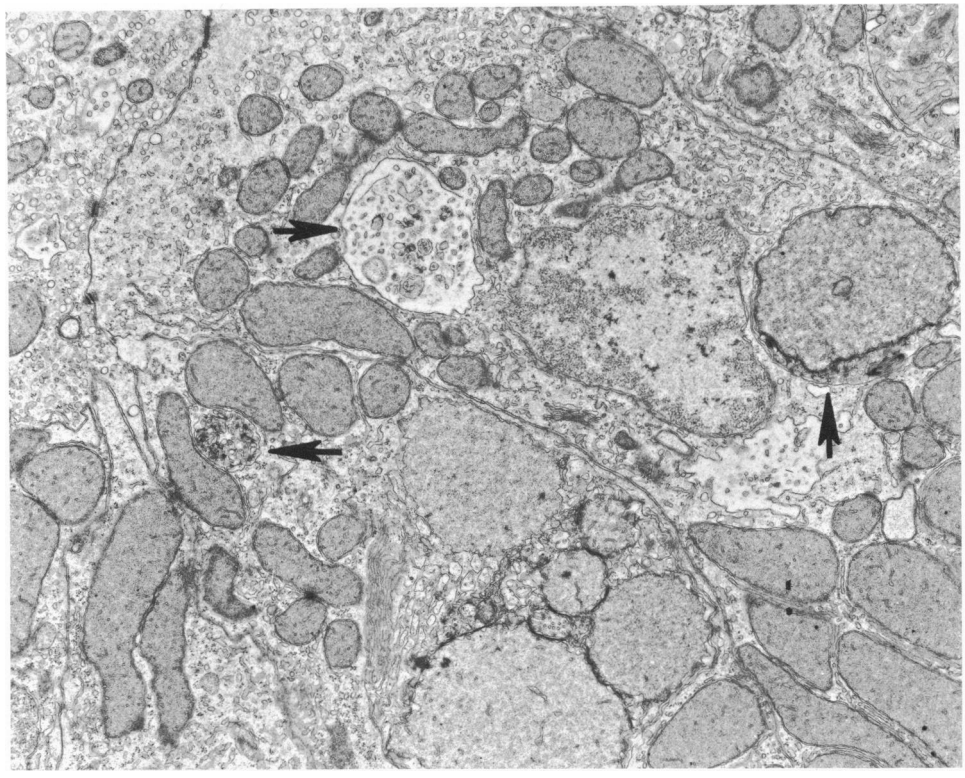


Fig 5—Apical region of flounder tubule incubated 5 hours in 10^{-5} M cycloheximide. Numerous autophagic vacuoles (*arrows*) are seen. Good preservation of ultrastructure is noted (× 31,200).



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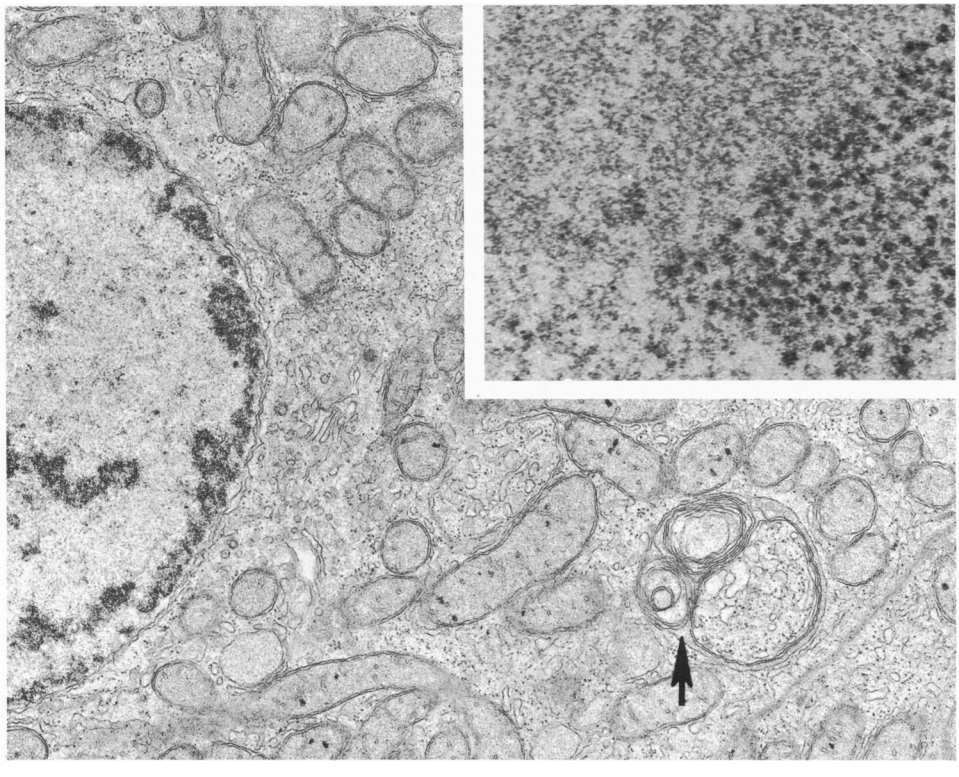


