Effect of Cycloheximide on Glucagon-Induced Autophagy

Quantitative Examinations on Hepatocytes in the Rat

H. J. Rumpelt, MD, and T. Weisbach, MD

By means of fine structural evaluation, a highly significant inhibitory effect of cycloheximide (20 mg/kg) on glucagon-induced autophagy is demonstrated in rat hepatocytes, which also presumably affects physiologic autophagic activities. Contrary to other investigators, we think *in vivo* global inhibition experiments using cycloheximide as an inhibitor of protein synthesis do not provide substantial information on the origin of the segregating membranes. (Am J Pathol 91:49–56, 1978)

DETERMINATION OF THE ORIGIN of the segregating membrane, one of the major problems in the process of cellular autophagy, has been the object of considerable investigative efforts in the past. Basically, two possible sources of vacuole membrane material have been proposed: a) membrane formation by *de novo* synthesis [1,2] and b) the utilization of preexisting cytoplasmic membranes.[3-6] The latter alternative gained considerable support from a detailed experimental study presented by Arstila and Trump in 1968.[3] Using cycloheximide as a potent inhibitor of protein synthesis, the authors failed to observe an inhibitory effect of cycloheximide on glucagon-induced autophagocytosis when applied to native rat liver slices. This observation was interpreted as indicative of vacuole membrane formation independent of actual protein synthesis. Additional fine structural and cytochemical examinations [3] suggested that the segregating membranes should likely be derived from endoplasmic reticulum membranes.

Recently, however, some contrary observations were reported [7-10] under different experimental conditions. We therefore speculated that reexamination of the cycloheximide effect on glucagon-induced autophagy might be useful. As the result of our efforts, this article presents significant evidence for a powerful inhibitory effect of cycloheximide on autophagic vacuole formation *in vivo*.

From the Department of Pathology, University of Mainz Medical School, Langenbeckstr. 1, D-6500 Mainz, West Germany.

Accepted for publication December 9, 1977.

Address reprint requests to Dr. H. J. Rumpelt, Department of Pathology, University of Mainz Medical School, Langenbeckstr. 1, D-6500 Mainz, West Germany.

0002-9440/78/0410-0049$01.00

Materials and Methods

All 15 animals used were male rats weighing between 170 and 200 g (strain: Wistar WU, obtained from Iwanowas Farms, Kisslegg, Germany). Prior to the experiment the rats were not fed overnight, but free access to water was permitted. Three groups (A, B, and C) of 5 animals each were formed; Group A served as controls. The experiment was started at 9 AM. Group C rats received 20 mg/kg cycloheximide (Actidone, Serva, Heidelberg) intraperitoneally, and Group B rats received a similar amount of saline solution. Thirty minutes later the rats of Groups B and C were injected with a single dose of 2 mg/kg glucagon (Serva, Heidelberg) intraperitoneally. Group A rats were given intraperitoneal saline injections twice.

Sixty minutes after glucagon administration, the rats were laparotomized under ether anesthesia. The middle liver lobe of each animal was then fixed *in situ* by a 4-minute application of a cooled 2% phosphate-buffered OsO_4 solution (pH 7.2) on the liver surface [11] while the liver blood circulation continued undisturbed. Small tissue blocks were taken from the prefixed superficial tissue layers, postfixed for 60 additional minutes in OsO_4 solution, and embedded in Epon 812. From methylene-blue-stained 1-μ sections, peripheroacinar liver areas were chosen for evaluation. The thin sections were mounted on 300-mesh copper grids and stained with uranyl acetate and lead citrate. The grid squares had a constant lateral length of 45 μ and were used as standard areas.

For each animal, thin sections from five randomly chosen tissue blocks were examined, and from each block three squares were evaluated. Autophagic vacuoles were counted directly when they appeared on the fluorescent screen of the electron microscope (Siemens Elmiscope EM Ia) using a primary magnification of × 15,000. Thus, 75 squares per animal group, representing a total area of 151,875 sq μ, were evaluated.

