Ciba Foundation Symposium

LYSOSOMES

Edited by A. V. S. de REUCK, M.Sc., D.I.C., A.R.C.S.
and
MARGARET P. CAMERON, M.A.

With 79 illustrations

1963

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LYSOSOMES

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Preface

IN ADDITION to symposia, which last for at least three days, the Ciba Foundation also organizes one-day study groups, which are generally held in honour of individual members of its Scientific Advisory Panel, on a subject of their own choosing. The possibility of holding such a study group in his honour had been discussed by the Director with Professor Christian de Duve in 1961, and naturally the topic was to have been "Lysosomes", but this proposal was delayed by a rebuilding programme undertaken at the Foundation, and by the time this was completed it was inescapable that the rapidly growing importance of the subject required a full symposium to do it justice.

It was with particular pleasure therefore that the Deputy Director of the Ciba Foundation undertook the arrangement of this meeting, with a strong Belgian contingent in the membership, to do honour both to Professor de Duve as a valued member of the Foundation's Advisory Panel, and to the importance of his work. The success of the symposium owes much to the warm and generous collaboration of Dr. Alex Novikoff, whose help and advice not only in devising the programme, but also in assisting the editors in preparing the proceedings for publication, have been invaluable.

Professor Danielli, who is among those recently lost by Great Britain to the United States, was recalled across the Atlantic to take the Chair at the meeting. Under his skilful guidance the proceedings here recorded will, it is hoped, fully demonstrate the present position of the lysosome concept in the words of many of those who have laboured to create it.

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R. Wattiaux	Laboratoire de Chimie Physiologique, Université de Louvain
R. Weber	Abteilung für Zellbiologie, Zoologisches Institut, Universität Bern

GENERAL PROPERTIES OF LYSOSOMES

THE LYSOSOME CONCEPT

CHRISTIAN DE DUVE

Laboratory of Physiological Chemistry,
University of Louvain, Belgium
and Rockefeller Institute, New York

THE BIOCHEMICAL CONCEPT

RAT-LIVER LYSOSOMES AS BASIS OF THE CONCEPT

THE name "lysosome" was proposed when it was found that, in rat liver, as many as five distinct acid hydrolases with widely differing specificities appeared to be associated together within a special group of cytoplasmic particles (de Duve *et al.*, 1955). As is obvious from the etymology of the word, this choice reflected the belief that the observed association is biologically meaningful. Subsequent work has greatly strengthened this belief, since rat-liver lysosomes are now known to contain at least twelve separate hydrolytic activities, some of which may be due to more than one enzyme species, all showing an acid pH optimum.

Rat-liver lysosomes are characterized by a second property which, though not explicit in their name, has always been considered of fundamental importance: the structure-linked latency of their enzymes. This property is a general one, extending to all the enzymes which have been identified so far as belonging to the particles. It has been attributed to the existence around the lysosomes of a membrane-like barrier of lipoprotein nature restricting the accessibility of their internal hydrolases to external substrates.

These two properties have been embodied in a schematic model of which several versions have already appeared (de Duve, 1958, 1959*a*; Novikoff, 1961; de Duve, Wattiaux and Wibo, 1962) and which is shown in its most recent form in Fig. 1. To some

