

A MORPHOMETRIC STUDY OF CELLULAR AUTOPHAGY INCLUDING DIURNAL VARIATIONS IN KIDNEY TUBULES OF NORMAL RATS

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ABSTRACT

Cellular autophagy in convoluted tubules of kidney was studied in 24 rats, killed in pairs at constant time intervals during one diurnal cycle, by (a) morphometric evaluation of tubular cells by the point-counting method in randomly sampled micrographs, and (b) selective search for autophagic vacuoles (AV) directly on the electron microscope screen.

The total area of tubular cells recorded in the electron microscope sections was $93 \times 10^4 \mu\text{m}^2$. Since the distal convoluted tubules, covering about 12% of the whole tubulocellular area, contained only 3–4% of all AV, they were omitted from the main calculations.

The number of AV per area unit and the total amount of segregated material showed a distinct diurnal rhythm, synchronous for the different types of AV which were distinguished from each other according to their contents. The minimum was found during the night, the maximum during the day. This rhythm appears similar to that described elsewhere in liver cells.

The mean segregated fractions were calculated from the relation of segregated to nonsegregated material in proximal convoluted tubular cells. The segregated fraction of the mitochondria was 4.4×10^{-4} . This value could account for the degradation of all mitochondria in a cell within 15 days, i.e., the upper limit of the lifetime of mitochondrial DNA in the cortex of the kidney, if one assumes that a mitochondrion is destroyed within 10 min after being segregated. The segregated fraction of microbodies was 11.7×10^{-4} . This suggests a shorter lifetime of these organelles. It is concluded that cellular autophagy plays a significant role in the turnover of cytoplasmic constituents, including the membranes of the endoplasmic reticulum.

Cellular autophagy, defined as the ability of a cell to segregate and to degrade portions of the cytoplasm, is a well-known phenomenon (5, 7, 15, 20). Certain basic questions, however, are still unanswered. In particular there are available only few papers which are concerned with the quantitative

evaluation of the frequency with which autophagic vacuoles (AV) occur under normal conditions. Only in the liver cell has the quantitative aspect of cellular autophagy so far been studied (4, 6, 21). As far as other cells are concerned, statements to the effect that cellular autophagy is enhanced

under certain conditions, have been based mainly on qualitative assessments. It was, therefore, considered of interest to investigate morphometrically the tubular cells of the kidney in which the morphology of AV is well known (10).

Since previous studies on the liver cell (19–21) had shown that the diurnal rhythm always must be considered as a factor determining the physiological occurrence of autophagic vacuoles, the influence of this factor was also studied here. It will be shown that in the tubular cells of the kidney of normal rats AV occur predominantly during the day. In retrospect it would, therefore, seem that the discovery of autophagic vacuoles in the tubular cells of the normal animal had been a chance finding, dependent on the fact that rats are active mainly during the night. If the data presented here are interpreted from a dynamic point of view, the results are compatible with the suggestion that cellular autophagy plays an important role in the turnover of cytoplasmic constituents of the normal cell.

MATERIALS AND METHODS

Animals and Preparation of Tissues

24 male Sprague-Dawley rats (WIGA, Sulzfeld, Germany) were used. They were kept at constant room temperature (24°C) and under controlled lighting conditions (light period: 7 a.m. to 7 p.m.; dark period: 7 p.m. to 7 a.m.). The animals had free access to a standard rat pellet diet (Herilan) and tap water. After a period of habituation of 3 wk, the body weight of the animals varied from 260 to 320 g. Two rats at a time were then killed at 7, 9, 11 a.m., 1, 3, 5, 7, 9, 11 p.m., 1, 3, 5 a.m. During the dark period the animals' room remained dark even at the times when selected animals were removed, so as not to disturb the diurnal cycle of the remaining rats. For that reason, also, the selected rats were carried to a separate operating room where, under light ether anesthesia, the left kidney was removed. Small cubes from the outer cortex were fixed for 2 h in 1.5% phosphate-buffered osmium tetroxide, pH 7.2, dehydrated, and embedded in Epon. One Epon block per rat was chosen after examination, under the light microscope, of semithin sections stained with Giemsa solution, and an area containing convoluted tubules was then selected for electron microscopy. Thin sections (Reichert OM U₂) were stained with lead citrate and viewed in the Siemens Elmiskop I A.

Morphometric Analysis of Tubular Cells

Six micrographs were randomly chosen from the selected specimen of each animal at a primary magnifica-

tion of $\times 4,200$. A point lattice was superimposed onto the negatives which were then enlarged threefold. With the aid of the point-counting method (28) the proportion between proximal and distal tubules was determined. By the same method the relative areas, a measure of the relative volumes, were determined for the following cellular components: nuclei, mitochondria, microbodies, the brush border, and the remaining cytoplasm including endoplasmic reticulum, Golgi fields, and ground substance (ERGS). Vacuoles and lysosomes were treated together as one entity. In addition, the number of sectioned profiles of mitochondria and microbodies per area was ascertained. The area of tubular cells recorded in this manner was about $2.5 \times 10^4 \mu\text{m}^2$.

Quantitative Evaluation of Autophagic Vacuoles

Since in untreated rats AV are found only in small numbers, an excessive number of randomly sampled micrographs would have been required to yield an adequate number of AV. It was therefore preferred to search for the AV directly on the electron microscope screen. In the quantitative evaluation, the square holes of the copper grids supporting the specimens were used as area units. The length of the side of randomly sampled squares from the 24 grids was measured with an ocular micrometer in the light microscope. The mean thus obtained was 91.2 (SEM = 0.8) μm . The mean area of one quadratic hole was therefore 8,320 (SEM = 104) μm^2 . The part occupied by tubular cells in this area was determined with the help of a point lattice superimposed on micrographs of low magnification (primary magnification $\times 600$, final magnification $\times 1,800$), taken from each of the 144 examined squares (six per animal). The mean of the proportion taken up by tubular cells was 0.776 (SEM = 0.008) μm^2 . One square unit, therefore, corresponded to a tubular cell area of 6,452 (SEM = 109) μm^2 . Six area units per animal were carefully examined for AV by scanning at a primary magnification of $\times 6,000$, using in addition a stereomagnifier $9\times$. The specimen was moved up and down in a systematic manner. Each AV was photographed at $\times 15,000$. The sectioned areas of the AV were determined at a final magnification of $\times 45,000$ by means of a point lattice, each point representing an area of 0.03 μm^2 . The total area of tubular cells examined for AV in this study was about $93 \times 10^4 \mu\text{m}^2$.

