

# INHIBITION BY INSULIN OF THE FORMATION OF AUTOPHAGIC VACUOLES IN RAT LIVER

## A Morphometric Approach to the Kinetics of Intracellular Degradation by Autophagy

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### ABSTRACT

Electron microscopic morphometry has demonstrated a rapid decrease in the fractional volume of autophagic vacuoles (AV) in hepatocytes of adult male rats after the intraperitoneal administration of insulin (5 U/kg of body weight). Except for a significant decrease in glycogen to about one-half its initial value, no major changes in the composition of the remaining cytoplasm, or in the average volume of the single hepatocyte, were seen. The decrease found in the AVs is attributed to an inhibition of the formation of new AVs—probably the morphologic counterpart of the well-known anticatabolic effects of insulin.

The decay of the fractional volume of the AVs appeared to follow first-order kinetics. Thus, the termination of the “life” of an AV by destruction of its contents may not depend directly on the “age” of the AV. The average half-life of the AVs amounted to ~9 min. Similar values were found for the different types of AVs, except for those containing glycogen. The half-life of these AVs was ~18 min. From the half-life values and from the “segregated fractions” at time zero, which were different for the different cytoplasmic components, rates of removal from the cytoplasm by autophagy were calculated. Expressed as “percent per day”, the following rates were found: whole cytoplasm, 2.3; mitochondria, 3.9; microbodies, 8.9; and glycogen, 1.1. The results indicate that autophagy, to some extent, is selective and plays an important, but not an exclusive, role in intracellular turnover.

**KEY WORDS** autophagy · inhibition of  
autophagic vacuole formation · insulin ·  
intracellular turnover · liver cell · morphometry

Fifteen years ago, Ashford and Porter (4) first reported that the formation of what is now known

as “autophagic vacuoles” (AV) can be induced in parenchymal cells of the liver by glucagon. Since then, this model has repeatedly been used to study mechanisms involved in cellular autophagy, such as the formation of AVs (3, 17, 37), or the interaction between preexisting lysosomes and the

newly formed, prelysosomal autophagosomes (2, 11-14, 16). Later, it was shown that cellular autophagy takes place even under physiological conditions (18, 19, 36), and is subject to circadian variations (37, 38, 40, 42). It was, however, not known whether, in addition to factors which would stimulate, there existed physiological agents which could inhibit the formation of AVs, and thus acted as antagonists of glucagon. Only Amherdt et al. (1) had shown that the pathologically increased number of AVs in liver cells of severely diabetic rats could be brought to normal by treatment with insulin. It therefore was of interest to see whether insulin could also suppress the basal autophagic activity in otherwise untreated animals.

If such a property of insulin could be demonstrated, this hormone might then be considered a true physiological inhibitor of autophagic breakdown. On the other hand, the inhibition of the formation of AVs may well serve also as a convenient tool for calculating the average lifetime of AVs from the rate of their decay. A morphometric analysis of the effect of insulin on the AVs of liver cells was, therefore, considered desirable. It held out the promise of offering an appropriate base line for considering the question whether there existed a correlation between the biochemists' measurements of the degradation of cellular constituents, such as proteins (for review, see references 23 and 24), and the degree of AV formation which, conceivably, might represent the morphological expression of the biochemists' determinations.

## MATERIALS AND METHODS

### *Experimental Conditions*

In the main experiment, a total of 45 male Sprague-Dawley rats (Wiga, Sulzfeld, Germany) weighing an average of 290 g were given a single intraperitoneal injection of 5 U of insulin (Hoechst, Germany) /kg of body weight. For every animal killed immediately after the injection, two others were killed at arbitrary time intervals ranging from 7 to 33 min after the administration of insulin. Because of circadian variations (37, 38, 40, 42), the experiments were restricted to the time interval between 10:30 and 11:30 a.m. This meant that repeated separate experiments had to be performed, in each of which six or nine animals, divided into groups of three, were used.

In an additional experiment, 12 rats were divided into groups of three. They were given a single intraperitoneal

injection of 5, 0.5, or 0.05 U of insulin/kg of body weight and were killed 15 min after the injection.

### *Tissue Preparation and Selection of Parenchymal Areas*

A thin slice of tissue was excised from the right half of the median lobe of the liver and cut into small cubes of ~1-mm length. 10-15 cubes were fixed in cold 1.5% phosphate-buffered osmium tetroxide for 2 h. Dehydration was started in 70% ethanol; the specimens were embedded in Epon following treatment with propylene oxide. Semithin sections were cut from five blocks per animal, and were examined with the light microscope. From sections containing both a periportal tract and a central vein, the distance between these two structures was measured by using a calibrated micrometer screw in the ocular of the light microscope. The average value was found to be 0.49 mm (SEM 0.02). This value seems to be consistent with the recent morphometric data of Jones et al. (27). Between a portal tract and a central vein, these authors have found cell cords of 24 hepatocytes on the average, each cell having a mean volume of  $6,536 \mu\text{m}^3$ . Assuming a cubic shape for these cells, an average side length of  $18.7 \mu\text{m}$  and, consequently, a distance of 0.45 mm between the periportal tract and the central vein can be calculated.

For each animal, one block containing a periportal tract was chosen, and a parenchymal area adjacent to the periportal tract was selected and trimmed out for electron microscopy. The side lengths of these final sectioning areas were measured by using the micrometer screw in the ocular of a stereoscopic lens, and were found to amount to an average value of 0.31 mm (SEM 0.01). According to the definition given by Loud (31), only periportal and midzonal parenchymal cells could be assumed, therefore, to be present in the samples obtained for electron microscopy, but not centrilobular cells; it is the latter cell type to which the most significant differences in organelle composition by comparison with the other cells in the liver lobule have been shown to be confined (31).

Silver-grey thin sections were stained with lead citrate and examined in the Elmiskop I A (Siemens, Germany), which was operated with a double condenser at an acceleration voltage of 60 kV.

### *Morphometry*

By light microscopy, the semithin sections of those blocks from which the thin sections for electron microscopy had also been obtained, were evaluated in step I for the number of nuclear profiles per test area and, by point counting (52), for the fractional area of hepatocellular cytoplasm in relation to the whole parenchyma.

In step II, eight micrographs per block and animal were taken at random for electron microscopic morphometry at a primary magnification of 4,200. Photographic prints, enlarged to a final magnification of

12,600, were evaluated by point counting for the volume fractions of the following cytoplasmic components: (a) mitochondria; (b) microbodies (=peroxisomes); (c) "ERGS", which is a rather loosely defined compartment (42) comprising endoplasmic reticulum, ribosomes, Golgi areas, and the cytoplasmic ground substance; (d) glycogen: this was identified as heavily stained particles mainly of the  $\beta$ -type; the very narrow spaces between aggregates were included in this compartment; (e) dense bodies: all structures which, according to the generally accepted criteria (8), could be assumed to be lysosomes were included, except those which fell within the definition for the AVs, as given below. Fat droplets were not considered of interest in the context of the present experiment.