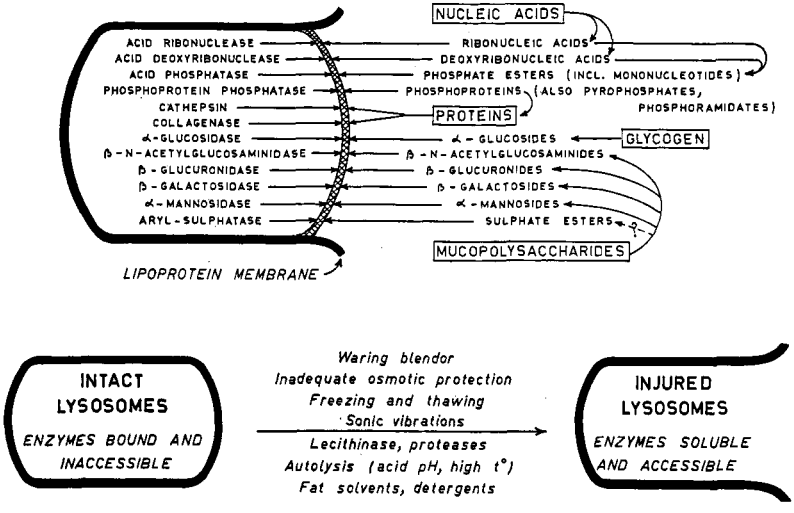

FIG. 1. Lysosomes as a biochemical concept. The model shown applies to rat liver lysosomes and is based mainly on investigations which have been summarized in preceding reviews (de Duve, 1959*a*; Novikoff, 1961) to which may be added the result of Frankland and Wynn (1962) on collagenase, those of Lejeune, Thinès-Sempoux and Hers (1963) on α-glucosidase, and those of Rose (1962) indicating that the lysosomal phosphoprotein phosphatase is also active on pyrophosphates and phosphoramidates.

extent, this model is either hypothetical or interpretative with respect to the biochemical data on which it rests. In addition, it includes details which may be peculiar to rat liver and are irrelevant with respect to its general applicability as a basis for a useful biochemical concept of lysosomes. Before attempting to formulate this concept, it is therefore necessary to examine first in the light of present knowledge whether the results obtained on rat

liver have been correctly interpreted and to what extent they are applicable to other biological materials.

BIOCHEMICAL CRITERIA OF IDENTIFICATION OF LYSOSOMES

Presence of acid hydrolases. The acid hydrolases which are found in hepatic lysosomes are widely distributed in nature, but their absolute as well as their relative concentrations may vary considerably from one tissue to another, and within the same tissue, according to its previous history.

It seems quite clear that, whichever their localization, these enzymes do not behave as a genetically linked group and are capable of considerable individual variations. For instance it has been found by Conchie, Findlay and Levvy (1959) that the tissues of C_3H mice, which are characterized by low levels of β-glucuronidase, contain several other glycosidases in normal amounts. On the other hand, according to the same authors, the preputial gland of both male and female rats or mice shows an extremely high activity of β-glucuronidase, though not of the other glycosidases. The complete absence of one or more enzymes of the group, though never of all, has occasionally been noted in some tissues.

In many cases, however, some kind of loose correlation appears to exist between the various acid hydrolases, in that tissues rich in one enzyme generally also contain high levels of many of the others. Such, for instance, is the case for liver, kidney, spleen, leucocytes and macrophages, which are good sources of most enzymes of the lysosome group.

Sedimentability of acid hydrolases. The first step in ascertaining the existence of lysosomes in a given tissue consists in finding out to what extent the acid hydrolases are sedimentable in homogenates of this tissue. In the experience of several workers and in our own, it is generally profitable to conduct such experiments on the assumption that lysosomes are present, and, accordingly, to investigate carefully the homogenizing procedure with, as

criterion, the degree of sedimentability of acid hydrolases. In many cases, the forces applied to disrupt the cells and, especially, the connective framework of the tissue, approach dangerously and even exceed the mechanical resistance of the particles, with the result that, unless great precautions are taken to minimize this damage, a large proportion of the acid hydrolases will be found in soluble form in the homogenate.

Liver is somewhat exceptional in this respect, and we have found that all grinding devices, if gentle enough, give about the same ratio of sedimentable to unsedimentable activity. However, excessive homogenization or the use of very high shearing forces, such as are produced in a Waring blendor turning at full speed, may cause considerable damage to the lysosomes, even in liver. In many other tissues the situation is more critical and even a small change in technique may alter significantly the yield in intact particles. For instance, it has been found by Greenbaum, Slater and Wang (1960) that homogenates from lactating rat mammary glands prepared with a Chaikoff-Emanuel homogenizer contained 70–80 per cent of the total activity of acid ribonuclease, β-glucuronidase and cathepsin in sedimentable form, whereas less than 30 per cent of these enzymes were sedimentable in homogenates prepared with a conventional homogenizer of the Potter-Elvehjem type. They found the time of homogenization with the latter device to have a great influence on the integrity of the particles. Spleen particles are also very sensitive to mechanical breakage, as noted by Conchie, Hay and Levvy (1961), who recommend the use of homogenizers with a clearance no smaller than 0·23 mm. We have made similar observations and found that distinctly better results are obtained on spleen with a Dounce than with a Potter-Elvehjem homogenizer.