A note concerning the statistical analysis of the data obtained is required. The analysis is based on the following model: measurements of the number and of the area of the AV per unit of cytoplasmic area show that these values are dependent on the time of the day at which the measurements are taken. This becomes apparent by a comparison of the "daytime" with the "night-time" values, assessed by the Student *t*-test (see Results). Hence it would seem reasonable to assume that the

values obtained are realizations of independent, and normally distributed, random variables x_{ij} ($i = 1, \dots, 12$; $j = 1, 2$) with identical variances σ^2 , but with different expectations $E[x_{ij}] = \mu_i$ for the 12 time intervals chosen for the measurements. Since

$$\bar{x} = \frac{1}{24} (x_{1,1} + x_{1,2} + \dots + x_{12,1} + x_{12,2})$$

is an unbiased estimator of the mean value

$$\mu = \frac{1}{12} (\mu_1 + \dots + \mu_{12}),$$

and since the variance

$$\frac{\sigma^2}{24} \text{ of } \bar{x}$$

is estimated without a bias by

$$\frac{1}{24} \cdot \frac{s_1^2 + \dots + s_{12}^2}{12} = s_{\bar{x}}^2,$$

it follows that the interval

$$(\bar{x} - t_{95\%} \cdot s_{\bar{x}}; \bar{x} + t_{95\%} \cdot s_{\bar{x}})$$

represents a confidence interval for μ of a probability of 95%; $t_{95\%} = 2.18$ here is the two-sided 95% limit (= the 97.5% point) of the t distribution with 12 degrees of freedom (24). It should be noted that in terms of variance analysis $s_{\bar{x}}^2$ is essentially the component of variance "between the groups."

RESULTS

Cellular Components of the Convoluted Tubules

The morphology of tubular cells in the rat kidney has been described in detail by others (9, 17, 26). For the purposes of the present paper it was necessary to distinguish the cells of the proximal from those of the distal convoluted tubules in randomly sampled micrographs in which cells are sectioned in all possible directions. The most useful criteria for this distinction were: the occurrence of a brush border, of endocytotic vesicles and vacuoles, of large lysosomal bodies, and of microbodies was taken to indicate a proximal tubular cell. No attempt was made to distinguish between the different segments of the proximal tubule. Distal tubular cells were recognized by their more uniformly appearing cytoplasm without microbodies (3), and by the sometimes deep infoldings of the basal labyrinth.

The results of the morphometric analysis are presented in Fig. 1. It can be seen that the

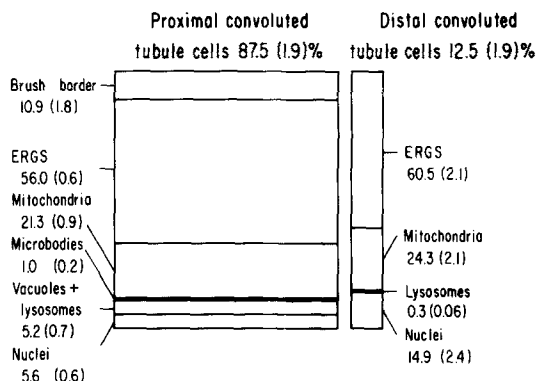


FIGURE 1 Showing the quantitative composition of the proximal and distal convoluted tubules. The relative areas, respective volumes are expressed as percentages; SEM in parentheses.

proximal convoluted tubules make up 87.5 (SEM = 1.9) % of the total area occupied by the tubular cells. The mean area taken up by proximal tubular cells in one square unit as defined above is therefore 5,646 (SEM = 156) μm^2 . Fig. 1 also shows the relative areas, respectively volumes, of the cytoplasmic components in proximal and distal tubular cells. An additional point to be made here is that no significant effect of the diurnal cycle on the composition of the tubular cells has been found.

As far as the number of organelles is concerned 3,910 (SEM = 220) mitochondrial profiles and 290 (SEM = 27) profiles of microbodies per $10^4 \mu\text{m}^2$ cell area were seen in the proximal tubular cells. From these values and from the proportion of the area taken up by the mitochondria and microbodies (Fig. 1), the mean area of a mitochondrial profile was calculated to be 0.55 (SEM = 0.04) μm^2 , and that of a profile of a microbody 0.34 (SEM = 0.08) μm^2 .

Autophagic Vacuoles

As far as the occurrence of AV in the proximal tubules is concerned the findings presented in the paper here are in agreement with those of Ericsson et al. (10). An additional finding, however, was the observation that very few AV were found in the distal tubules (Fig. 2) where they make up only about 3-4% of the total number of AV seen in tubular cells. In the calculations which follow, the cells of the distal convoluted tubules and their AV were, therefore, not taken into account. Since

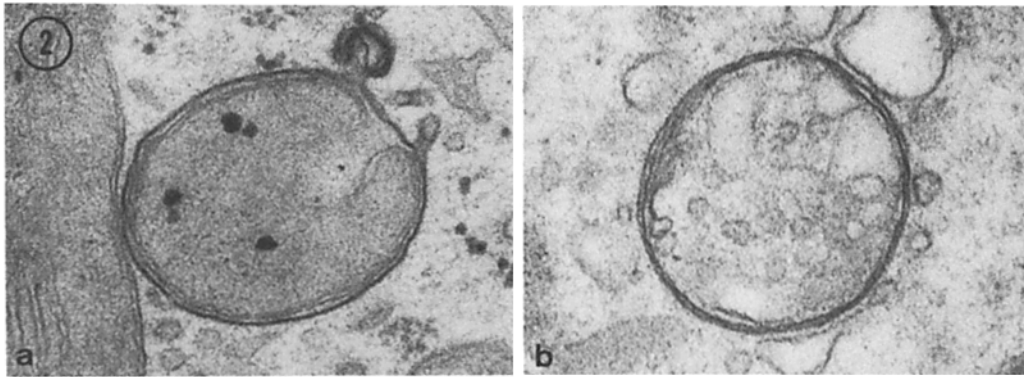


FIGURE 2 Showing examples of autophagic vacuoles in cells of distal convoluted tubules. The vacuole in Fig. 2 *a* contains a mitochondrion, the vacuole in Fig. 2 *b* a portion of cytoplasmic ground substance together with small cisternae and vesicles. $\times 70,000$.

it is well known that various stages of the process of segregation and lysosomal degradation of portions of the cytoplasm can be observed at any given time, only those vacuoles were included in the quantitative evaluation of the AV studied here whose contents could be clearly identified as being of cytoplasmic origin. These vacuoles, therefore, correspond to the cytosegresomes described by Ericsson et al. (10). Based on the principles used by Deter (6) in the liver, the following types of AV were accepted for inclusion in the calculation used here:

(a) VACUOLES CONTAINING MITOCHONDRIA (Figs. 3 *a-c*; 5 *a-c*): Segregated mitochondria were identified by the paired outer and inner membranes and by the cristae. The latter may become the only criterion, when the segregated mitochondrion was sectioned tangentially or when the outer membrane was already destroyed. In the early stages the matrix of segregated mitochondria shows no alterations (Figs. 3 *a, b*; 5 *a-c*). Later the matrix may be either denser or looser than normal. Electron-dense granules of the matrix are often found in a segregated mitochondrion, but such granules alone are not characteristic enough to warrant the conclusion that the vacuolar contents were of mitochondrial origin. Such an example shows in Fig. 3 *g* an intravacuolar structure, which could be derived from a mitochondrion, but which has not been included in the calculations presented here because it does not conform to the strict criteria adopted.