The total number of test points applied per sample was varied depending on the expected values for the volume fractions of the different cytoplasmic components. 840 points with a distance of 1.5 cm in a square lattice were counted per animal for determining the volume fractions of mitochondria, glycogen, and ERGS. 7,560 points with a distance of 0.5 cm served for the evaluation of microbodies, whereas 21,000 points, whose distance was 0.3 cm, were needed for the evaluation of the lysosomes. On the basis of the formula given by Weibel and Bolender (53), the expected error of the volume fraction in each sample was estimated to be about 0.05 for mitochondria, 0.07 for microbodies, and 0.09 for lysosomes.

The rather high number of animals used in the present study could be expected to compensate for the sampling error which results from evaluating only one tissue block per animal. The reason for this manner of sampling was that, as far as the volume fraction of AVs (the most interesting parameter in this study) was concerned, the variability is much greater between different animals than between the different blocks of one animal. This has been found in another experimental series.<sup>1</sup> The high variability between different animals could possibly be explained by the fact that, as will be shown in this study, the lifetime of autophagic vacuoles is very short compared to the lifetimes that can be assumed for most of the other cytoplasmic organelles.

In step III, the AVs were evaluated. Because of their very low cytoplasmic volume fraction, they were counted and measured directly with the electron microscope. The square openings of the grid supporting the specimens were used as area units. The surface area of each individual square was determined by a method described elsewhere (39), in which the movements are registered by the digital counters of the driving mechanism. To test the reproducibility of this method, which has the advantage of not being dependent on the actual magnification, a set of square openings was measured on several occasions. For the relatively large areas amounting to  $\sim 8,300 \mu\text{m}^2$  each, the coefficient of variation  $s/\bar{x}$  was

<sup>1</sup> Unpublished observations.

found to be  $<4\%$ . For purposes of comparison, the square openings were subsequently measured in the light microscope by means of a calibrated micrometer screw in the ocular. The differences between the average values obtained by electron or light microscopic measurements were found to be  $<5\%$ . Six square openings were evaluated per animal. From the total test area and the respective fractional area occupied by cellular cytoplasm, the surface area of cytoplasm evaluated could be calculated. It amounted to  $\sim 4 \times 10^4 \mu\text{m}^2$  per animal on the average.

For the identification of AVs, strict morphological criteria were used. AVs were defined as a transitional stage between segregation and destruction; hence vacuoles were included in the evaluation only if their demarcation from the remaining cytoplasm could at least in part be identified as a membranous structure, and if one of the cytoplasmic components already defined - mitochondria, microbodies, ERGS, and glycogen (Fig. 1) - could be clearly seen in their contents. Depending on their main content, the number and the surface area of the individual profiles of AVs encountered during the systematic search were classified and registered. Two diameters  $a$  and  $b$  of each profile were measured by means of a crossed millimeter scale engraved onto the fluorescent screen of the electron microscope. The surface area of the profile was estimated as  $a \times b \times \pi/4$  after correcting for the distortion by the oblique position of the screen during the measurement under the stereoscopic lens (39). By dividing the total surface area of the profiles of all AVs, or of the different types of AVs, by the total surface area of cytoplasm evaluated, the respective fractional volumes of the AVs could be calculated. The number of the different types of AVs per unit volume of cytoplasm was estimated by relating the number of profiles per surface area of cytoplasm to the fractional volume according to the formula given by Weibel and Gomez (described in reference 52). A spherical shape of the AVs was assumed for that calculation in which no correction was made for the size distribution.

Since the present study refers mainly to fractional volumes and marginally, as a derivative of this parameter, to numerical densities, but not to surface densities, only the Holmes effect had to be taken into consideration among the possible systematic errors that are known to bias the results of stereological evaluations (53). Except for ERGS which, as the "translucent" component, is subject to underestimation, the fractional volumes of the components evaluated in step II and also the different types of the AVs evaluated in step III tend to be overestimated to a small degree; on the assumption of a constant section thickness, the extent of this overestimation varies depending on the respective particle diameter. A correction for the Holmes effect was, however, not considered necessary, because relative rather than absolute changes of the fractional volumes were of major interest in this study. Even for the absolute values of "segregated fractions" (see Results and Table I), a

correction for the Holmes effect would have been unnecessary, because these figures are quotients of two parameters both of which could be expected to be overestimated to the same extent. Only ERGS has to be mentioned as an exception. AV's containing this component tend to be overestimated, whereas ERGS as the free, "translucent" component can be expected to be underestimated.

## RESULTS

Insulin had no significant effect on the morphometric parameters obtained by light microscopic evaluation (Fig. 2). As shown by linear regression, the number of nuclear profiles per unit area (Fig. 2, upper part) increased during the experimental time interval slightly, but not significantly (Student's *t* test), by a factor of  $\sim 1.04$ . The volume fraction of hepatocellular cytoplasm in relation to the parenchymal tissue showed a small, but significant, increase by a factor of 1.02 (Fig. 2, lower part). Taking the number of nuclear profiles as proportional to the number of sectioned hepatocytes, by using the formula given by Weibel and Gomez (described in reference 52) it could be calculated that the number of hepatocytes per tissue volume increased by a factor of 1.05 within the time interval of the present experiment. The change in the average volume of the cytoplasm of a single hepatocyte could be calculated by dividing the factor of increase of the volume fraction of hepatocellular cytoplasm by the factor of increase of the number of hepatocytes per tissue volume. A decrease to  $\sim 0.97$  of the initial cytoplasmic volume per single hepatocyte was thus obtained. This decrease, even if it is not statistically significant, is possibly due to a loss of glycogen as shown in the next paragraph (Fig. 3). Since, however, the decrease in the average cytoplasmic volume of the single hepatocyte is rather small, the changes in the volume fraction of AVs, to be described later, can be taken as representative not only of the unit volume of hepatocellular cytoplasm but also of the average single hepatocyte.

The volumetric composition of the hepatocellular cytoplasm with respect to its major components ( $V_{\text{cyto, comp}}/V_{\text{cyto}}$ ) found at time zero (Table I, column 1) was in agreement with the data reported for liver cell cytoplasm fixed by immersion (44). The fractional volume of dense bodies, which are not listed in Table I, was found to be 0.31% (SEM 0.03). Under the influence of insulin (Fig. 3) the fractional volumes of mitochondria, of microbodies, and of dense bodies did