Another variable which has rarely been investigated so far, but may be of great importance, is represented by the composition of the homogenizing medium. Most workers use unbuffered sucrose solutions of concentrations varying between 0·25 and

0·88 M. Such media generally give satisfactory results with mammalian tissues, but they are not necessarily optimal. They may even be totally inadequate in some cases and it is quite possible that, in some materials, lysosomes require a high ionic strength, a well-defined pH, specific ions or an osmotic protector of larger molecular weight than sucrose to maintain their integrity. This point deserves to be kept in mind when homogenates with largely soluble or accessible acid hydrolases are obtained under the most favourable grinding conditions.

Finally, it is important to point out that all acid hydrolases do not belong to lysosomes. In liver, part of the β-glucuronidase activity is clearly non-lysosomal and associated with microsomes (de Duve *et al.*, 1955; Paigen, 1961), whereas the soluble fraction contains a phosphoprotein phosphatase and an acid phosphatase which are different from the lysosomal enzymes. The former can be distinguished by its insensitivity to inhibition by molybdate (Paigen and Griffiths, 1959), and the latter by its high activity on *p*-nitrophenyl phosphate, susceptibility to alloxan and lack of sensitivity to tartrate inhibition (Neil and Horner, 1962).

When the literature is examined with the above consideration in mind, it is found that there is at present no well-authenticated example of a biological material, at least of animal origin, which does not yield a homogenate in which acid hydrolases are partly sedimentable. On the contrary, many preparations have been described, which, when suitably homogenized, contain between 50 and 90 per cent of these enzymes in sedimentable form. Whether the hydrolases are entirely particle-bound in the intact cells cannot be decided at the present time, but it is likely that, in many cases, the proportion of bound activity is higher in the cells than in the homogenate. It has also been observed that great sensitivity to the homogenization procedure goes together, as in spleen, with high levels of unsedimentable activity. Consequently, it is not unreasonable to suppose that the acid hydrolases

are largely or completely associated with cytoplasmic particles in most normal tissues.

Specificity and physical properties of particles containing acid hydrolases. One of the greatest difficulties encountered with liver has been the demonstration that the particles containing the acid hydrolases form a distinct group, different from mitochondria, microsomes and other intracellular components. It is only recently that fractions of relatively high purity have been isolated, though still with a very low yield.

However, the characterization of lysosomes as a separate group of particles did not have to await their quantitative purification, and a great deal of information has been obtained by considering simply the shape of the distribution curves of the particle-bound acid hydrolases in a number of centrifugal systems. These experiments, though resting on the assumption that the distribution of a bound enzyme reflects fairly faithfully that of its host-particles, have acquired greater demonstrative power from the fact that all the assayed hydrolases behave in a similar fashion whichever the system investigated. It has even been possible, simply by analysing the results of such experiments in the framework of an elementary mathematical theory, to derive information concerning the magnitude and statistical distribution of a number of physical parameters of the particles, such as their size, density, hydration ratio, accessibility to sucrose and osmotic properties (Beaufay and Berthet, 1963). When compared against similar quantities, calculated in an analogous manner for enzymes present in other cytoplasmic particles, the results of such computations appear clearly as characteristic of a separate population.