(b) VACUOLES CONTAINING ERGS (Fig. 3 *d-f*): This heterogeneous group of AV contains

membranes of the granular or agranular (Fig. 3 *e*) endoplasmic reticulum, vesicles or cisternae of the Golgi complex (Fig. 3 *d, f*), and free ribosomes (Fig. 3 *e*). Intravacuolar membranous structures which did not present characteristics of the cytoplasmic components mentioned (Fig. 3 *h*) were not counted. A large proportion of these vacuoles contain cytoplasmic ground substance. Conceivably, AV containing nothing but ground substance may exist, but they cannot be adequately interpreted as being AV. Occasionally AV were found containing ERGS together with a segregated mitochondrion (Fig. 3 *b*). In these instances the vacuole was classified as belonging to group *a*, but an additional correction was made for the ERGS group when the areas of the AV were determined.

(c) VACUOLES CONTAINING MICROBODIES (Fig. 4): This type of AV was least common. The criteria used to identify segregated microbodies were: the fine floccular matrix, the cylindrical structures (Fig. 4 *a, b*) described in detail by Langer (16), and in some instances nucleoidlike densities (Fig. 4 *a*). Two vacuoles were encountered which contained osmium-resistant tubular structures within the matrix of a segregated microbody (Fig. 4 *c*). These tubules had the same shape and size as tubules shown to have been derived from the matrix of segregated microbodies in the liver cell (19, 20).

The structure demarcating the contents of AV from the remaining cytoplasm is variable and may appear either as a single (Figs. 3 *c*; 4 *c*) or as a double membrane (Figs. 2 *b*; 4 *e, f*; 5 *a*). Frequently a rather thick osmiophilic layer can be found par-

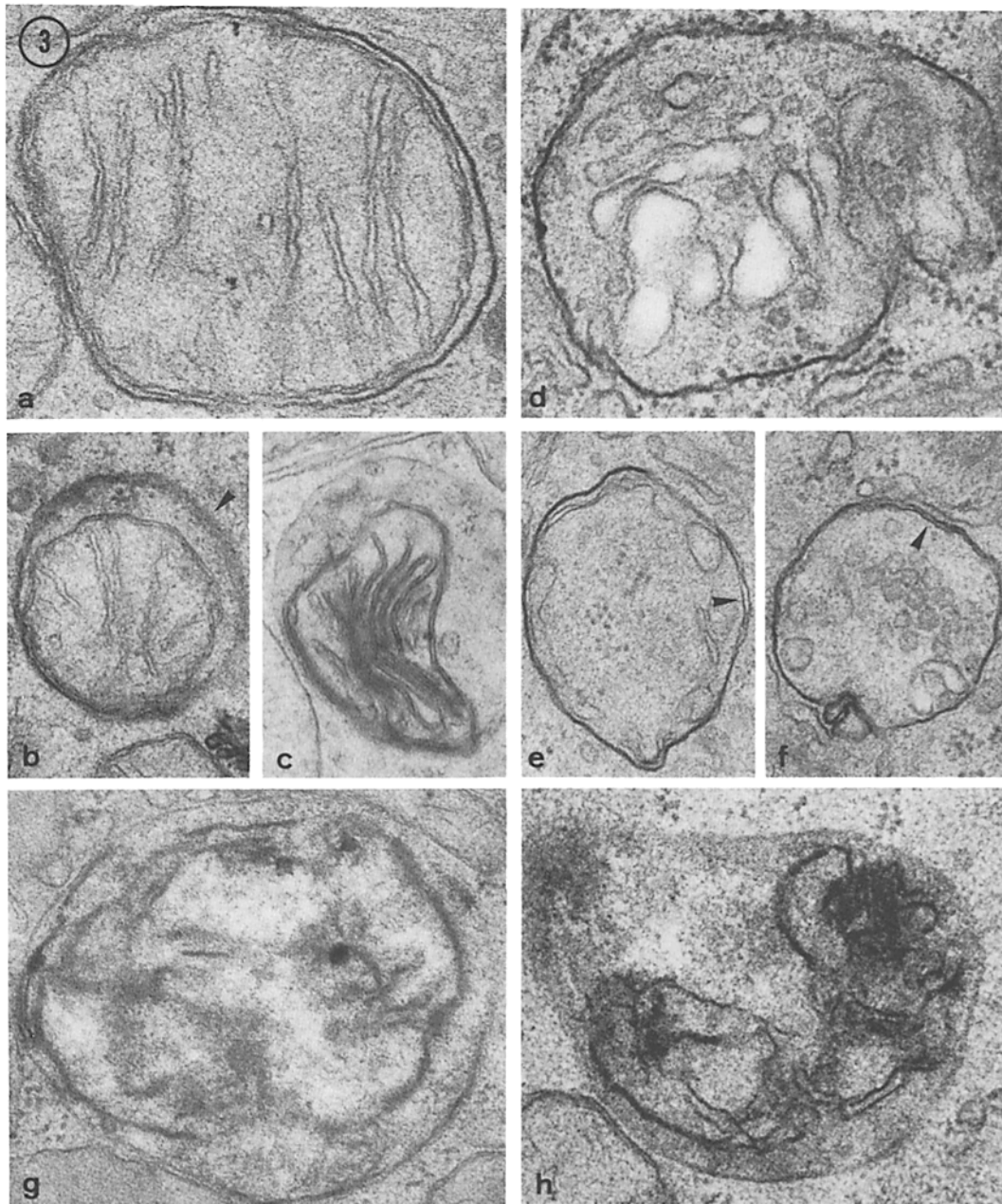


FIGURE 3 Showing autophagic vacuoles in proximal tubular cells containing mitochondria (*a-c*) and ERGS (*d-f*). In Fig. 3 *g* and *h* later stages of destruction are seen. The segregating structure appears as a thick osmiophilic layer (*a, d-f*) or as a double membrane (arrows, *e* and *f*). When sectioned tangentially the segregating structure appears as a dark blurred zone (arrow, *b*). The segregated mitochondria (*a, b*) and the ERGS portions (*d-f*) show no striking alterations, whereas the mitochondrion in Fig. 3*c* is partly destroyed. In Fig. 3 *g* and *h* the contents of the vacuoles may result from destruction of a mitochondrion (*g*) or of a portion of ERGS, but are not clearly identifiable. This type of vacuole was not included in the quantitative evaluation. *a, d*, $\times 80,000$; *b, c, e, f*, $\times 55,000$; *g, h*, $\times 65,000$.

ticularly in very early stages of segregation (Figs. 3 *a, d-f; 5 b*). Rarely the segregating structure was incomplete and appeared as an open circle. In these cases the incomplete vacuole was regarded as being an AV, if at least three-quarters of the circle was formed by the segregating structure (Figs. 3 *a; 4 a*). The latter may appear as a blurred dark zone

when sectioned tangentially. Such pictures were included only when a part of the border clearly showed a membranous appearance (Fig. 3 *b*).