Interpreted and counted as autophagic vacuoles were those organelles limited by a single membrane and containing recognizable cytoplasmic elements (Figure 1). Very early stages of autophagic vacuole formation (Figure 1A) were exclusively seen in those animals which had received glucagon only (Group B). This type of vacuole (Figure 1A) accounted for 5% of all autophagic vacuoles of this group and characteristically displayed a more irregular boundary with double- or triple-layered or thick condensed portions of the limiting membranes.[cf 2,3,12] Residual bodies (Figure 1F) were excluded from the evaluation.

Statistical Procedure

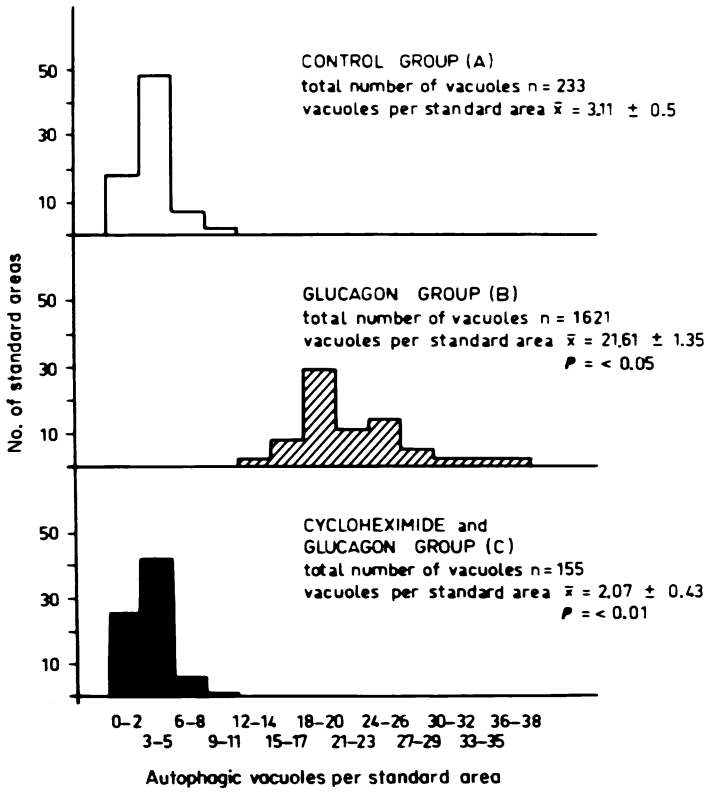
The mean values as well as the standard errors of frequency of autophagic vacuoles were calculated separately for each experimental group. Differences between the groups were assessed using the Kruskal and Wallis test.

Results

The 225 squares evaluated (a total of 455,625 sq μ) included cross sections of 346 hepatocytes of the control group (A), 357 hepatocytes of the glucagon group (B), and 344 hepatocytes of the cycloheximide–glucagon group (C).

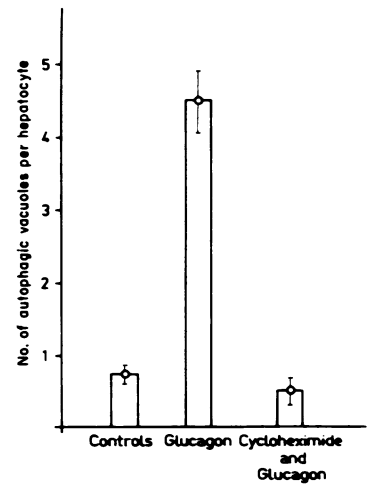
The numeric distribution of autophagic vacuoles found in the three experimental groups is shown in Text-figure 1:

1. In the control group (A) 233 vacuoles were counted. The mean value was 3.11 ± 0.5 vacuoles per standard area or 0.7 ± 0.13 vacuoles per cross-section of each hepatocyte (Text-figure 2).



TEXT-FIGURE 1—Numeric distribution of autophagic vacuoles per standard area (45 sq μ).

TEXT-FIGURE 2—Mean values of autophagic vacuoles per cross-section of each hepatocyte.



2. Sixty minutes after glucagon administration (Group B), the number of vacuoles had significantly ($P < 0.05$) increased and exceeded that of the controls approximately 7-fold. The exact vacuole number was 1621, which gave a mean value of 21.61 ± 1.35 vacuoles per standard area or 4.53 ± 0.4 vacuoles per cross-section plane of each liver parenchymal cell (Text-figure 2).