Such detailed experiments have not been carried out on other preparations, but a number of tissues are already known in which more or less marked differences in the centrifugal behaviour of the acid hydrolases with respect to other particulate enzymes have been observed. In the case of kidney, especially after injection of egg-white, which causes a considerable enlargement of

the particles (Straus, 1954, 1956), of neutrophil polymorphonuclear leucocytes (Cohn and Hirsch, 1960*a*), of thymus after X-irradiation (Rahman, 1962*b*), characterization has been pursued right up to a considerable purification of the lysosomes. In other materials, such as brain (Beaufay, Berleur and Doyen, 1957; Whittaker, 1959) pituitary (La Bella and Brown, 1958), pancreas (Van Lancker and Holtzer, 1959) and HeLa cells (Watiaux, 1962), present data are limited to differences in distribution in one or more centrifugal systems. To the author's knowledge, adequate results are not available for other tissue or cell types, but it should at least be mentioned that except for the presence of acid phosphatase in a purified Golgi fraction from rat epididymis (Kuff and Dalton, 1959), there is as yet no case on record in which, for instance, the acid hydrolases have been shown to be constituents of mitochondria or of other known cytoplasmic particles. Also, their levels in isolated nuclear preparations have always been found to be very low. Finally, the results of cytochemical staining experiments have provided valuable additional information, in that they have invariably revealed on a wide variety of material a distribution pattern for acid phosphatase different from that of mitochondria (see, for instance: Novikoff, 1961).

On the whole, present evidence lends strong support to the hypothesis that the acid hydrolases, which, in liver, have been shown to belong to lysosomes, are also located in a special group of particles in most, if not all, tissues in which they are present. When investigating a new type of material, it seems at least advisable to start on this assumption and to act accordingly, if necessary by varying media and fractionation methods until evidence of separability is obtained.

In conducting or discussing such experiments, it is important to keep in mind that the physical properties, such as size, density, hydration ratio, permeability, osmotic behaviour and sedimentation coefficient, which determine the manner in which lysosomes become distributed in a given centrifugal system, may and do vary

to a considerable extent from one tissue to another, and, within the same tissue, according to the circumstances to which the animal has been exposed. Also, their dispersion within the particle population is often very large. In fact, it seems to be a general property of lysosomes that they are both heterogeneous and variable in their physical characteristics. Therefore, the quantitative information available on liver lysosomes can in no way serve as guide for the separation of similar particles from other materials, and the conditions set for the isolation of a lysosome-rich fraction from rat liver are not necessarily optimal for other tissues. The guiding criterion should, of course, be the behaviour of the enzyme activities themselves and not a preconceived notion of the physical properties of their host-particles. This point, which is obvious, is only mentioned here because some authors have investigated the presence of lysosomes simply by applying the centrifugal scheme of de Duve and co-workers (1955) and have relabelled the latter workers' L fraction (termed such for light-mitochondrial) as lysosomal, even when their experimental results belied a selective concentration of lysosomes in it.

Structure-linked latency of acid hydrolases. In the model of Fig. 1, the structure-linked latency of the enzymes in rat-liver lysosomes has been attributed to the lack of permeability of a surrounding membrane both to the internal enzymes and to external substrates. This sac-like representation of the lysosomes has been deduced largely from early experiments showing that the acid phosphatase-containing particles behave as osmotic systems (Berthet *et al.*, 1951). It was concluded that "any representation of these granules must at least include the essential features of an osmotic system: a limiting barrier capable of depressing to a greater or lesser extent the diffusion of solutes present within or outside the granule; an interior medium containing a number of osmotically active components, to which the barrier is not permeable. The simplest model obeying this description is that of a sac, consisting of

fluid enclosed in a semi-permeable membrane". Subsequent experiments reinforced this representation, by showing that various lysosomal enzymes were released in an essentially parallel fashion, whichever the agent used. These results appeared to argue against an actual binding of the enzymes, since one would expect the strength of the bonds to vary for different proteins. For similar reasons, the possibility that the lack of activity of the hydrolases may be a direct consequence of their binding was discarded as improbable and it was always implicitly assumed that the hydrolases are present in diffusible form within the lysosomes and are therefore capable of acting on internal substrates provided the right pH conditions are realized. In particular, the hypothesis has been put forward that the particles' own cathepsin may be responsible for their autolytic breakdown, both *in vitro* and *in vivo*, when the ambient pH is lowered (Beaufay and de Duve, 1959; de Duve and Beaufay, 1959).