One point has to be stressed. When the basal interdigitations of tubular cells are sectioned perpendicularly, the resulting appearance may simulate an autophagic vacuole (Fig. 6) surrounded by

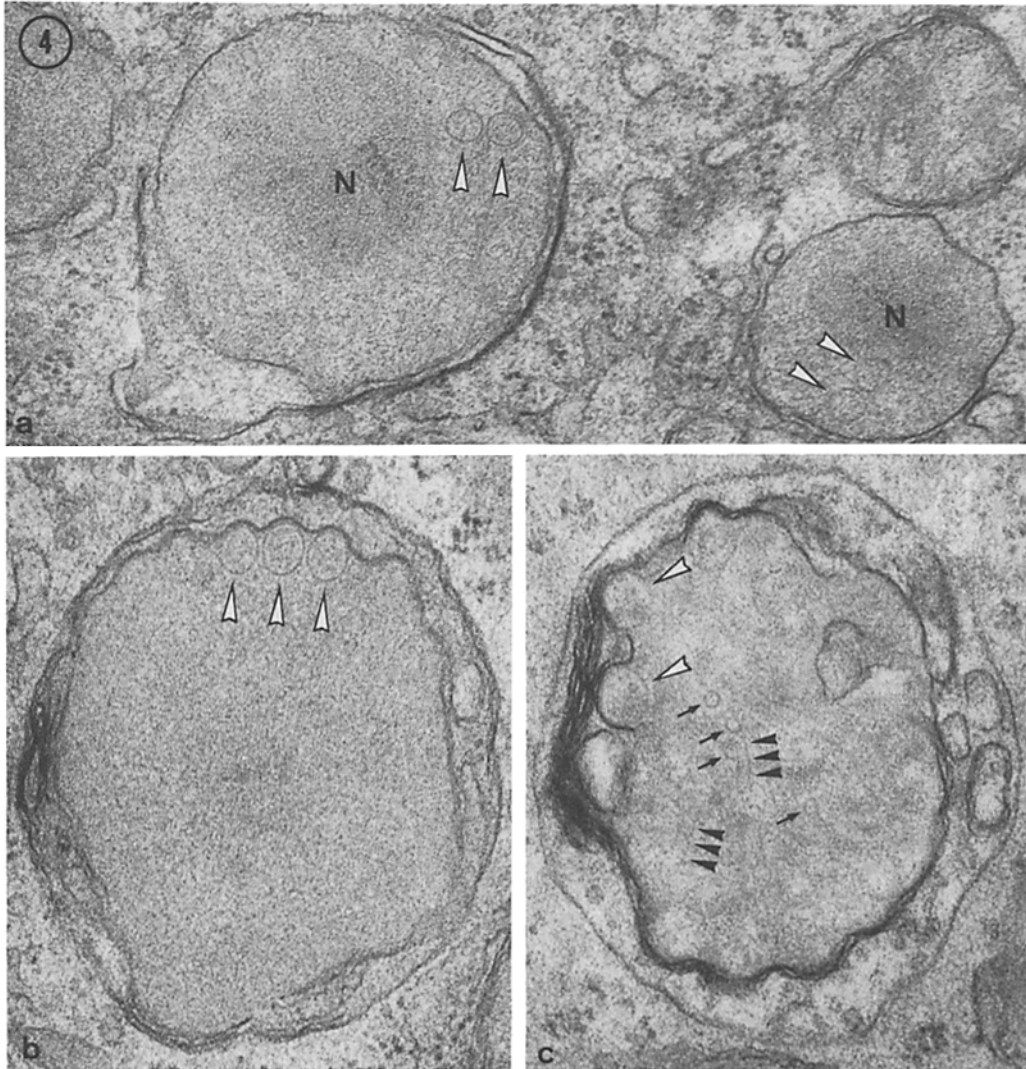


FIGURE 4 Showing microbodies in different stages of segregation and destruction. In Fig. 4 *a* three-quarters of the microbody on the left is surrounded by the segregating structure which appears partly as a double membrane, partly as a thick osmiophilic layer. As in the nonsegregated microbody on the right a nucleotidlike density (*N*) and cyndric structures (white arrowheads) can be seen. In Fig. 4 *b* the cyndric structures are seen also in a microbody which is completely segregated or, as in Fig. 4 *c* partly destroyed. The matrix of the microbody in Fig. 4 *c* shows in addition microtubular structures which have been sectioned transversely (arrows) or longitudinally (black arrowheads). *a*, $\times 50,000$; *b, c*, $\times 68,000$.