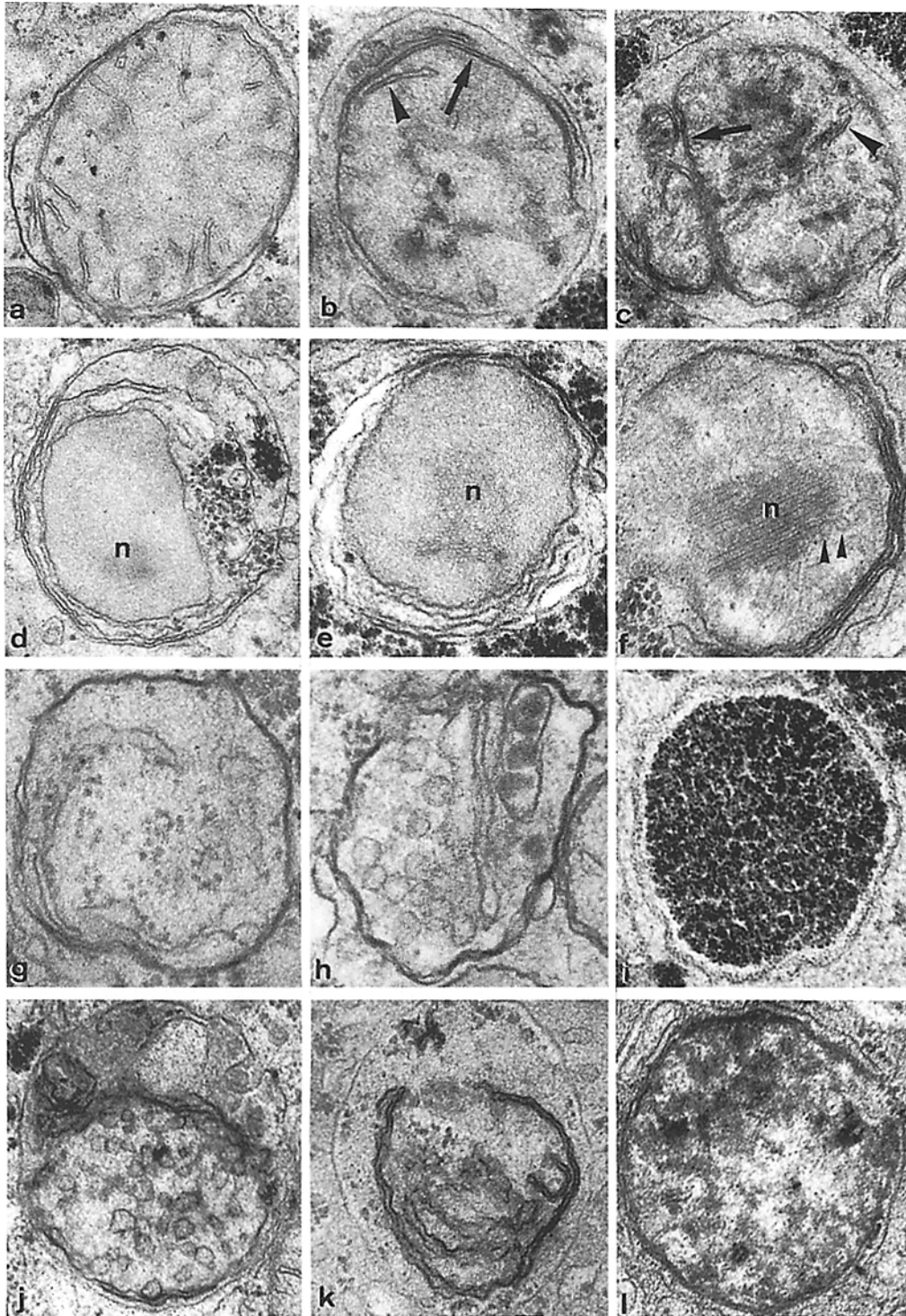
not show any significant changes. The fractional volumes of the ERGS compartment and of glycogen, however, changed significantly in opposite directions. Glycogen decreased from an average fractional volume of 21 to  $\sim 12\%$  at the end of the experiment, whereas ERGS increased from 55 to 62%.

If all the AVs are considered one compartment, the fractional volumes of the different components within this compartment ( $V_{\text{AV, comp}}/V_{\text{AV}}$ ) (Table I, column 2), were found at time zero to be different from the fractional volumes within the compartment of free cytoplasm. There were relatively more mitochondria, and particularly microbodies, in the AVs than in the cytoplasm, whereas the opposite held true for ERGS. The proportion of glycogen was about the same in both compartments (Table I, columns 1 and 2).

The fractional volumes of the AVs, and of the different types of AVs in relation to the whole cytoplasm ( $V_{\text{AV, comp}}/V_{\text{cyto}}$ ), found at time zero, are shown in Table I, column 3. Despite a rather high variation of the single values (Figs. 4 and 5), the values for the standard error of the mean (Table I) could be kept at a tolerable level because of the relatively large number of experimental animals used. The fractional volume of all AVs amounted to a value of about  $2.1 \times 10^{-4}$ .

After the administration of insulin, a rather rapid, highly significant decrease of the fractional volume of AVs could be seen. When the values were plotted on a linear scale (Fig. 4 *a*), the regression line calculated by the method of least squares did not seem to fit the set of single points in an optimal manner, because many more single points could be found below the regression line than above it. This suggested that decay possibly followed not a linear, but rather an exponential function. When the values were plotted on a semilogarithmic scale, the line obtained by the method of least squares seemed more appropriate (Fig. 4 *b*). This was also suggested by the values for the coefficient of correlation,  $-0.65$  in the linear but  $-0.68$  in the semilogarithmic plot. From the regression line in the semilogarithmic plot, the time required for the fractional volume of AVs to decrease to one-half the preceding value could be estimated as  $\sim 9$  min.

The values for the fractional volume of the different types of AVs, shown in Fig. 5, decreased under the influence of insulin to about the same extent as the whole AV compartment. This decrease again was always statistically significant.



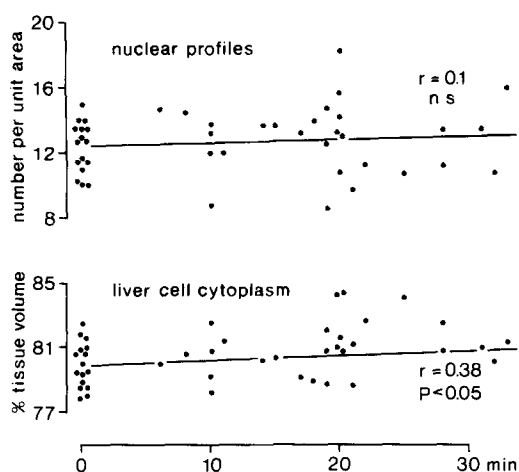


FIGURE 2 Fig. 2 shows that the parameters obtained by morphometric evaluation in the light microscope are not subject to major changes after the administration of insulin. The number of nuclear profiles per test area (upper part of the figure) increases during the experimental time interval by a factor of 1.04. The fractional volume of hepatocellular cytoplasm in relation to the whole lobular parenchyma (lower part of the figure) shows an increase by a factor of 1.02.

On the linear scale (Fig. 5, left side), the slope of the regression lines was similar for AVs containing mitochondria, microbodies, or ERGS, whereas the fractional volume of the AVs containing glycogen seemed to decrease more slowly. To plot the values of the fractional volumes of the different types of AVs on semilogarithmic scales, the values had to be grouped in a certain manner. The reason for this was that a number of zero values had been obtained which could not be used in a semilogarithmic plot. This was particularly so when the AVs containing microbodies were evaluated. In all these considerations, the animals at

time zero were taken as one group. The other three groups consisted of all the animals killed between 6 and 14 min, between 15 and 24 min, and between 25 and 33 min after the injection of insulin, respectively. The logarithms of the mean fractional volumes of each group were calculated and coordinated to the time intervals of 0, 10, 19.5, and 29 min, respectively. Values for the half-life were calculated from the linear regression lines which are shown in Fig. 5 (right side).