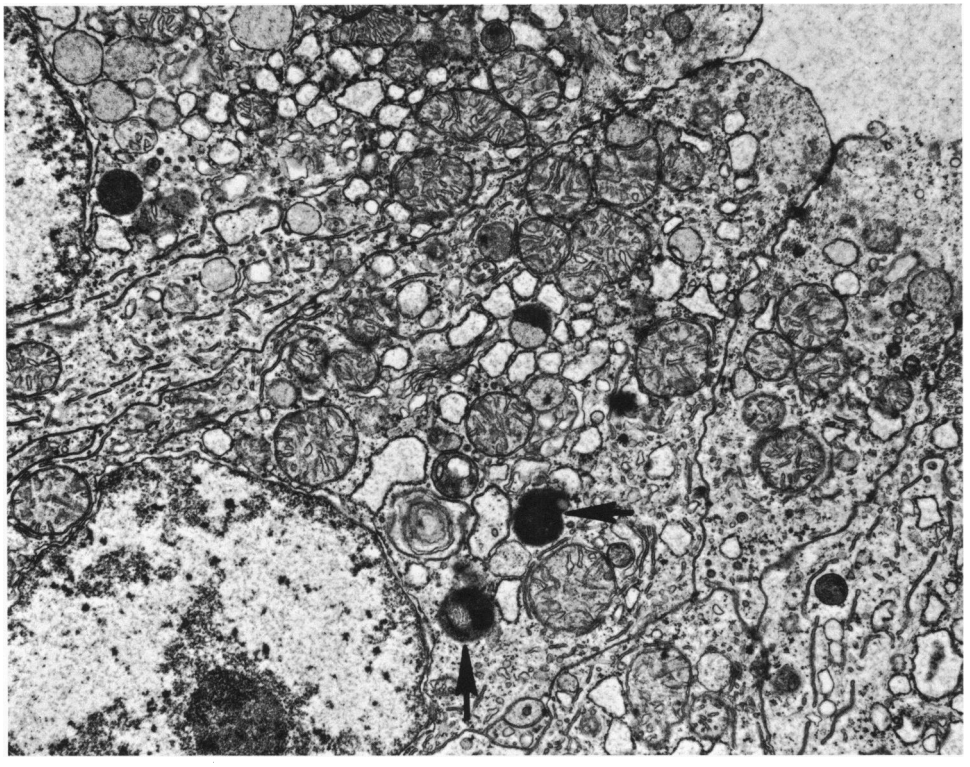
7A

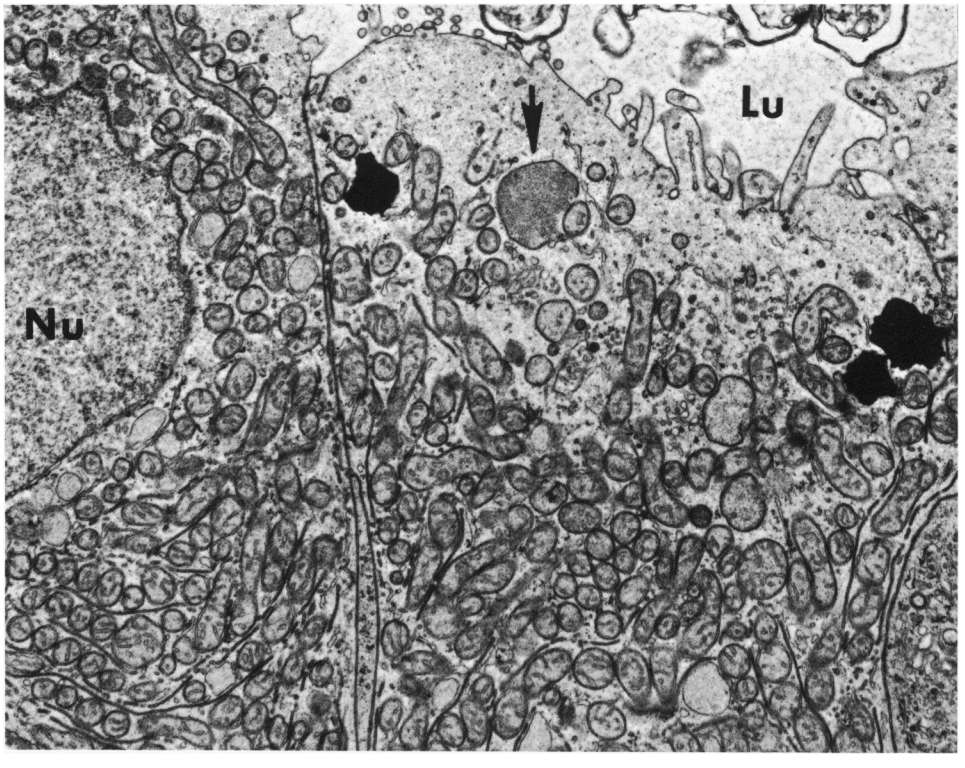
7B

Fig 6—Apical region of flounder tubule after 4 hours incubation in 2 × 10^{-4} M puromycin. Several large autophagic vacuoles (*arrows*) are noted (2% Osmium, × 20,000). **Fig 7A**—Large autophagic vacuole (*arrow*) in tubule treated 5 hours with 50 μg/ml actinomycin D. The nucleus at left shows marked chromatin clumping, and ribosomal scattering is evident. Mitochondria are intact (× 14,200). **B**—High power view of a nucleolus which has undergone stratification into particulate areas (*right*) and fibrillar areas (*left*). Tubule treated 5 hours with 5 μg/ml actinomycin D (× 75,800).

Fig 8—Apical region of flounder tubule incubated 4 hours anoxically by bubbling with nitrogen. The *arrows* indicated two residual bodies, but no autophagic vacuoles are seen. Mitochondria have undergone high-amplitude swelling, the nucleus shows marked chromatin clumping and margination, and the endoplasmic reticulum is dilated (× 7800).

Fig 9—Apical region of flounder tubule incubated for 24 hours at 0 to 4 C without gassing. Excellent preservation of normal ultrastructure is noted, and autophagic vacuoles are not present. Very few pinocytic vesicles are noted in the apical region. Mitochondria are intact; the dense areas are lipid droplets (× 8400).