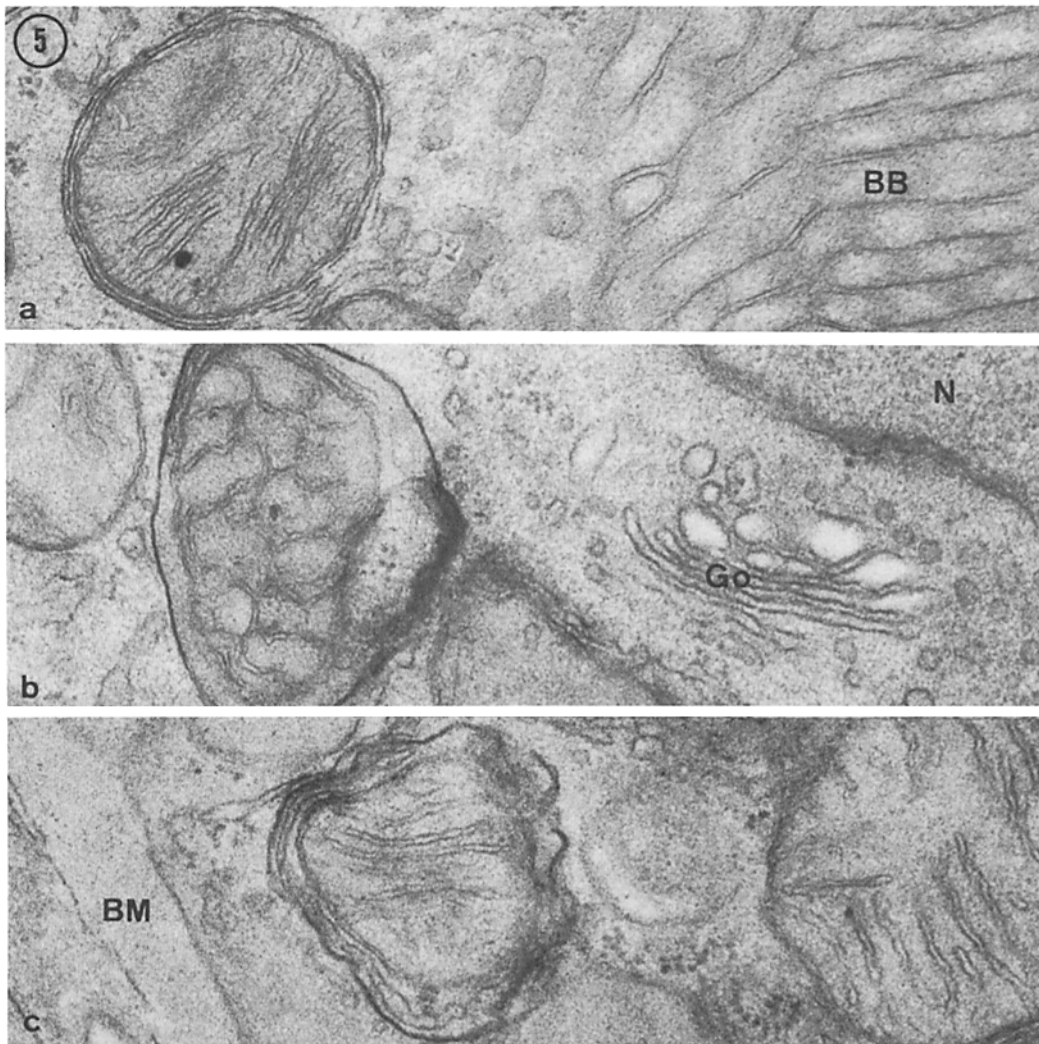


FIGURE 5 Showing autophagic vacuoles in different regions of cells of the proximal convoluted tubules. In Fig. 5 *a* a vacuole is seen near the brush border (*BB*), in Fig. 5 *b* in the vicinity of the nucleus (*N*) and the Golgi complex (*Go*), and in Fig. 5 *c* close to the basement membrane (*BM*). The mitochondria in these vacuoles are still well preserved. The segregated structure appears as a double membrane (*a*) or as a thick osmiophilic layer (*b, c*). *a, b*, $\times 57,000$; *c*, $\times 69,000$.

a double membrane. This misinterpretation can be avoided by paying attention to the relatively wide and rather regular distance between the two membranes, which in reality are the plasma membranes of two neighboring cells. Similar "pseudovacuoles" have also been noted in hepatocytes (19).

Autophagic vacuoles were found in all regions of the tubular cells. An example of an AV near the brush border is shown in Fig. 5 *a*. AV were also

found near the nucleus and the Golgi complex (Fig. 5 *b*), and in the basal part of the cell close to the basement membrane (Fig. 5 *c*). Nevertheless, AV occur preferentially in the perinuclear area. The reason for this suggestion can be found in the following calculations: if the distance between the center of an AV and the nuclear membrane is measured, a figure of $1.8 \mu\text{m}$ and less is obtained in 25% of all AV seen. The area of cytoplasm within

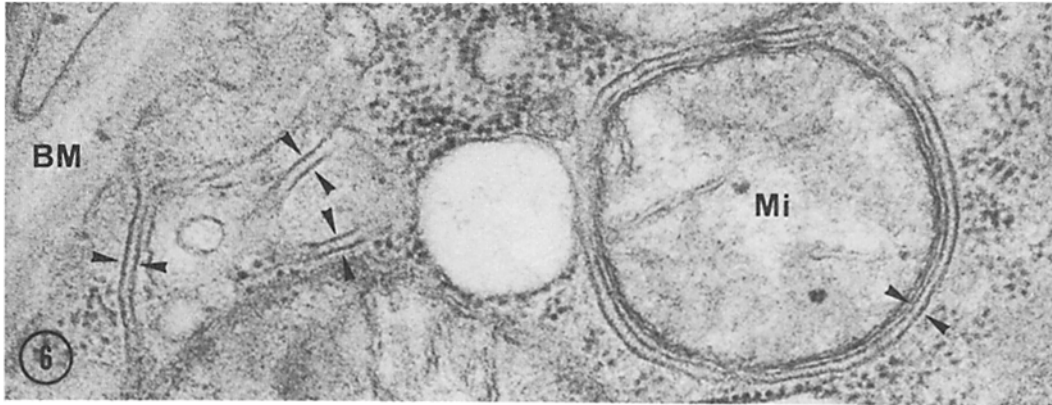


FIGURE 6 Showing a "pseudovacuole." An interdigitation between two cells may appear as a double-walled autophagic vacuole. The two membranes surrounding the mitochondrion (*Mi*) are the plasma membranes of two neighboring cells. These membranes have the same appearance as the paired membranes of the basal labyrinth (arrowheads) near the basement membrane (*BM*). $\times 54,000$.

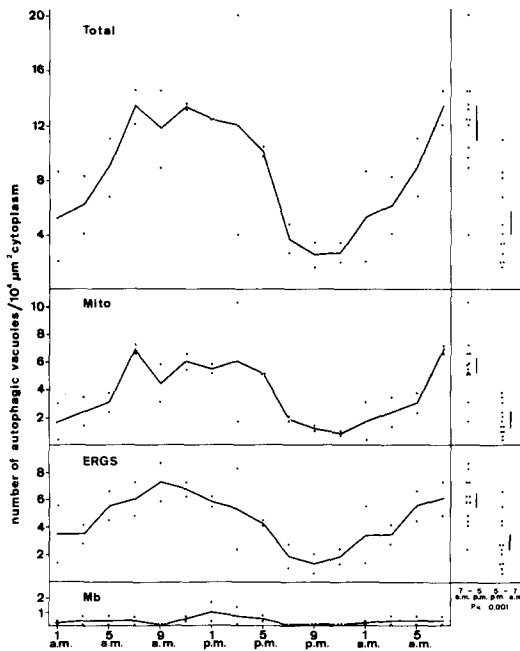


FIGURE 7 Showing the number of autophagic vacuoles per $10^4 \mu\text{m}^2$ of cytoplasm of cells of the proximal convoluted tubules. A diurnal variation with a maximum during the day and a minimum during the night exists for the different types of autophagic vacuoles containing mitochondria (*Mito*), *ERGS*, and microbodies (*Mb*). The column on the right shows that the differences between the values for the daytime and the nighttime are statistically significant.

circles drawn around the nuclei at a distance of $1.8 \mu\text{m}$ was calculated after measuring the diameters of 530 nuclear profiles in the low magnification micrographs. This area was about 11% of the whole cytoplasm, which did not include the brush border. The number of AV situated in this small perinuclear area was, therefore, about twice that which could have been expected.

