COMMUNICATIONS

Short papers submitted expressly for this section, reporting original and significant findings of immediate interest and judged to be acceptable without major revision, will be published within approximately three months. See inside back cover for details.

PARTICIPATION OF LYSOSOMES IN CELLULAR AUTOPHAGY INDUCED IN RAT LIVER BY GLUCAGON

RUSSELL L. DETER, PIERRE BAUDHUIN, and CHRISTIAN DE DUVE. From the Laboratoire de Chimie Physiologique, University of Louvain, Belgium. Dr. Deter's present address is Department of Anatomy, Baylor University College of Medicine, Houston, Texas

INTRODUCTION

Numerous observations made over the last five years have established the widespread occurrence of cellular autophagy as well as its importance in the processes whereby living cells achieve the degradation of their own constituents (For a review, see reference 5). In all the cases that have been investigated in this respect, autophagic vacuoles have been found to stain positively for acid phosphatase, suggesting strongly that lysosomal enzymes are the main agents of the digestive processes taking place within them. However, the mechanism of the segregation phenomenon and the manner in which hydrolases become associated with the sequestered area are still far from being understood. One theory, proposed by Novikoff et al. (9), considers areas of endoplasmic reticulum, assumed to be involved in the intracellular transport of newly synthesized hydrolases, as providing both the surrounding membrane of autophagic vacuoles and their enzymic complement. According to this theory, cellular autophagy represents a mechanism, though not necessarily the only one, whereby new lysosomes may arise in a living cell. Other workers have expressed similar ideas concerning the origin of lysosomes (1, 8). An alternative hypothesis, put forward by de Duve and Wattiaux (5), considers preexisting lysosomes as the source of the enzymes that are found in autophagic vacuoles, on the assumption, either that the particles actually participate in the sequestration phenomenon, or that they fuse with initially hydrolase-less "autophagosomes" as they are known to do with the "heterophagosomes" formed by endocytosis.

In a recent investigation, Deter and de Duve (7) have found that the injection of a large dose of glucagon, a powerful inducer of autophagy in liver, causes a transient, but manifest increase in the mechanical and osmotic fragility and in the median sedimentation coefficient of rat-liver lysosomes. These changes were tentatively attributed to an enlargement of the lysosomes, possibly reflecting extensive participation of these particles in the autophagic process. But in the absence of morphological data, no firm conclusion could be reached. The present communication reports the first results of a quantitative morphological study correlating the appearance of autophagic vacuoles with a marked decrease in the number of recognizable dense bodies present in particulate fractions separated from hepatic homogenates 45 min after glucagon injection.

METHODS

The biochemical part of the experiments was conducted as described by Deter and de Duve (7). Male rats, fasted overnight, were killed 45 min after an intraperitoneal injection of 50 μg of glucagon (Eli Lilly Co., Indianapolis) per 100 g body weight, or of solvent. Homogenization of the livers in 0.25 M

sucrose was combined with fractionation into a nuclear fraction and a cytoplasmic extract. The extracts were further divided into a particulate M + L fraction and a P + S supernatant by centrifugation at 250,000 *g*. min (2). Washing of the sedimented particles was omitted for better preservation of the fragile lysosomes. The preparations were assayed for free and total acid phosphatase before and after exposure to 0.15 M sucrose.

Samples of the M + L fractions were fixed with glutaraldehyde (approximately 3 hr after isolation), filtered through a Millipore membrane of 0.01-μ pore size, and further processed for electron microscopy by the methods described by Baudhuin, Evrard, and Berthet (4). A number of micrographs of each preparation were scanned systematically and the surface area of all profiles of recognizable dense bodies and autophagic vacuoles was measured according to Baudhuin and Berthet (3). The methods of these authors, which are based on the principle of Delesse (6) and on the work of Wicksell (10), were used to evaluate: (*a*) the proportion of the total pellicle volume occupied by the particles under study; and (*b*) their size distribution. These data were used to compute the mean volume of the particles as well as their absolute number, and the results were further related to the liver weight by taking into account the volume of the pellicle and the amount of M + L fraction included in it. Except for the autophagic vacuoles in the control preparations, which were too rare, the scanning was continued until about 100 profiles of each type had been measured in each preparation.