3. With cycloheximide administration 30 minutes prior to glucagon (Group C), the number of autophagic vacuoles totaled only 155: approximately one third less than in the control group. The mean value of vacuoles was 2.07 ± 0.43 per standard square and 0.43 ± 0.2 per hepatocyte (Text-figure 2). The difference in comparison to the glucagon group (B) was highly significant ($P < 0.01$), while the wide dispersion of values in the control group (A) rendered statistical comparison uncertain.

Discussion

The results of this quantitative examination clearly demonstrate that autophagic vacuole formation can be completely inhibited by cycloheximide administration. This supports earlier observations (not quantitatively determined): Kovács and Réz [7] reported inhibition of neutral-red–induced krinom formation and autophagocytosis by cycloheximide in liver, pancreas, and seminal vesicle epithelial cells of mice and in liver and pancreas epithelial cells of cockerels, as determined by light microscopic studies. Réz and Kovács [8] reported inhibition of neutral-red–induced formation of autophagic vacuoles in mouse pancreatic acinar cells, as determined by electron microscopic studies. Verbin et al [9] reported cycloheximide-induced inhibition of cytolysosome formation in 1-β-D-arabinosylcytosine-induced intestinal lesions, as determined by light and electron microscopic studies. Rumpelt et al [10] reported prevention of D-glactosamine-induced hepatocellular autophagocytosis in the rat, as determined by electron microscopic studies. All these observations were based on *in vivo* systems (rats, mice, and cockerels) and were established after administration of relatively large doses of the antibiotic (10 to 100 mg/kg). Why Arstila and Trump,[3] although they were working with concentrations even larger than those used in the present experiment (10^{-4} M = 28.14 mg/liter), failed to produce a similar effect in their *in vitro* system (rat liver slices incubated in Krebs–Ringer–bicarbonate buffer) is not easily explainable.

Cycloheximide is a well-known and widely used potent inhibitor of nuclear-directed protein synthesis.[13–16] Concentrations not higher than 5 mg/kg produce maximal inhibition of protein synthesis. This is as high as

98% in rats;[17] the remaining 2% presumably involves mitochondrial protein synthesis, which is not affected.

The effect of cycloheximide on autophagic activity is obviously dose-dependent. According to Réz and Kovács,[8] 10 mg/kg injected into mice will evoke no effect. Shelburne et al [18] observed that after the administration of 1.5 mg/kg cycloheximide to rats, when the protein synthesis was depressed to approximately 90%, no inhibition of autolysosome formation occurred. However, our experiments with rats [10,19] showed an inhibitory effect in hepatocytes when 5 mg/kg cycloheximide was used, but this was far from complete. Thus, it seems that the inhibitory effect of cycloheximide might not be limited to its effect on cytoplasmic protein synthesis.

Horgan and Griffin [20] could demonstrate an inhibitory effect of cycloheximide on the specific RNA polymerase I in the nucleolus of the aquatic mold *Blastocladiella emersonii*. According to Faber and Farmar,[17] concentrations of cycloheximide which exceed the 5 mg/kg limit will additionally bring about a dose-dependent inhibition of RNA synthesis. In rats the RNA synthesis inhibition amounts to 70% when doses as used for this study are given. Whether this effect is linked with the mechanism of the inhibition of autophagic vacuole formation or whether other primary or secondary still unknown cycloheximide-induced effects on metabolism may interfere with autophagy remains to be determined.