The quantitative evaluation of AV showed clear-cut diurnal variations. As far as the number of AV is concerned (Fig. 7) there was seen a synchronous oscillation of all the different types of AV as defined above. A rather broad, plateau-like "maximum" was found between 7 a.m. and 5 p.m. and a somewhat narrower "minimum" between 7 p.m. and 3 a.m. When the values obtained during the light period (7 a.m. to 5 p.m.) were compared with those obtained during the dark period (7 p.m. to 5 a.m.) the differences were found to be highly significant by means of the *t*-test (Fig. 7, right side). As far as the area of segregated material per area of cytoplasm is concerned (Fig. 8) a diurnal rhythm was again encountered which was similar to that found for the number of AV. In fact the differences between the minimum and the maximum are, if anything, even a little more obvious, suggesting that the AV tend to be smaller during the period of minimum than they are during the period of maximum.

Some of the results of the morphometric analysis of the cytoplasm of proximal tubular cells and

of the quantitative evaluation of AV are summarized in Table I. In the upper part of Table I the areas of the whole cytoplasm are evaluated, and the areas of its components are shown, as well as

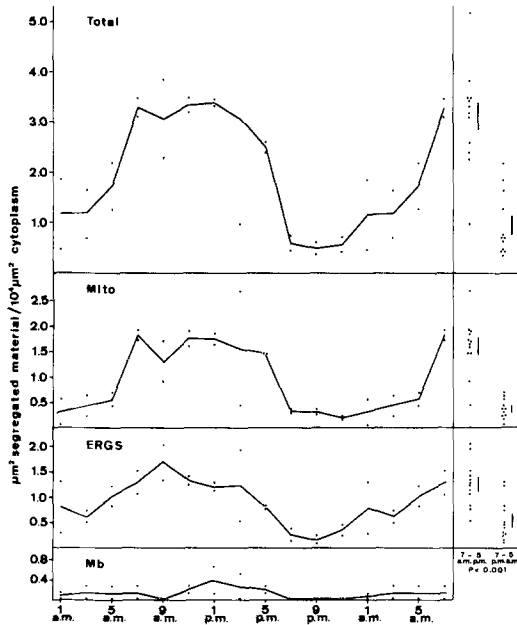


FIGURE 8 Showing the area of segregated material per area of cytoplasm in cells of the proximal convoluted tubules. The curves are essentially the same as in Fig. 7, indicating that the number of autophagic vacuoles is proportional to the amount of segregated material. The upper curve represents the segregated fraction for the whole cytoplasm, the mean being 2.1×10^{-4} (cf. Table I). The column on the right again shows that the differences between the values for the daytime and the nighttime are statistically significant.

the areas obtained for the material segregated within AV. The mean values of the segregated fractions of the cytoplasm and of its components, and the 95% confidence intervals, were calculated from the single values of the segregated fractions found in each animal. The mean values of the segregated fractions are not identical for the different cytoplasmic components. In the lower part of Table I the segregated fractions are shown which have been calculated from the number of the sectioned profiles of mitochondria and microbodies. The mitochondria now present a segregated fraction which is about twice that calculated from the areas. This difference is due to the fact that the mean area of a segregated mitochondrion was found to be only half that of a nonsegregated mitochondrion. In contrast to this, however, the microbodies are about the same size whether they are free or segregated. The segregated fraction of the microbodies is influenced, therefore, by the manner of calculation, be it from the area or from the number of sectioned profiles.

DISCUSSION

In order to draw from the quantitative data presented here valid conclusions concerning the dynamics of cellular autophagy, it is necessary to keep in mind that AV represent a transitional stage between the phase of segregation and the phase of destruction of segregated contents. The amount of material that has been segregated can become a measure of the intensity of the process of cellular autophagy, if the time interval between the segregation and the destruction (henceforth called the "destruction time") is, on the average, constant.

TABLE I
The Segregated Fractions (= Segregated/Nonsegregated Material) of the Whole Cytoplasm and of its Components Calculated as Mean Values of the Diurnal Cycle

Basis of calculation	Component	Nonsegregated	Segregated	Segregated fraction $\times 10^{-4}$	95% confidence interval
Area	Cytoplasm	$76.6 \times 10^4 \mu\text{m}^2$	$155.9 \mu\text{m}^2$	2.1	(1.6; 2.6)
	ERGS	$45.4 \times 10^4 \mu\text{m}^2$	$70.6 \mu\text{m}^2$	1.6	(1.2; 2.0)
	Mitochondria	$17.3 \times 10^4 \mu\text{m}^2$	$75.9 \mu\text{m}^2$	4.4	(3.3; 5.5)
	Microbodies	$0.8 \times 10^4 \mu\text{m}^2$	$9.4 \mu\text{m}^2$	11.7	(3.1; 20.3)
Number of sectioned profiles	Mitochondria	31.8×10^4 Mean area 0.55 (SEM 0.04) μm^2	289 Mean area 0.26 (SEM 0.01) μm^2	9.1	(6.7; 11.5)
	Microbodies	2.4×10^4 Mean area 0.34 (SEM 0.08) μm^2	26 Mean area 0.36 (SEM 0.03) μm^2	10.8	(5.5; 16.1)

Obviously, if the destruction time varies, the amount of segregated material will vary too, even if the rate of segregation and destruction is unaltered. This relationship between the destruction time and the number of AV in a given area is illustrated in Fig. 9.

The destruction time cannot be measured directly. An attempt to calculate the destruction time indirectly from some data derived here will be described later on. So far there has been no evidence available to show that the destruction time in fact is subject to significant variations. After stimulation of cellular autophagy in liver cells by glucagon, for instance, the destruction of the segregated material can be observed within a short time (6). Moreover, the increase in number of AV after treatment with glucagon cannot be explained on the basis of a prolonged destruction time (20). Despite certain reservations in the calculations presented here a constant destruction time has been assumed.