This view has recently been challenged by Koenig (1962), who proposes, as an alternative, that lysosomes may be solid complexes in which the various enzymes are retained by ionic conjugation with acidic glycolipids and that this binding is responsible for their latency in intact particles. In support of this hypothesis, Koenig mentions the fact that lysosomes do not look like bags in the electron microscope, as well as a number of experimental results obtained mostly on brain lysosomes, but also on liver and kidney particles, and indicating that the enzymes can be released by relatively low concentrations of various organic and inorganic cations (Koenig and Jibril, 1963).

As mentioned in the sentence quoted above, the bag model was adopted as the simplest one compatible with the experimental results; it was never claimed to be the only one. For instance, one could equally well imagine the particles as clusters of miniature bags, and, at the limit, as a sponge-like structure, provided it possessed the observed osmotic properties. Similarly, the picture of a bag does not necessarily imply a structureless interior,

but simply requires that the hydrolases have enough freedom of movement to leave the particles once the limiting membrane has been injured. Such models are compatible with the known morphological aspect of lysosomes. They are also compatible with the results described by Koenig, if it is assumed that the ionic bonds which appear to be responsible for the integrity of the particles link together the components of the membrane or matrix of the lysosomes, without necessarily involving the enzymes themselves. This distinction is of some importance, since Koenig's model, by ascribing the latency of the enzymes to their binding, seems to imply that the enzymes may be intrinsically inert as long as they remain associated with the particles. This question is relevant to some of the biological implications of the lysosome concept.

Except for this point, we feel that the actual mechanism responsible for the phenomenon of structure-linked latency is of less importance with respect to the lysosome concept than the phenomenon itself. Here again, when looking for the latter, it is necessary to approach the problem with an open mind and to give up only when all experimental possibilities have been exhausted. It must be remembered that the conditions under which acid hydrolases are assayed are often by themselves highly injurious to the particles and that the demonstration of structure-linked latency may require special precautions. It must also be kept in mind that the sensitivity of lysosomes to disrupting treatments may vary greatly from one material to another.

In our own work on liver, we have repeatedly stressed the necessity of running free activity assays in the presence of an adequate osmotic protector and at a pH no lower than 5 and for a time no longer than 10 min. when the enzymes are determined at 37°C. In our opinion, lack of observance of some of these precautions may be largely responsible for the finding by Walker (1952) that the latency of β-glucuronidase in mouse-liver particles can be overcome by means of high substrate concentrations in

one-hour assays, for the results of Weber and Niehus (1961), who failed to demonstrate an activation of acid phosphatase by Triton X-100 in homogenates of tadpole tails in three-hour assays, and for those of Greenbaum, Slater and Wang (1960), who observed a large difference between the latency of cathepsin (measured at pH 3·7) and that of acid ribonuclease and β-glucuronidase (measured at pH 5) in homogenates of lactating rat mammary gland.

At the present time, evidence of structure-linked latency has been obtained on numerous tissue preparations and there are already solid grounds for the belief that this property is a fairly general one. It has also been found that latency may be very difficult to demonstrate in some cases and that lysosomes may differ greatly in this respect from those from rat liver, especially in animals other than mammals. For instance, in homogenates of the tail of *Xenopus* larvae, Mr. Eeckhout in our laboratory has found latent acid phosphatase to be very sensitive to Triton X-100 and to temperature, being best demonstrated at 0°C, but to be resistant to prolonged homogenization, freezing and thawing or exposure to hypotonic media.

Association of acid hydrolases within the same particles. One question which has frequently been raised and has not yet been answered in an unequivocal manner is whether all lysosomal enzymes are present together in all particles or whether there exist several kinds of lysosomes, differing qualitatively in their enzymic equipment. It has been stressed that rat-liver lysosomes do not behave as enzymically homogeneous particles and that practically each of their constituent enzymes shows a slightly different distribution pattern when the particles are subfractionated in a system of high resolution. While this fact complicates the biochemical assay of lysosomes and invalidates to some extent the postulate of biochemical homogeneity on which much of the work on these particles was initially based, it does by its very complexity weaken its own value as an argument against the

unicity of lysosomes. For, if we start by assuming that individual particles differing slightly in their relative content of two or more enzymes are unlikely to exist, then we are led to conclude that the liver cells contain almost as many kinds of lysosomes as they contain lysosomal hydrolases. This possibility, although not incompatible with present biochemical data, is not easily reconciled with the cytochemical observations indicating that a considerable proportion of the particles which have been identified biochemically as lysosomes stain positively for acid phosphatase. Unless this enzyme occupies a much larger number of lysosomes than the other dozen or so already localized in these particles, the presence of more than one enzyme species in each particle must be accepted and, with it, the existence of a certain degree of enzymic heterogeneity within the particle population.