References

1. Ashford TP, Porter KR: Cytoplasmic components in hepatic cell lysosomes. J Cell Biol 12:198–202, 1962
2. Pfeifer U: Probleme der cellulären Autophagie. Morphologische, enzymcytochemische und quantitative Untersuchungen an normalen und alterierten Leberepithelien der Ratte. Ergeb Anat Entwicklungsgesch 44:1–74, 1971
3. Arstila AU, Trump BF: Studies on cellular autophagocytosis: The formation of autophagic vacuoles in the liver after glucagon administration. Am J Pathol 53:687–733, 1968
4. Arstila AU, Trump BF: Autophagocytosis: Origin of membrane and hydrolytic enzymes. Virchows Archiv [Cell Pathol] 2:85–90, 1969
5. Ericsson JLE: Mechanism of cellular autophagy. Lysosomes in Biology and Pathology, Vol 2. Edited by JT Dingle, HB Fell. Amsterdam, North-Holland Publishing Co., 1969, pp 345–394
6. Oledzka-Slotwinska H, Desmet V: Participation of the cell membrane in the formation of "autophagic vacuoles." Virchows Archiv [Cell Pathol] 2:47–61, 1969
7. Kovács J, Réz G: Prevention of neutral red-induced krinom formation and autophagocytosis by cycloheximide in epithelial cells. Acta Biol Acad Sci Hung 23:407–408, 1972
8. Réz G, Kovács J: Prevention by cycloheximide of neutral red-induced formation of autophagic vacuoles and krinom granules in mouse pancreatic acinar cells. Virchows Archiv [Cell Pathol] 12:123–132, 1973

9. Verbin RS, Diluiso G, Farber E: Protective effects of cycloheximide against 1-β-D-arabinosylcytosine-induced intestinal lesions. Cancer Res 33:2086–2093, 1973
10. Rumpelt HJ, Albring M, Thoenes W: Prevention of D-galactosamine-induced hepatocellular autophagocytosis by cycloheximide. Virchows Archiv [Cell Pathol] 16:195–203, 1974
11. Thoenes W, Langer KH: Die Endocytose-Phase der Eiweissresorption im proximalen Nierentubulus. Untersuchungen am Ferritin-resorbierenden Einzeltubulus der Rattenniere. Virchows Archiv [Cell Pathol] 2:361–379, 1969
12. Glinsmann WH, Ericsson JLE: Observations on the subcellular organization of hepatic parenchymal cells. II. Evolution of reversible alterations induced by hypoxia. Lab Invest 15:762–777, 1966
13. Kerridge D: The effect of actidione and other antifungal agents on nucleic acid and protein synthesis in Saccharomyces carlsbergensis. J Gen Microbiol 19:497–506, 1958
14. Colombo B, Felicetti L, Baglioni C: Inhibition of protein synthesis by cycloheximide in rabbit reticulocytes. Biochem Biophys Res Commun 18:389–395, 1965
15. Colombo B, Felicetti L, Baglioni C: Inhibition of protein synthesis in reticulocytes by antibiotics. I. Effects on polysomes. Biochim Biophys Acta 119:109–119, 1966
16. Lodish HF, Housman D, Jacobsen M: Initiation of hemoglobin synthesis. Specific inhibition by antibiotics and bacteriophage ribonucleic acid. Biochemistry 10:2348–2356, 1971
17. Farber JL, Farmar R: Differential effects of cycloheximide on protein and RNA synthesis as a function of dose. Biochem Biophys Res Commun 51:626–630, 1973
18. Shelburne JD, Arstila AU, Trump BF: Studies on cellular autophagocytosis: The relationship of autophagocytosis to protein synthesis and to energy metabolism in rat liver and flounder kidney tubules *in vitro*. Am J Pathol 73:641–670, 1973
19. Rumpelt HJ, Albring M: Autophagocytosehemmung durch Cycloheximid in D-Galaktosamin geschädigten Rattenhepatocyten. Verh Dtsch Ges Pathol 58:522, 1974
20. Horgen PA, Griffin DH: Specific inhibitors of the three RNA polymerases from the aquatic fungus *Blastocladiella emersonii*. Proc Natl Acad Sci USA 68:338–341, 1971

Acknowledgments

We wish to express our gratitude to Mrs. K. Schulze and Mrs. M. Müller for expert technical assistance.