Diurnal Rhythm of Cellular Autophagy

With that assumption of a constant destruction time, the diurnal variations of both the number of AV and the amount of the segregated material

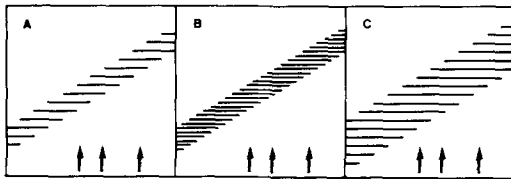


FIGURE 9 Explaining the relationship between the destruction time, the rate of segregation, and the frequency of AV. Each horizontal bar represents the history of an AV from segregation (left end of each bar) to degradation (right end of each bar). The length of each bar, therefore, indicates the destruction time. The number of end points in an arbitrary interval of time, represented by the width of each of the rectangles A-C, corresponds to the rate of segregation, respective to the rate of destruction. In Fig. 9 A the destruction time and the rate of segregation are arbitrarily chosen in such a manner that at any given time of observation (vertical arrows) three bars, i.e., three AV, can be observed. In Fig. 9 B the destruction time is the same as in Fig. 9 A, but the rate of segregation, i.e., the number of AV formed in the given time interval, is doubled. In this case, six AV can be seen at any given time of observation (vertical arrows). In Fig. 9 C, again six AV are encountered at any given time of observation, but the rate of segregation now is the same as in Fig. 9 A, whereas the destruction time is doubled.

indicate that the segregation and the destruction of cytoplasmic components in the cells of the proximal tubules of the kidney is not a process which continues at the same rate throughout the cycle, but that it takes place preferentially during the daytime, that is, during the inactive phase of the animals. On the other hand, the process of cellular autophagy appears to abate at night, during the period of food intake and maximal activity.

The question arises as to how this diurnal rhythm can be explained. That the tubular cells of the kidney are subject to diurnal changes is well known and can, for instance, be seen when the phenomenon of compensatory hypertrophy after unilateral nephrectomy is studied (14). No functional correlation between factors similar to those which influence compensatory hypertrophy and cellular autophagy, however, can for the present be postulated. Furthermore, experimental conditions known to influence the process of cellular autophagy have to be considered. While it is known that sublethal cell injury (7, 27) and functional overload by excessive protein reabsorption (18) do indeed stimulate the process of cellular autophagy in tubular cells of the kidney, in the context of the diurnal rhythm these factors cannot be considered as being as relevant as a simple alteration of the metabolic state, for instance by glucagon (1, 2, 6) or cyclic AMP (23) which induces autophagic vacuolation in liver cells. Cyclic AMP produces a rise in the number of autophagic vacuoles also in isolated fragments of kidney tubules (unpublished data). The possibility that the diurnal changes of cellular autophagy are influenced by hormonal stimuli can not, therefore, be ruled out. Since the diurnal rhythm in the cells of the proximal convoluted tubules is essentially synchronous to that described in parenchymal cells of the liver (20) or, for that matter, also in exocrine pancreatic cells (unpublished data), the question arises whether the changes described here cannot be viewed as an expression of a regulatory mechanism of cellular autophagy common to the whole body.

The decrease of cellular autophagy during the night, i.e., during and after food intake, can be correlated with the biochemical observation that the protein degradation in the liver ceases after food intake (11). This leads to the general question as to what extent cellular autophagy is responsible for the degradation of cellular constituents, which can be measured biochemically as degradation of

proteins or of lipids. This problem can only be discussed if due regard is paid from a kinetic point of view to the proportion of the segregated cytoplasmic components in relation to the whole cytoplasm, i.e., the segregated fraction.

Segregated Fraction and the Turnover of Cytoplasmic Constituents

Quantitative evaluation of AV in normal control animals has been undertaken, until now, in only a few papers. From an electron microscope analysis of fractions of liver homogenates, Deter (6) calculated that the relative volume of autophagic vacuoles was in the region of 1.8×10^{-4} , whereas studies in electron microscope sections gave a value of 3.6×10^{-4} (4). In both these studies the values obtained were calculated on the basis of total liver volume. If, however, only the cytoplasm of the hepatocyte is used as a basis for this calculation, the corrected values which correspond to the segregated fraction, as defined in the present paper, are 2.3×10^{-4} and 4.6×10^{-4} , respectively.

Although these two values obtained in liver cells are of about the same magnitude as the total segregated fraction calculated for cells of the proximal convoluted tubules which, as shown in the present paper, yields a value of 2.1×10^{-4} (Table I), these figures are, nevertheless, not directly comparable to each other for the following methodological reasons: (a) The values of the segregated fraction in the paper of Deter (6) and Bolender and Weibel (4) have been obtained in rats starved overnight, whereas in the present study the mean value during the diurnal cycle of animals having free access to food throughout was determined. (b) In the two papers mentioned, later stages of degradation than the ones acceptable by the limits defined in this paper may have been included. (c) In the present calculation the brush border of the proximal tubular cells was included as part of the cytoplasmic area, but it is still a matter of dispute whether or not this component should be regarded as properly belonging to the cytoplasm in the context presented here, for it does not appear to be directly affected by cellular autophagy. If the brush border is excluded the corrected value for the segregated fraction would be 2.3×10^{-4} .

It, therefore, is apparent that a strict comparison between the segregated fractions of the liver

and kidney cells cannot be made at the present time.

It has been the purpose of the present paper to arrive at some conclusions concerning the significance of cellular autophagy for the turnover of the components of the cytoplasm. The question, therefore, arises to what extent such cytoplasmic components can be degraded in the course of physiological autophagy. The static parameter "segregated fraction" is not sufficient for this purpose and an additional parameter, a parameter of time, must be included.

The destruction time, as defined earlier on, cannot be measured directly. However, some indications can be obtained from the heterophagic uptake and destruction of cellular components by phagocytic cells. It has been reported that if isolated liver mitochondria are injected into the portal vein they are destroyed in heterolysosomes of Kupffer cells within 20 min after injection (8). In the case of autophagy, however, such a clearly defined starting point is, at the present time, not available. A further calculation of the kinetics of cellular autophagy will, therefore, depend on another parameter of time, namely the "lifetime" of cellular structures.

This question of the lifetime can be illustrated by the example of the mitochondria which, in this context, have to be viewed as individual entities. The values for the half-life of various components of the mitochondria have been determined biochemically (25). Such values, however, are representative of the lifetime of mitochondria only if the component which is being investigated remains stable, i.e., is not subject to "turnover" during the life history of the mitochondrion. From this point of view mitochondrial DNA can be assumed to be the most likely component. Its half-life in the cortex of the kidney was shown to be 10.4 days (13); this value, however, has to be considered as the upper limit for a reutilization of labeled DNA precursors cannot be excluded. From the proportion

$$\text{lifetime} = \text{half-life} / \ln 2$$

one can calculate a value of 15 days as the upper limit of the mean lifetime of the mitochondria. This value will be adopted here as a basis for further calculations.