As can be seen in Fig. 6, the number of different types of AVs per unit volume of hepatocellular cytoplasm, i.e., their numerical density, decreases to nearly the same rates as the respective fractional volumes (Fig. 5). It can be concluded that the decrease in the fractional volume of AVs after insulin administration is due mainly to a decrease in the number of AVs, but not to a decrease in the volume of the individual AV.

The results of the separate experiment, in which different doses of insulin were given, show that a considerably lower dose (0.5 U) than that used in the main experiment (5 U) reduced the volume fraction of AVs within 15 min to about the same extent (Fig. 7). The very low dose of 0.05 U of insulin/kg of body weight, however, did not appear to be effective. 15 min after giving this low dose, the volume fraction was found to be of the same order of magnitude as in the main experiment at time zero (Fig. 3). It should be noted that the behavior of the animals receiving different doses of insulin did not show any striking differences. No signs of shock were observed even in the rats receiving the high dose of 5 U of insulin/kg of body weight.

## DISCUSSION

Two points require discussion: (a) What is the meaning of the decrease in AVs and how is it

FIGURE 1 Different types of AVs are shown (a-i). Mitochondria enclosed in an AV present either a normal appearance (a) or a partial destruction of their membranes (b and c). As long as parts characteristically arranged as paired leaflets of the outer and the inner mitochondrial membrane (arrows), or of cristae (arrowheads), were recognizable, these vacuoles were included in the morphometric evaluation. Microbodies (=peroxisomes) were regularly found in the contents of AVs (d-f). They are identified by their single bordering membrane, their homogeneous matrix, and their crystalloid nucleoid (n) (d and e). When destruction takes place (f), the nucleoid (n) as well as tubular structures (arrowheads) derived from the matrix (34, 39) still allow identification. AVs containing ERGS appear as segregated portions of cytoplasmic ground substance together with membranes of the endoplasmic reticulum and ribosomes (g), or with elements of the Golgi apparatus (h). Glycogen as the content of an AV is shown in Fig. 1 i, and can also be seen as an additional component in the AV shown in Fig. 1 d. Vacuoles were not included in the present evaluation, if their contents were no longer identifiable (j-l) because of advanced destruction.

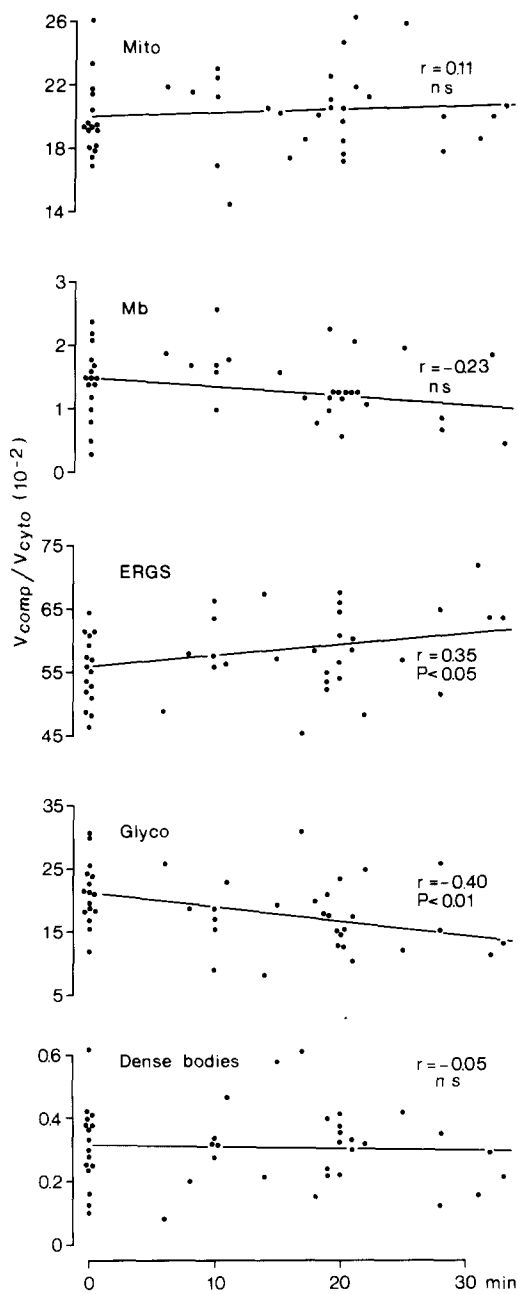


FIGURE 3 Fig. 3 shows the fractional volumes in cytoplasm of the main cytoplasmic components, at various time intervals after the administration of insulin. It can be seen that the volume fractions of mitochondria, microbodies, and dense bodies are not significantly changed. The increase in the ERGS compartment is probably a result of the significant decrease of glycogen to about half its initial value.

related to insulin? (b) What bearing have these findings on the morphological interpretation of intracellular degradation of cellular proteins and other components?

#### *Insulin as an Inhibitor of the Formation of AVs*

The size of the AV compartment depends on the rate at which AVs are formed by the process of segregation, and on the rate at which the "life" of AVs is terminated by destruction (Fig. 8a). A decrease in the size of the AV compartment could result either from an increased rate of destruction (Fig. 8b) or from a decreased rate of segregation (Fig. 8c).

The factors responsible for destruction of the contents of an AV are not yet well understood. It has been suggested (2, 13, 37) that destruction can occur even at the prelysosomal, or autophagosome (10), stage of an AV. It appears to take place, however, largely at the lysosomal stage of AV (13). The AV compartment could, therefore, decrease if autophagosomes, formed at a normal rate, fused more rapidly with lysosomes. There is no evidence that insulin stimulates this process.

The alternative explanation that the decrease of the AV compartment is due to a decreased rate of segregation (Fig. 8c) appears to be more attractive: an inhibited formation of AVs could be related to the biochemical observation that the osmotic sensitivity of lysosomes decreases in the perfused liver under the influence of insulin (34). Autolysosomes, representing a large proportion of AVs, are known to be more sensitive to osmotic stress than the later lysosomal stages (11, 14). Insulin and glucagon are known to act as antagonists (for review, see reference 21) for a variety of processes. One could now also point to the degradation of cellular proteins which can be greatly reduced by adding insulin to the liver perfusion fluid (33). This inhibition of protein degradation is probably a direct consequence of the inhibited formation of AVs.