RESULTS

The biochemical results obtained on three pairs of animals are summarized in Table I. As observed before (7), glucagon injection does not alter significantly the total acid phosphatase content of the liver; it causes only minor changes in the distribution of the enzyme, which is recovered to the extent of 70–75% in the M + L fraction. The enhanced fragility of the lysosomes following glucagon treatment is evidenced by the increase in free acid phosphatase activity of the cytoplasmic extract and of the M + L fraction, and especially by their greatly augmented sensitivity to osmotic shock in these fractions. These observations are in agreement with previous findings showing marked biochemical alterations of the lysosomes between 45 and 75 min after injection of the hormone (7). A somewhat higher free activity is also found in the P + S fraction, but the small lysosomes present in this fraction are essentially resistant to osmotic shock, whether glucagon has been given or not.

Illustrative low-power micrographs of ultrathin sections through M + L fractions from a treated and untreated animal are shown in Fig. 1. The results of the quantitative measurements are summarized in Table I and represented graphically in Fig. 2. Most conspicuous is the presence of numerous autophagic vacuoles in the M + L fractions from glucagon-injected rats, as opposed to their very infrequent occurrence in the control preparations. These vacuoles were of several types. Some contained mitochondria, often with small amounts of other cytoplasmic components. Others contained only membranous structures of both the rough- and the smooth-surfaced variety. Some were packed mostly with dense amorphous material within which many ribosomes could be recognized. Occasionally, a microbody was found segregated within an autophagic vacuole. In contrast with most of the free mitochondria, which showed a somewhat condensed matrix with swollen intracristal and intermembrane spaces, the sequestered mitochondria generally had closely apposed membranes. In some cases, their internal structure looked essentially normal; in others, it showed evidence of more or less advanced alterations. The same was true of the other segregated cytoplasmic constituents. The computed size distribution of autophagic vacuoles in preparations from glucagon-treated rats is veiy irregular (Fig. 2). This is not surprising in view of their heterogeneous nature. Profiles of autophagic vacuoles in the control preparations were too rare to allow a similar analysis.

In confirmation of preliminary observations made on intact tissue sections and referred to briefly previously (5), glucagon injection was found to cause a considerable reduction in the number of dense bodies. As shown clearly in Fig. 2, this reduction appears to be caused by a net loss, affecting preferentially the smaller members of the population. Consequently, the mean volume of the remaining dense bodies is increased and their total volume decreased (Table I).

It is interesting that the control preparation No. 2 contained distinctly more autophagic vacuoles than the other two controls. It also contained fewer dense bodies and showed a somewhat higher osmotic sensitivity of the lysosomes, suggesting that the changes produced by glucagon may also take place when autophagy occurs spontaneously. For an unknown reason, glycogen particles oc-

TABLE I

Properties of Fractions

Object	Measurement	Unit	Control 1	Control 2	Control 3	Control Mean	Glucagon 1	Glucagon 2	Glucagon 3	Glucagon Mean
Liver (E + N)	Total acid phosphatase	Units/g liver	8.39	7.39	7.06	7.61	8.99	7.80	7.38	8.06
N Fraction	Total acid phosphatase	% of E + N	6.8	4.5	4.5	5.3	3.4	4.1	4.1	3.9
M + L Fraction			73.2	73.3	74.0	73.5	75.0	69.5	74.3	72.9
P + S Fraction			22.9	21.9	21.0	21.9	23.4	28.1	25.4	25.6
Extract (E)	Free acid phosphatase	% of total activity found in fraction								
	Untreated		21.0	20.1	19.9	20.3	23.0	29.4	29.3	27.2
	Osmotic shock*		32.7	40.3	31.9	35.0	50.5	64.5	62.9	59.3
M + L Fraction	Untreated		11.2	13.4	10.3	11.6	16.3	20.7	16.7	17.9
	Osmotic shock*		22.6	32.8	26.1	27.2	50.8	64.4	59.8	58.3
P + S Fraction	Untreated		58.3	63.0	61.1	60.8	65.5	73.9	71.0	70.1
	Osmotic shock*		66.8	63.0	61.1	63.6	67.0	72.9	74.3	71.4
M + L Fraction Dense bodies	Total volume	cm^3/100 g liver	0.27	0.23	0.29	0.26	0.21	0.19	0.18	0.19
	Mean volume	μ^3	0.051	0.075	0.056	0.061	0.096	0.130	0.075	0.100
	Number	10^9/g liver	47.9	27.9	47.7	41.2	19.3	13.1	21.6	18.0
M + L Fraction Autophagic vacuoles	Total volume	cm^3/100 g liver	~0.03	~0.06	~0.01	~0.03	0.24	0.25	0.24	0.24
	Mean volume	μ^3	—	—	—	—	0.245	0.314	0.284	0.281
	Number	10^9/g liver	—	—	—	—	9.9	8.0	8.6	8.8