On the assumption that the formation and the destruction of mitochondria are in equilibrium,

and that destruction is carried out exclusively by cellular autophagy, formula (a) given here would appear to be valid (21):

$$\frac{\text{Segregated mitochondria}}{\text{nonsegregated mitochondria}} = \frac{\text{destruction time}}{\text{lifetime}} \quad (a)$$

The relation expressed by this formula can be applied not only to mitochondria, but also to other cytoplasmic components which are destroyed by autophagy. This is illustrated in Fig. 10. Since in formula (a) the expression on the left is identical with the segregated fraction as defined earlier, it follows that

$$\text{Destruction time} = \text{lifetime} \times \text{segregated fraction} \quad (b)$$

and

$$\text{lifetime} = \frac{\text{destruction time}}{\text{segregated fraction}} \quad (c)$$

If in formula (b) as pointed out earlier, the lifetime is assumed to be 15 days and the segregated fraction of mitochondria, calculated on the basis of areas, respectively volumes, is assumed to be 4.4

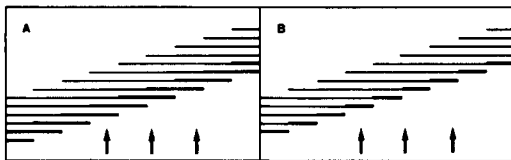


FIGURE 10 Explaining the relationship between the lifetime, the destruction time, and the segregated fraction. Each horizontal bar represents the life history of an organelle from its origin to its segregation (= lifetime, thin part of the bar), and from its segregation to its destruction (= destruction time, thick part of the bar). In Fig. 10 A, the arbitrary ratio between the destruction time and the lifetime is 1:2. At any given time of observation (vertical arrows) two thick bars, i.e., two segregated organelles, and four thin bars, i.e., four nonsegregated organelles, can be observed resulting in a segregated fraction of 0.5. In Fig. 10 B, the lifetime is the same as in Fig. 10 A, whereas the destruction time is shortened to such an extent that the ratio between the destruction time and the lifetime is 1:4. In that case the segregated fraction at any given time of observation (vertical arrows) is 0.25.

$\times 10^{-4}$ (Table I), a calculated destruction time of about 10 min is obtained. This value is lower than that calculated elsewhere for the mitochondria of the liver; that calculation, however, was based on the number of profiles and not, as is the case in the present paper, on the relative volumes of the AV. In addition the value then adopted for the lifetime of liver mitochondria was probably too high.

At first sight a value of 10 min for the destruction time of the mitochondria would appear rather short. In this connection, however, it has to be stressed that this value, by definition, applies only to the time interval in which segregated mitochondria can still be identified. It does not apply to the time interval which includes the final degradation of the mitochondria into low molecular components.

Certain factors, however, may lead one to underestimate the segregated fraction and, consequently, also the destruction time: (a) AV may be overlooked during sampling, particularly if they are tangentially sectioned. (b) The organelles may shrink after they have been segregated.

While the latter point may play a part, it can hardly be of sufficient significance to account for the fact mentioned earlier, that the area of a segregated mitochondrion is only half that of a nonsegregated one (Table I). This may perhaps be explained by postulating a selective segregation of smaller mitochondria. In favor of this assumption is the fact that none of the large mitochondria having a length of several micrometers and seen particularly in the basal part of the cells, have in the present study been found within an AV. Against shrinkage as a general and important factor speaks the fact that, as pointed out earlier, the majority of segregated mitochondria does not display an increase in density of their matrix (cf. 1). Furthermore, the mean areas of profiles of segregated and nonsegregated microbodies are not different (Table I). For that reason the values calculated on the basis of areas, respectively volumes, are preferred for kinetic calculation used here rather than those calculated on the basis of the number of sectioned profiles.

A factor which may lead, on the other hand, to an overestimation of the destruction time is the fact that the lifetime of cortical mitochondria, based on biochemical calculations (12, 13), can only be calculated from a mixture of mitochondria derived from both proximal and distal convoluted tubular cells. The possibility, however, cannot be

excluded that the lifetime of mitochondria in cells of the proximal convoluted tubules is shorter than that in the distal ones because the frequency of AV is higher in proximal convoluted tubules. It must again be pointed out that only 3-4% of all the AV found were seen in distal tubular cells, which make up about a total of 12% of the total tubular cell area, respectively volume (Fig. 1).

Provided that the destruction time of segregated microbodies is the same as that of mitochondria a lifetime of about 8 days can be calculated from the segregated fraction 11.7×10^{-4} (Table I). This would imply that the turnover of the microbodies is higher than that of mitochondria. Biochemical turnover studies of renal microbodies, however, are still lacking. In the liver the half-life of peroxisomal constituents has been shown to be considerably shorter than that of mitochondria (12, 22). A quantitative study of AV in the normal liver cells (21) is in keeping with the suggestion that this shorter half-life can be accounted for by a higher rate of degradation of microbodies by autophagy.

Based on these considerations the following suggestions can now be made: (a) Cellular autophagy does not proceed in a purely random manner, but is a discriminating process capable of selecting different kinds of cytoplasmic components for segregation and degradation. It should be pointed out that a similar conclusion has been arrived at on the basis of several other experiments (4, 6, 20). (b) The data presented here are compatible with the assumption that the degradation of mitochondria and of microbodies takes place to a large extent, if not exclusively, by cellular autophagy. There is no contradiction between this suggestion and the many biochemical observations showing that different constituents of an organelle have different turnover rates. This fact can be explained by the assumption that such constituents are exchanged or degraded as an essential part of the function of an organelle during its lifetime.

It can be seen from Fig. 8 and from Table I that the area of segregated ERGS nearly equals that of segregated mitochondria. It, therefore, seems that even the turnover of the membranes of the endoplasmic reticulum cannot be easily interpreted without invoking cellular autophagy. The fact that the segregated fraction of the ERGS measuring 1.6×10^{-4} is obviously smaller than that of the mitochondria measuring 4.4×10^{-4} (Table I) does not contradict this assumption; for it is by no

means a priori certain that all components which, according to the definition have to be designated as "nonsegregated ERGS," also contribute material for cellular autophagy. It, therefore, could be possible that the compartment from which ERGS vacuoles derive is smaller than the ERGS compartment which has been surveyed by the method used here. In that case the "true" segregated fraction would be correspondingly larger. Another difficulty stems from the fact that the life history of endoplasmic reticulum membranes is complicated by their involvement in secretory processes. Nevertheless, the view that cellular autophagy plays an important role in the degradation of endoplasmic reticulum membranes is supported by the morphological finding that membranes induced by the administration of phenobarbital are preferentially segregated in the phase of recovery (4).

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