It is true that a greater degree of heterogeneity has been found in some tissues, especially in spleen, in which the partition of various acid hydrolases of the lysosome group between the soluble and particulate fractions of the homogenate has been found to vary within relatively large limits from one enzyme to the other (Conchie, Hay and Levvy, 1961; Roth, Bukovsky and Eichel, 1962). On the basis of such and other results, Conchie and Levvy (1963) have expressed serious misgivings as to the existence of lysosomes as a separate group of cytoplasmic particles. In our opinion, such an extreme view is untenable in the face of all the accumulated evidence to the contrary. It is our feeling that the cellular heterogeneity of most tissues, and, possibly, the presence in some cells of more than one enzyme species with different intracellular locations, might already go far in explaining the observed heterogeneity of lysosomes. It has indeed been found by Wattiaux and co-workers (1956) that lysosomes from Kupffer cells contain more acid nucleases and cathepsin, and less acid phosphatase and β-glucuronidase, than those from parenchymal cells. Therefore, even if the lysosomes from both cell types were enzymically homogeneous, their mixture would already appear

heterogeneous if the centrifugal properties of the two populations are not superimposable.

However, it must be recognized that the association of the acid hydrolases within single individual particles, although highly probable, has not been established with certainty. In view of its crucial importance with respect to the functional implications of the lysosome concept, it is to be hoped that techniques will be found to settle this question in an unequivocal fashion. One must also keep in mind the possibility that in some specialized tissues, one or more of the enzymes usually found in lysosomes may be associated partly or totally with a special kind of particle unrelated to lysosomes.

FORMULATION OF A GENERAL BIOCHEMICAL CONCEPT OF LYSOSOMES

It will be clear from the preceding discussion that it suffices to strip the definition of hepatic lysosomes from all incidental details such as size and other physical characters, osmotic properties, centrifugal behaviour, mechanism of structure-linked latency and sensitivity to individual disrupting treatments, to arrive at a concept applicable to all investigated materials. The elements which are left are those which were considered essential in the earliest formulation of the concept: the association, within a special group of cytoplasmic particles, of a number of soluble acid hydrolases of widely differing specificity, in such a manner as to restrict to a considerable extent the accessibility of these enzymes to surrounding substrates, both the association and the latency of the enzymes being dependent on the structural integrity of the particles. Implicit in the concept are the assumptions that the enzymes coexist within single individual particles and that they are present in them in fully active form. Though not actually demonstrated by the biochemical data, these hypotheses are strongly supported by our present morphological and functional knowledge of lysosomes.