* Exposed to 0.15 M sucrose for 30 min at 0° and then brought back to 0.25 M sucrose before assay.

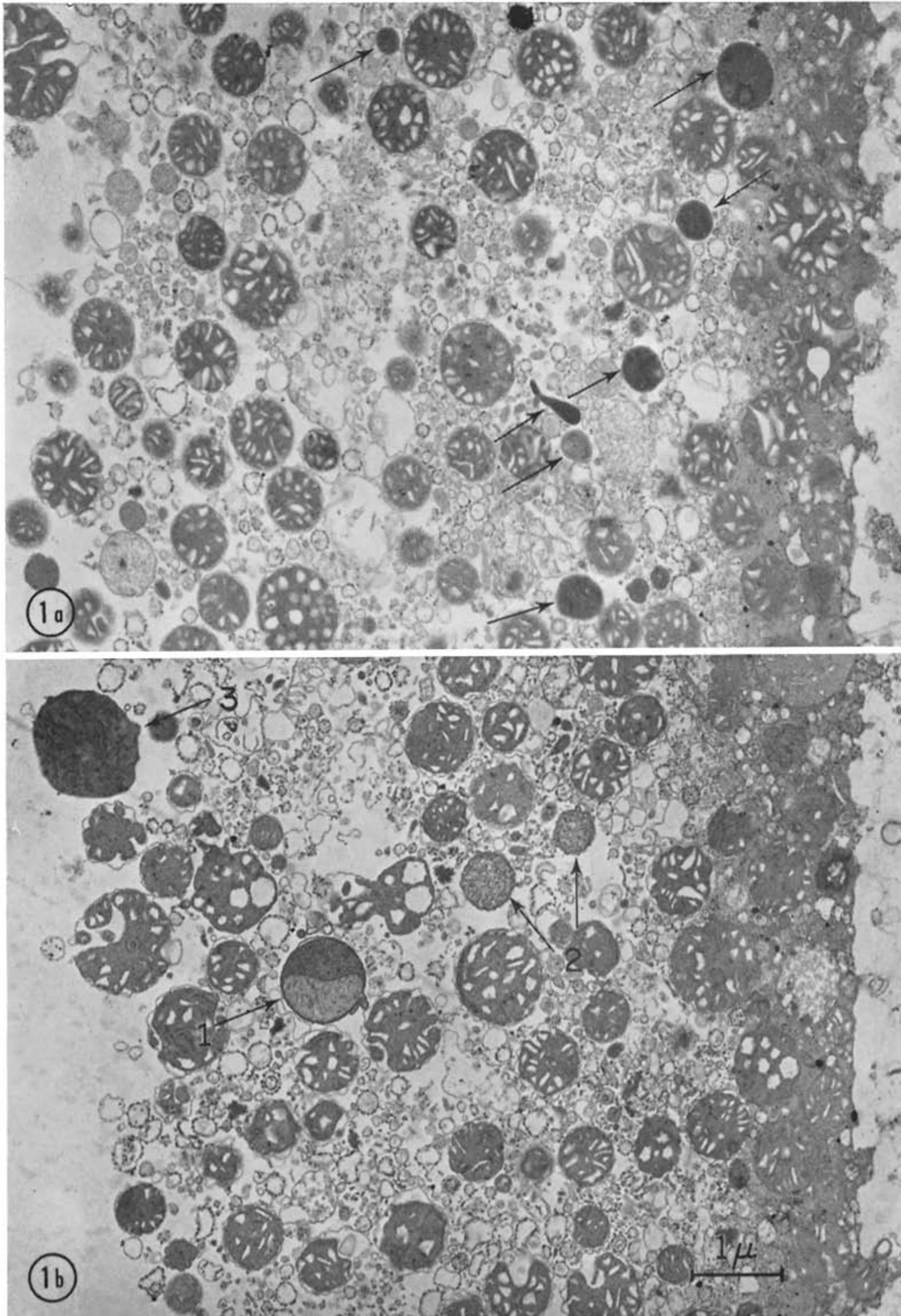

FIGURE 1 Sections through pellicles from M + L fractions from control (*a*) and glucagon-injected animal (*b*). Both preparations are similar with respect to the appearance of the mitochondria, which show a condensed matrix and dilated intermembrane spaces, and to their relatively high content of endoplasmic reticulum and amorphous material (denatured proteins?), which is due to the lack of washing. Numerous profiles of dense bodies are seen in control