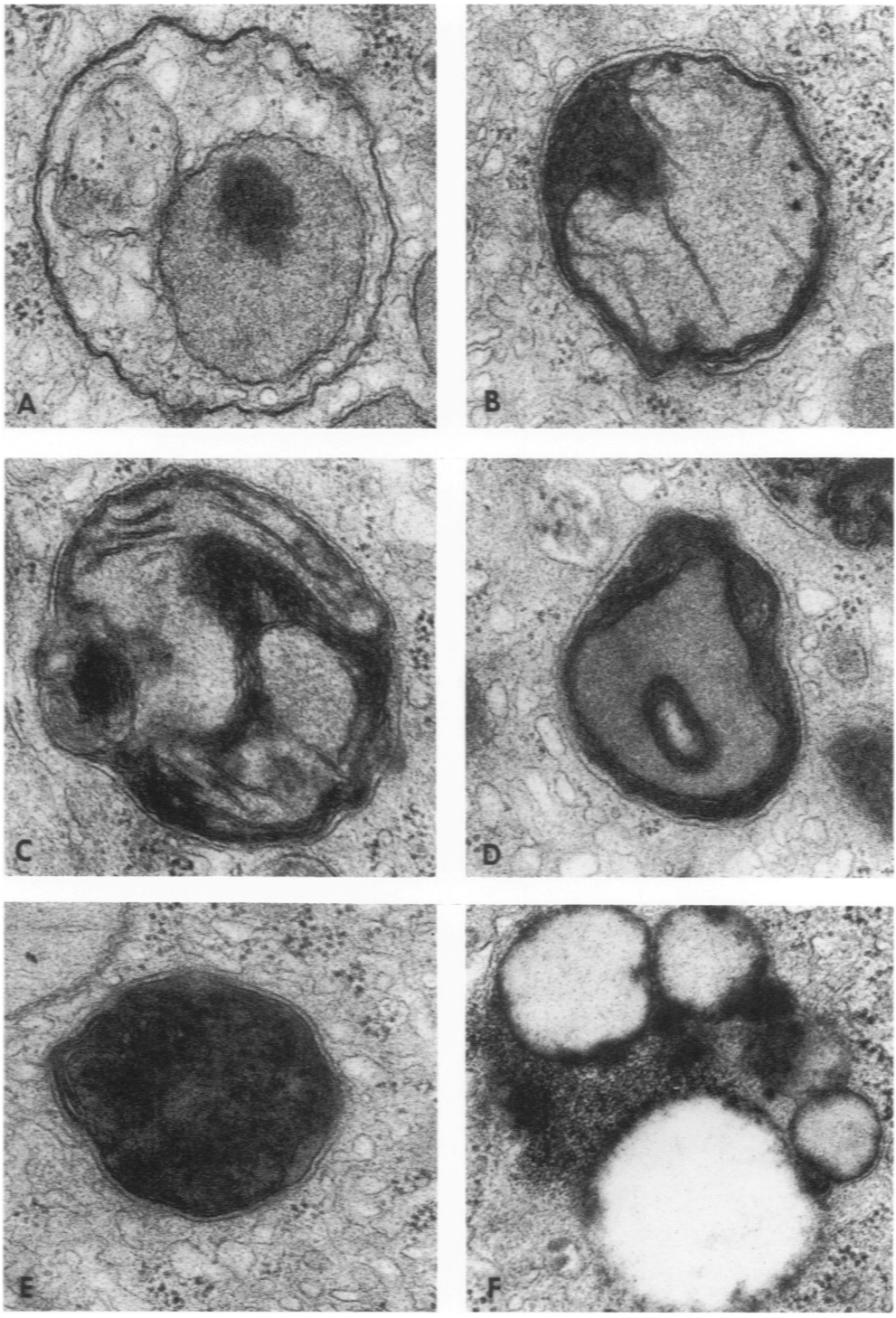


Figure 1—Ultrastructural spectrum of autophagic vacuoles in experimental groups A, B, and C. **A**—Very early stage of vacuole formation, exhibiting a characteristic thick condensed limiting membrane and ultrastructurally unaltered enclosed organelles. In glucagon-treated animals only. **B** through **E**—Autophagic vacuoles with an advancing degradation of cytoplasmic content, increasing electron density, and decreasing size. In all three experimental groups. **F**—Residual body, as it occurred in all three groups. These organelles were excluded from evaluation. (× 57,500)

[*End of Article*]