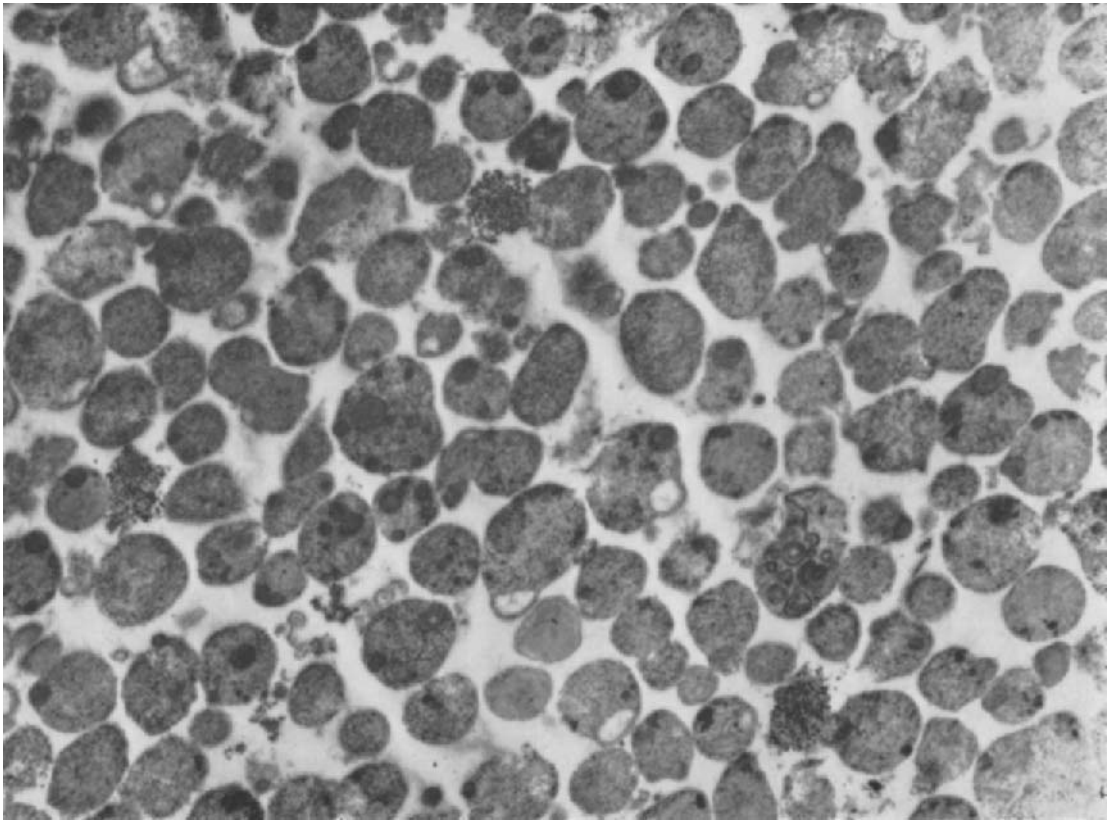

FIG. 2. Electron micrograph of a purified preparation of rat-liver lysosomes isolated by density equilibration in a gradient of glycogen in 0·5 M-sucrose. In agreement with biochemical measurements, which revealed some cytochrome oxidase but negligible urate oxidase activity, other parts of the pellet showed evidence of mitochondrial contamination but no microbodies. (Lead-stained. × 17,375.)

THE MORPHOLOGICAL CONCEPT

It is obvious that the biochemical concept of lysosomes implies a morphological one. Thus, the formulation of the lysosome theory immediately raised the problem of the identification of the postulated particles, and it was indeed very gratifying to find that mitochondrial preparations enriched in lysosomes did contain a high proportion of granules obviously different from mitochondria (Novikoff, Beaufay and de Duve, 1956). As we now know, the tentative identification of hepatic lysosomes with the pericanalicular dense bodies which was made at that time turned out to be correct, but more through luck than otherwise, since the preparations examined were very impure and must have contained a second type of rare component, which fortunately was not detected, the particles containing urate oxidase, catalase and D-amino acid oxidase (de Duve *et al.*, 1960). These have now been isolated in relatively pure form by means of density gradient centrifugation and identified with the so-called "microbodies" (Baudhuin and Beaufay, 1963). Lysosomes, largely freed from mitochondria and microbodies by similar techniques, showed the expected structure of peribiliary dense bodies (Fig. 2).

So far, correlated biochemical and morphological studies of this type have been carried out on very few tissues. Evidence has been reported suggesting the lysosomal nature of protein reabsorption droplets from kidney (Straus, 1954, 1956), of neutrophil granules from polymorphonuclear leucocytes (Cohn and Hirsch, 1960*a*) and of "dense bodies" from thymus (Rahman, 1962*b*), but in no case could the degree of purity achieved be considered sufficient in itself for unequivocal morphological identification. This point is stressed, not to cast doubt on the proposed identifications which, at least in kidney and leucocytes, are supported by considerable additional evidence, but to underline the fact that lysosomes, though defined biochemically, may be very difficult to recognize morphologically in various kinds of cells by following a purely biochemical approach.