preparation (arrows). Three types of autophagic vacuoles are recognized in preparation from glucagon-treated animal: one containing a mitochondrion and dense material (*1*), two containing membranous elements (*2*), one containing dense material, ribosomes, and some membrane remnants (*3*). × 13,000.

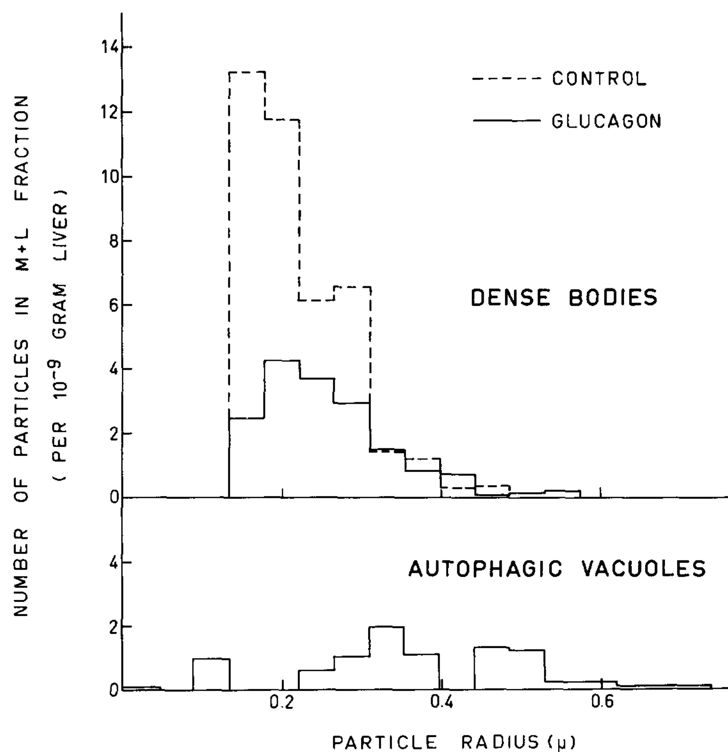

FIGURE 2 Size distribution of dense bodies and autophagic vacuoles in M + L fractions from control and glucagon-injected animals. Pooled results obtained on three animals in each group (Table I).

curred in abnormally high amounts in this preparation.