The question arises whether the decrease of the AV compartment is mediated directly by insulin or whether it is a secondary event in the regulatory response to the hypoglycemia induced by insulin (for review, see reference 25). Lowered plasma levels of glucose or of other low molecular weight substrates are unlikely to inhibit the formation of AVs, since the formation of AVs has been stimulated when the concentration of nutrients in the

TABLE I  
Morphometric and Kinetic Parameters of Cellular Autophagy

Component	(1)	(2)	(3)	(4)	(5)	(6)
	Fractional volume of the components in cytoplasm $V_{\text{cyto, comp}}/V_{\text{cyto}}$	Fractional volume of the components in AV compartment $V_{\text{AV, comp}}/V_{\text{AV}}$	Fractional volume of the AVs in cytoplasm $V_{\text{AV, comp}}/V_{\text{cyto}}$	Segregated fraction of the components $V_{\text{AV, comp}}/V_{\text{cyto, comp}}$	Lifetime of the AVs $t_{1/2\text{AV, comp}}/\ln 2$	Rate of autophagic breakdown $1/t_{\text{cyto, comp}}$
	$10^{-2}$	$10^{-2}$	$10^{-4}$	$10^{-4}$	min	day <sup>-1</sup>
All together	100.0	100.0	2.11 ± 0.25	2.11	13.0	0.023
Mitochondria	20.0 ± 0.6	37.4 ± 3.8*	0.79 ± 0.12	3.95	14.6	0.039
Microbodies	1.4 ± 0.2	4.3 ± 0.7*	0.09 ± 0.02	6.42	10.4	0.089
ERGS	55.4 ± 1.4	38.3 ± 4.6*	0.81 ± 0.14	1.47	16.6	0.013
Glycogen	21.4 ± 1.2	20.0 ± 5.5	0.42 ± 0.09	1.95	26.0	0.011

The morphometric parameters from rat liver parenchyma at time zero of the present experiment, i.e., immediately after the administration of insulin, are shown in columns 1-4. Each value represents the mean of 16 animals ± standard error of the mean. Except for glycogen, the fractional volumes in the AV compartment, shown in column 2, are different from the corresponding values in the cytoplasm, shown in column 1. The asterisks in column 2 indicate that by Student's *t* test the differences are statistically highly significant ( $P < 0.001$ ). In columns 5 and 6, the kinetic parameters calculated from the decay of the AVs after insulin administration are shown. The values for the average lifetime of the AVs (column 5) were obtained by dividing the values for the half-life (Fig. 5) by  $\ln 2$ . The rates of autophagic breakdown were calculated according to the model shown in Fig. 9.

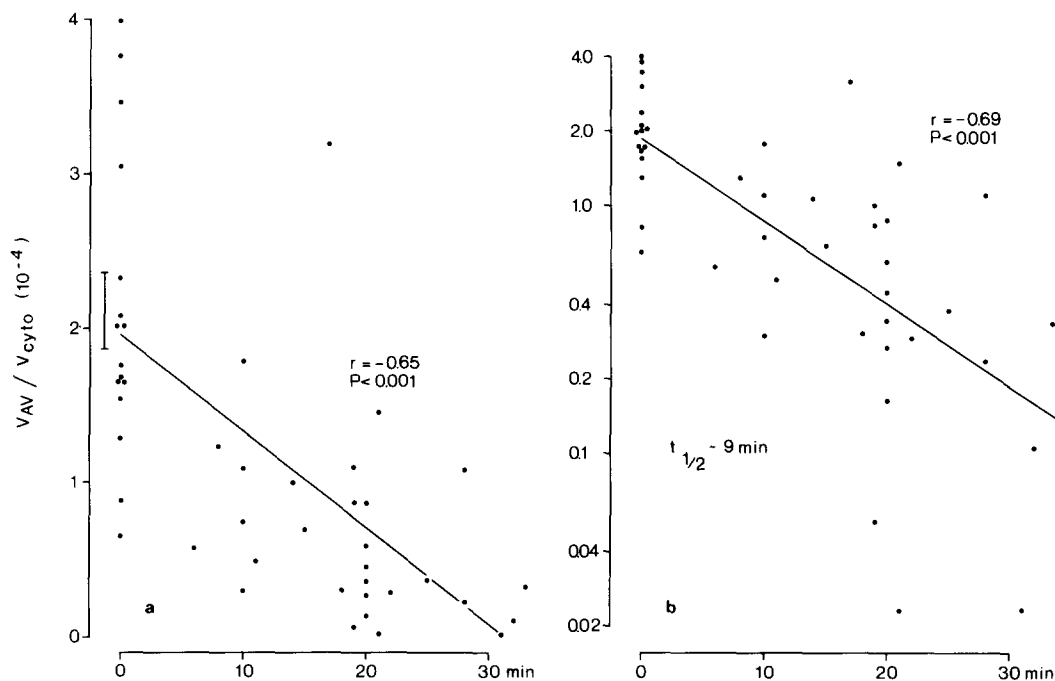
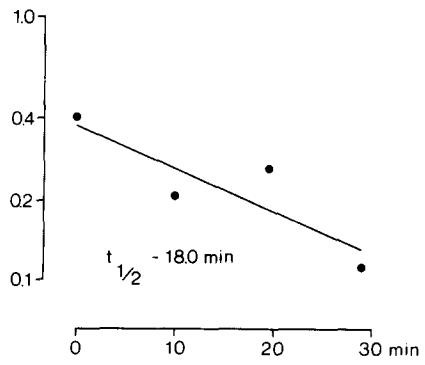
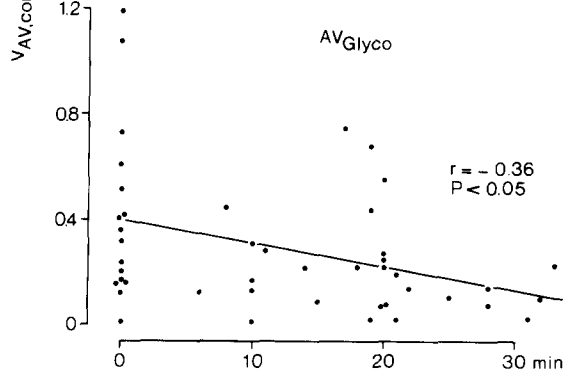
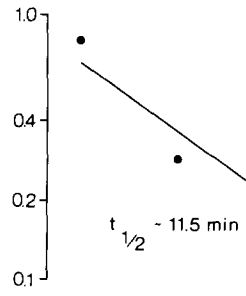
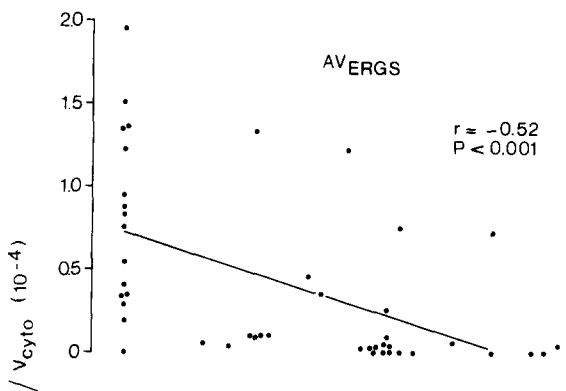
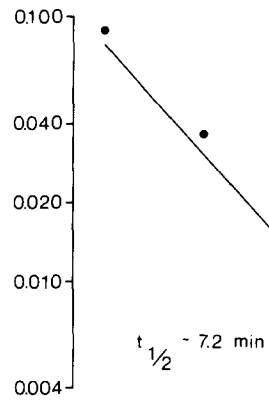
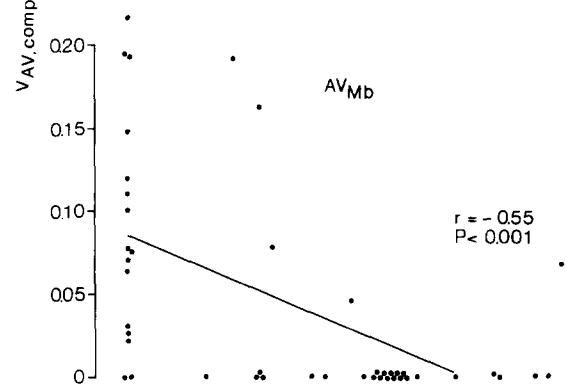
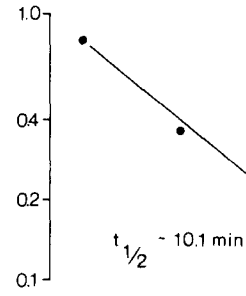
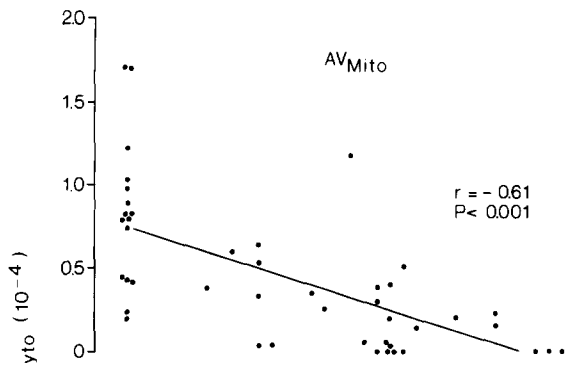