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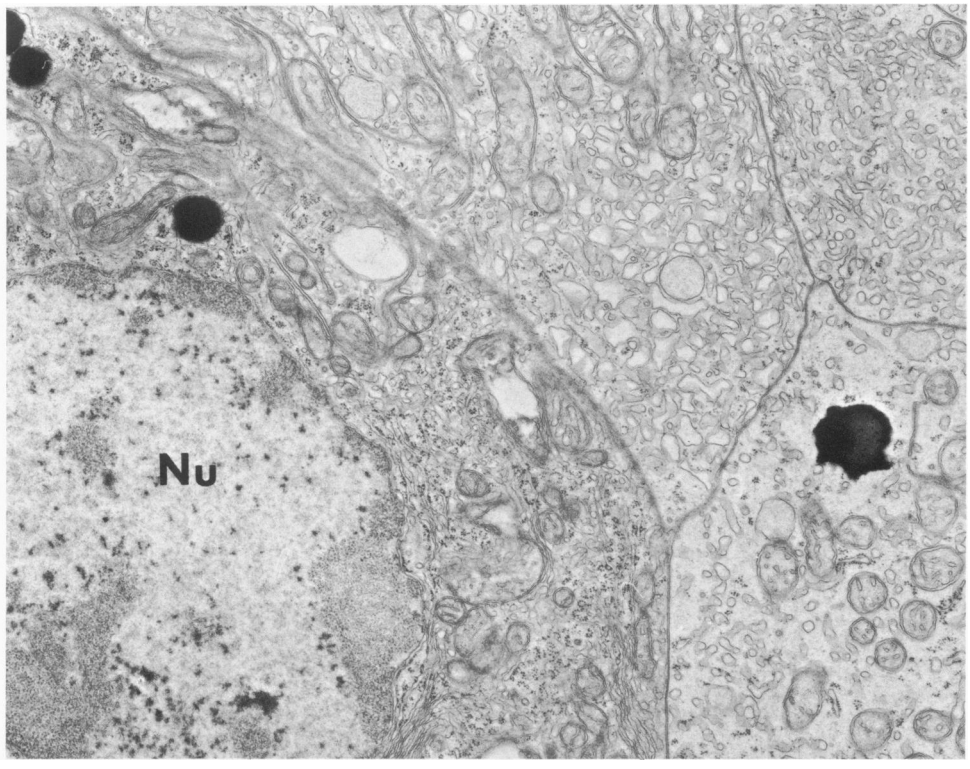

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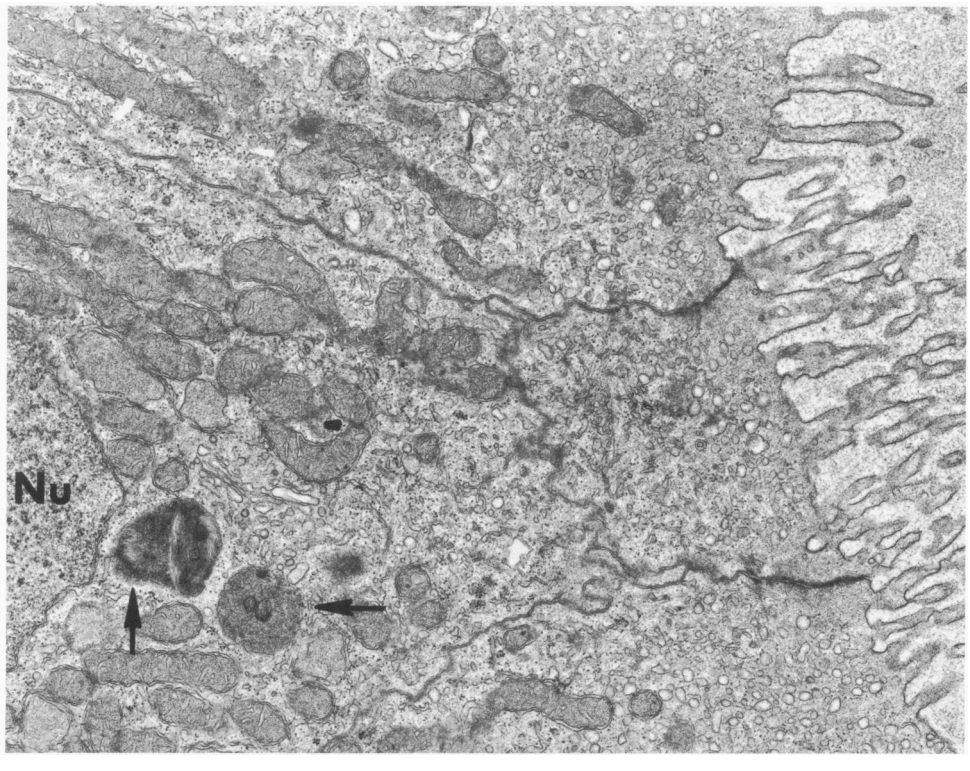

Fig 10—Dilated endoplasmic reticulum, chromatin clumping and absence of autophagic vacuoles in tubules treated with 10^{-4} M oligomycin (30 minutes). Dense lipid droplets are also present (× 13,700). **Fig 11**—Two residual bodies (*arrows*) in the apical region of a tubule treated 1 hour with 10^{-4} M 2,4-dinitrophenol. Autophagic vacuoles are not present (× 20,000).