DISCUSSION

As a result of glucagon injection, more than half the dense bodies recoverable in the M + L fraction, which are the main form of lysosome in normal rat liver (2), disappear in 45 min or less. Since the smaller members of the population are lost preferentially, the reduction in total dense body volume is smaller, but still quite marked, of the order of 30%. However, hardly any acid phosphatase is lost concomitantly from the M + L fraction and it follows, therefore, that, unless the smaller dense bodies are devoid of this enzyme, some 30% of it must have been transferred to another particulate component. We can exclude the possibility that small dense bodies contain no acid phosphatase. Staining experiments do not support it, and the biochemical changes produced by glucagon demonstrate clearly that acid phosphatase-containing particles are indeed affected to a considerable extent by injection of the hormone. Consequently, enzymic activity must have been lost together with the smaller dense bodies. Theoretically, it could have been transferred either to even smaller bodies of the same type, whose profiles would largely escape recognition in the sections (3), or to particles of a different morphological appearance. Although an increase in slowly sedimenting particles containing acid phosphatase has been noted after glucagon (7), this change cannot provide the sole explanation of our results since it would be reflected mostly in a shift of activity from the M + L to the P + S fraction, and it is unlikely to account for the increased fragility of the lysosomes. Furthermore, autophagic vacuoles, which appear in large number after glucagon injection, are known to stain positively for acid phosphatase. The conclusion seems inescapable that preexisting lysosomes or dense bodies, and preferentially the smaller ones, participate extensively in cellular autophagy, at least in the liver of glucagon-treated rats. These particles must be the main source of the enzymes that carry out the degradation of the segregated material.

It cannot yet be decided whether the lysosomes are involved in the actual segregation process, either directly, or indirectly through the existence of continuities with the endoplasmic reticulum (Novikoff, personal communication), or fuse secondarily with autophagic vacuoles formed by other membrane-bounded structures. As pointed out elsewhere (5), the existence of permanent connections between lysosomes and endoplasmic reticulum is not supported by the observations made on livers containing highly swollen lysosomes, filled for instance with Triton WR-1339, dextran, or sucrose. One image suggesting fusion between a lysosome and an autophagic vacuole has been published by de Duve and Wattiaux (5). Studies similar to those reported here, but carried out before the onset of the biochemical changes, which occur fairly suddenly about 30 min after glucagon injection (7), may help to settle this point.

According to our estimates, about 2.5 dense bodies seem to disappear for every autophagic vacuole formed. The true ratio could be smaller, since part of the autophagic vacuoles present in the liver are likely to be destroyed in the course of isolation. It is interesting that the volume occupied by the dense bodies and autophagic vacuoles together is increased by glucagon treatment by as much as 50%, or 0.14 cm^3 per 100 g liver. This presumably represents the volume occupied by the segregated material. Taking into account the probable loss of autophagic vacuoles by homogenization and fractionation, their apparently high density, and the fact that autophagy may affect only the cytoplasm of parenchymal cells, we may estimate that 0.5% of the parenchymal cytoplasm may suffer digestion as a result of a single injection of a large dose of glucagon.

These investigations were supported by a grant from the Fonds de la Recherche Fondamentale Collective. The authors are indebted to Professor J. Meinguet, Director of the Centre de Calcul, for providing computer facilities, to Dr. O. K. Behrens, of the Lilly Research Laboratories, Indianapolis, for his generous gift of glucagon, and to Dr. H. Beaufay for his valuable help in the beginning of this work. R. L. Deter is the holder of a 1966–1967 postdoctoral fellowship from the United States Public Health Service (1-F2-CA-29,337-01). P. Baudhuin is Chargé de Recherches du Fonds National de la Recherche Scientifique.

Received for publication 31 July 1967.

REFERENCES

1. Ashford, T. P., and K. R. Porter. 1962. *J. Cell Biol.* **12**:198.
2. Baudhuin, P., H. Beaufay, and C. de Duve. 1965. *J. Cell Biol.* **26**:219.
3. Baudhuin, P., and J. Berthet. 1967. *J. Cell Biol.* **35**. In press.
4. Baudhuin, P., P. Evrard, and J. Berthet. 1967. *J. Cell Biol.* **32**:181.
5. de Duve, C., and R. Wattiaux. 1966. *Ann. Rev. Physiol.* **28**:435.
6. Delesse, A. 1847. *Compt. Rend.* **25**:544.
7. Deter, R. L., and C. de Duve, 1967. *J. Cell Biol.* **33**:437.
8. Ericsson, J. L. E. 1964. *Acta Pathol. Microbiol. Scand. Suppl.* **168**:1.
9. Novikoff, A. B., E. Essner, and N. Quintana. 1964. *Federation Proc.* **23**:1010.
10. Wicksell, S. D. 1925. *Biometrika.* **17**:84.