FIGURE 4 Fig. 4 shows the significant decrease in the fractional volume in cytoplasm of the AVs dependent on the time interval after insulin administration. (a) The values are plotted on a linear scale. The mean value at time zero ± the standard error of the mean is indicated by the vertical bar. The regression line does not seem to fit the decay in an optimal manner. (b) The single values are plotted on a semilogarithmic scale. The coefficient of correlation now is higher when compared to that of the linear plot. A "half-life" of the compartment of AVs of ~ 9 min can be obtained from the regression line.





cellular environment was lowered (32, 35). Stimulation would also be expected if the regulatory secretion of glucagon was of importance in the morphological effects observed. Nor are other hormones, such as catecholamines, released in response to the insulin-induced hypoglycemia (25), likely to account for the inhibition of the formation of AVs. The only finding of the present experiments which can be attributed to secondary mechanisms is the loss of glycogen (Fig. 2). This could be due to glucagon or, more probably, to the  $\alpha$ -adrenergic action of catecholamines (20).

The following additional biological facts suggest that insulin may act by preventing the formation of AVs. In the course of the diurnal cycle of autophagy (37, 38, 42), the volume fraction of AVs reaches a minimum at about the same time interval at which the plasma level of insulin reaches its peak (26)—usually in the late part of the light period, when the experimental rats, active in the dark, begin to assimilate food. The parallelism between an inhibition of autophagy and elevated insulin levels is further exemplified by refeeding rats after starvation, where a long-term insulin “overshoot” has been described (7, 51). At the same time, the volume fraction of AVs was greatly reduced (41). Finally, the increased fractional volume of AVs in liver cells of severely diabetic rats returns to normal values after the administration of insulin (1).

Although, in biochemical terms, one merely speaks of an inhibition of protein breakdown, a distinction should be made morphologically between the different steps which can be inhibited in the course of cellular autophagy. Insulin appears to inhibit the formation of AVs, recalling a similar prelysosomal action by cycloheximide (29, 45), another inhibitor of protein degradation (54). The other mechanism of inhibiting protein degradation is an impairment of intralysosomal digestion, with an intralysosomal accumulation of undigested material as its morphological consequence (9). Under the influence of a lysosomotropic agent such as ammonia, an inhibitor of protein degradation, the

number of AVs was unchanged, but the lysosomal apparatus was enlarged (49). Since the process of segregation did not appear to be affected (49), it seems questionable whether under physiological circumstances ammonia can exert a true regulatory function on protein catabolism (48).

#### “Morphological” vs. “Biochemical” Degradation of Cytoplasmic Constituents

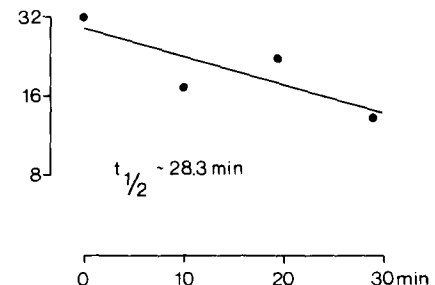
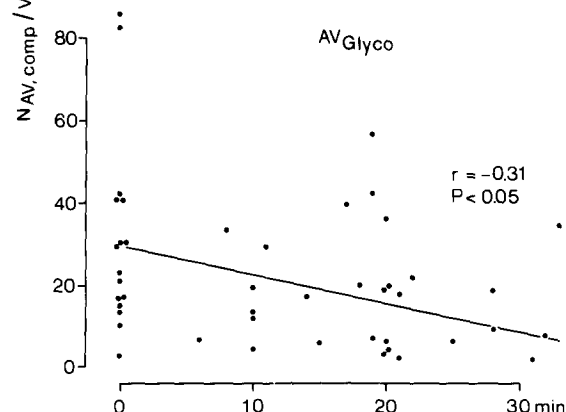
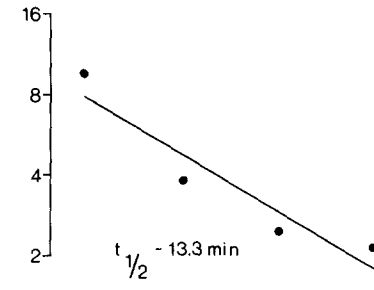
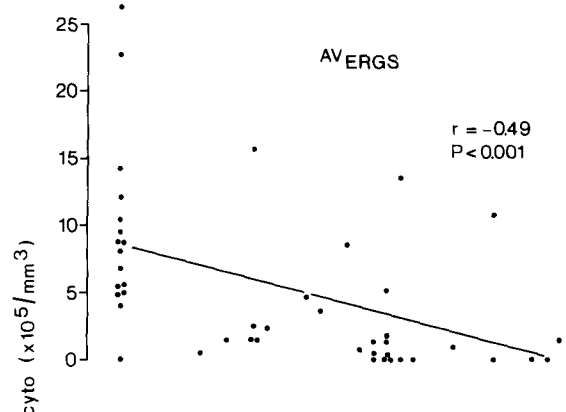
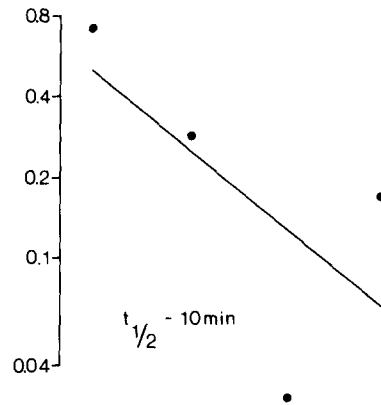
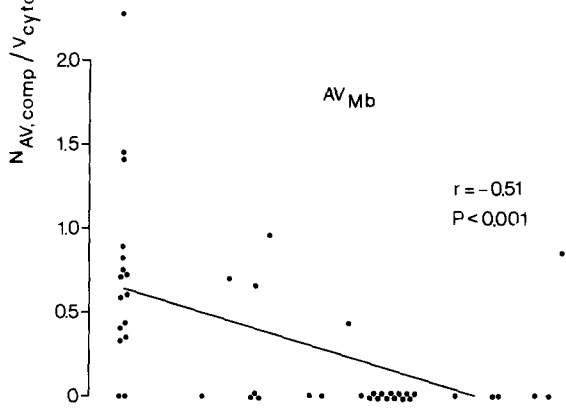
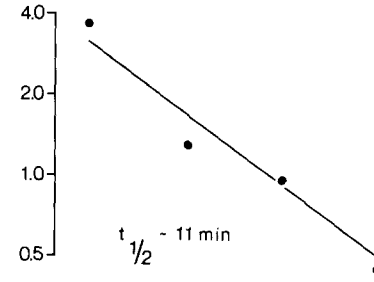
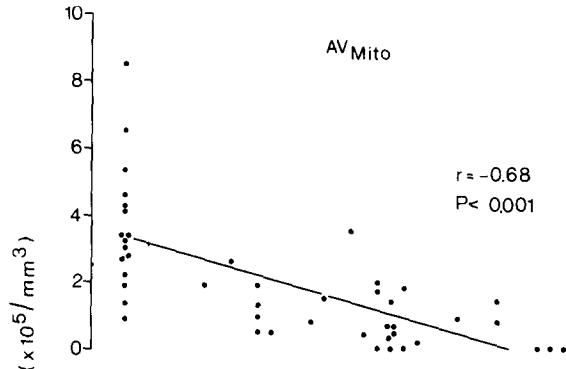
Biochemical turnover studies (for review, see reference 23) have not yet elucidated the exact mechanisms of intracellular degradation which may concern the organizational level of organelles (22) or primarily the level of molecules (46, 50).

The discovery of cellular autophagy revealed a well-defined morphological expression of the breakdown of portions of cytoplasm and of their degradation into small molecular compounds. Dynamic parameters of the extent to which cellular autophagy contributes to intracellular turnover have so far been estimated only by indirect calculations (42).

The decay of AVs found in the present experiments has offered a more direct way for a kinetic interpretation. The model of Fig. 8 was, therefore, modified to include the compartment “free cytoplasm”, i.e., that part of the whole cytoplasm that is not enclosed in the AVs (Fig. 9). The size of this compartment ( $V_{\text{cyto}}$ ) is assumed to depend on the rate at which cytoplasmic constituents are formed, and on the rate at which they are transferred to the compartment “AV” after having spent their average lifetime ( $t_{\text{cyto}}$ ). Assuming steady-state conditions, one can deduce from the completed model shown in Fig. 9 that the rate at which the cytoplasm is broken down by autophagy ( $1/t_{\text{cyto}}$ ) depends on the cytoplasmic volume fraction of the AVs ( $V_{\text{AV}}/V_{\text{cyto}}$ ) and on the average lifetime of an AV ( $t_{\text{AV}}$ ). This could also be used to calculate the rates of autophagic breakdown for the different cytoplasmic components ( $1/t_{\text{cyto, comp}}$ ), by replacing the unit volume of cytoplasm ( $V_{\text{cyto}}$ ) by the unit volume of the cyto-

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FIGURE 5 The fractional volumes in cytoplasm of the different types of AVs are shown in relation to the time intervals after the administration of insulin. The decay seen on the linear scale (left side) can be shown by regression analysis to be statistically significant for all types of AVs, but it appears to be slower for the AVs containing glycogen, compared to the other types. On the right side of the figure, the grouped values (see text) are plotted on semilogarithmic scales. From the regression lines the values for the “half-life” of the fractional volume of the different types of AVs are obtained.



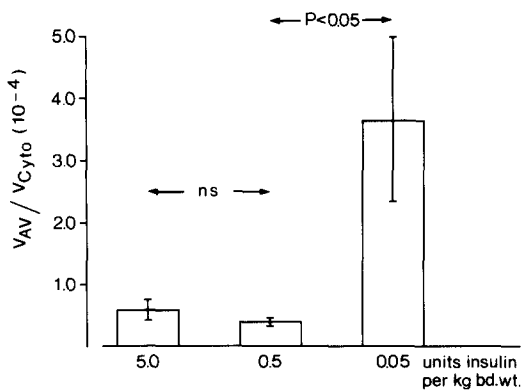


FIGURE 7 Fig. 7 shows the fractional volume of AVs 15 min after different doses of insulin. The values obtained were equally low after 5 or 0.5 U/kg of body weight, whereas after 0.05 U the values were found to be in the same order of magnitude as at time zero in the main experiment (Fig. 4).

plasmic component ( $V_{cyto, comp}$ ) and, correspondingly,  $V_{AV}$  by  $V_{AV, comp}$  and  $t_{AV}$  by  $t_{AV, comp}$  (Table I).

The finding that the decay of the volume fraction of the AV compartment follows approximately a semilogarithmic function seemed, at first, unexpected. Initially, it was assumed that the destruction of the AV content—the termination of its “life”—would depend on the “age” of an AV, i.e., on the time elapsed since the AV had been “segregated”. The decay of the volume fraction of the AVs would then be expected to be linear after the formation of new AVs had been blocked. The exponential decay found suggests, however, that destruction is independent of the “age” of an AV. The probability of undergoing destruction at any given moment would then be the same for all the cytoplasmic portions enclosed within AVs. This would be true if destruction depended largely on the change from the phagosomal to the lysosomal stage of an AV, and if the fusion responsible for this change took place at random, as proposed on the basis of the collision theory (12). Although there may be other possibil-

ities for the nonlinear decay of the volume fraction of the AVs, the values for  $t_{AV}$  could be estimated from the apparent half-life values by the equation:

$$t = t_{1/2} / \ln 2.$$

Except for the AVs containing glycogen, the values obtained in the present experiment (Table I, column 5) range from 10 to 16 min. These values are of the same order of magnitude as the delay, ~15 min, in the release of amino acids from intracellular proteins after the administration of such inhibitors of protein degradation as amino acids (55) or cycloheximide (28, 54). Presumably, if the formation of new AVs is blocked, a time interval of about the average lifetime of the AVs is required before the actual supply for intracellular degradation, i.e., the AV compartment, is exhausted.

The exponential decay of the volume fraction of the AVs does not reflect the exponential decay of proteins with labeled amino acids. The well-known first-order kinetics regularly found in biochemical turnover studies (23) have to be explained in relation to autophagy by the assumption

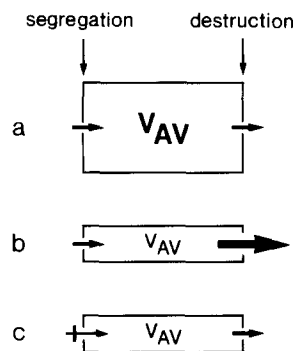
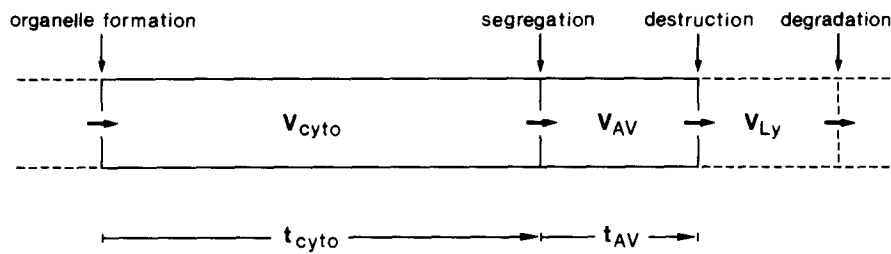


FIGURE 8 Fig. 8 shows the compartment of AVs as a dynamic balance between segregation leading to the formation of an AV and destruction which, as defined terminates the “life” of an AV (a). A decrease in volume of the compartment can be due either to an increased destruction (b) or to an inhibition of segregation (c).

FIGURE 6 The calculated numbers of the different types of AVs per unit volume of hepatocellular cytoplasm (=numerical density) are shown in relation to the time interval after insulin administration. The values were plotted in the same manner as in Fig. 5. The decay seen on the linear (left side and on the semilogarithmic scales (right side) is very similar to that of the fractional volume of the different types of AVs (Fig. 5).



$$V_{AV} / V_{cyto} = t_{AV} / t_{cyto}$$

$$1 / t_{cyto} = V_{AV} / V_{cyto} \times 1 / t_{AV}$$

FIGURE 9 Fig. 9 shows a model for the calculation of kinetic parameters of autophagic degradation. The volume of the compartment "free cytoplasm ( $V_{cyto}$ )" depends on the rate of formation of the cytoplasmic components and on the rate at which they are segregated after having spent their average lifetime ( $t_{cyto}$ ). The rate at which the cytoplasm is degraded by autophagy ( $1/t_{cyto}$ ) depends on the fractional volume of the AVs and on the lifetime of an AV ( $t_{AV}$ ).

that segregation, the step supposedly limiting the rate of autophagic degradation, takes place independent of the "age" of the organelles. Consistent with that assumption is the fact that there are no morphological (2, 19, 37) or cytochemical (2) changes in organelles to be found before or in the early stages after their segregation.

The concept of segregation in a random manner can, however, apply only to a defined population of organelles, e.g., mitochondria. For the different cytoplasmic components, segregation appears to involve a phenomenon of discrimination. This had already been concluded from the fact that the proportions of the different components found in the AV, that is, the "segregated fraction" (42) or the quotient  $V_{AV, comp} / V_{cyto, comp}$  of the present paper, show striking differences (6, 30, 42). These may, however, indicate, but by no means prove, the selectivity of autophagic segregation. The relatively high segregated fraction of microbodies (42), found again in the present study (Table I, column 4), could be explained also by assuming that these organelles are particularly resistant to destruction, in other words, that the lifetime of AVs containing microbodies is longer than that of AVs containing mitochondria. The finding that the lifetime of the AVs containing microbodies is even shorter than that of the other types of AVs (Table I, column 5) appears to invalidate this objection.

The rate at which microbodies are removed from the cytoplasm by autophagy has been calcu-

lated to be about twice that of the corresponding rate found for mitochondria (Table I, column 6). This ratio agrees well with biochemical observations showing that the half-life of peroxisomal proteins is about half that of mitochondrial proteins (15, 43). The statement that different turnover rates are easily compatible with degradation by autophagy invalidates one of the important arguments advanced against autophagy as an essential mechanism of intracellular turnover (46, 50).

The absolute rates of autophagic breakdown are considerably lower than the rates measured biochemically. Mitochondrial proteins, for instance, are degraded at a rate of ~10-15% per day (15) compared with a morphological degradation of ~5% found in this study (Table I, column 6). A similar disproportion applies also to the microbodies with a protein degradation of 20-30% per day (15, 43) and an estimated morphological degradation of ~10% per day (Table I, column 6). In view of these differences, some possible errors in the present calculations have to be considered. Because of the circadian rhythm of cellular autophagy (38, 40, 41), the only correct value of the volume fraction of AVs, which should be used for calculations on the proposed model (Fig. 9), would be the mean value of a circadian cycle (42). Depending on the time of day, the number of AVs in the liver is still increasing in the first part of the light period (37, 39), the time interval chosen here. Since the volume fraction of

AVs at time zero was found to be very similar to the mean value of a circadian cycle in kidney tubules (42), it was assumed, for the present calculation, that it was also close to the mean value of the circadian cycle in liver cells. Whether the volume fraction of the AVs has been over- or underestimated by such an assumption must remain an open question. As a factor contributing to an underestimation of the volume fraction of AVs and, consequently, of the rate of autophagic breakdown, the loss of AV profiles sectioned tangentially, however, has to be taken into consideration. An additional possible source of underestimating the rate of autophagic breakdown may reside in the fact that the values for the lifetime of AVs as calculated in the present experiment have to be regarded as maximal values, because the formation of AVs could a priori be inhibited only incompletely by insulin.

The factors mentioned here are unlikely to account for an underestimate of the rate of autophagic breakdown by a factor of two or three. Even if the morphological rates have to be corrected by a smaller factor, one still arrives at the conclusion that the contribution of autophagy to the overall degradation of the macromolecules of cellular organelles may be up to ~50%. This value would be in keeping with the observation that protein degradation is inhibited by ~50% in the regenerating liver after partial hepatectomy (47) where AVs are completely absent for at least 36 h (40).

It is beyond the scope of the present paper to discuss possible mechanisms other than autophagy, as defined in this and in other papers, by means of which cytoplasmic constituents could be degraded. It should be noted, however, that we have so far not found in our material convincing morphological evidence for "microautophagy" (10), which has been proposed as a possible mechanism to explain the different, and sometimes high, rates at which certain soluble proteins are degraded (5).

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*Note added in proof:* In a recent publication (Mortimore, G. E., and C. M. Schworer. 1977. Induction of autophagy by amino acid deprivation. *Nature (Lond.)*. **270**:174-176), it has been reported that autophagic vacuoles induced by amino acid-free liver perfusion regressed with a half-life of 8-9 min when amino acids were added to the perfusion medium. The half-life found in the present paper appears to be valid, therefore, not only for physiological, but also for stimulated autophagy.

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