COMBINED BIOCHEMICAL AND MORPHOLOGICAL STUDY OF PARTICULATE FRACTIONS FROM RAT LIVER

Analysis of Preparations Enriched in Lysosomes or in Particles Containing Urate Oxidase, D-Amino Acid Oxidase, and Catalase

PIERRE BAUDHUIN, M.D., HENRI BEAUFAY, M.D., and CHRISTIAN DE DUVE, M.D.

From the Laboratory of Physiological Chemistry, University of Louvain, Belgium

ABSTRACT

Six particulate preparations isolated from rat liver under different experimental conditions were analyzed biochemically and examined in the electron microscope. The results confirm the lysosomal nature of the pericanalicular dense bodies and demonstrate that the microbodies are the bearers of urate oxidase, catalase, and D-amino acid oxidase. Catalase, representing a major component of the particles, and D-amino acid oxidase appear to be associated with the structureless "sap" of the particles, urate oxidase with their crystalloid core or with their outer membrane.

INTRODUCTION

Biochemical studies carried out in this laboratory over the past ten years have demonstrated the presence, in conventional mitochondrial fractions from rat liver, of two distinct populations of cytoplasmic particles, in addition to the true mitochondria which form the main component of such preparations: (*a*) the lysosomes, characterized by a variety of acid hydrolases and apparently devoid of any other known enzyme so far searched for; (*b*) a second group of bodies, as yet unnamed, containing urate oxidase, catalase, and D-amino acid oxidase. The experimental evidence supporting the existence of these two types of particles as separate entities, different both from each other and from the mitochondria, has recently been reviewed in detail (11).

In 1956, Novikoff *et al.* (20) described the results of some preliminary electron microscope examinations of fractions that had been enriched in lysosomes by differential centrifugation. They noted that these preparations contained, in addition to numerous mitochondria, a relatively large number of particles of a different type, clearly identifiable as the pericanalicular dense bodies described shortly before by Rouiller (25). They suggested tentatively that these bodies might correspond to the lysosomes identified biochemically. This suggestion has since been strengthened considerably by cytochemical staining studies which have shown clearly that at least one of the lysosomal enzymes, acid phosphatase, is virtually restricted in liver sections to the pericanalicular dense bodies of the

parenchymatous cells and to bodies of somewhat similar appearance occurring in the Kupffer cells (9, 13, 15, 28). It is worth mentioning that the preparations examined by Novikoff *et al.* (20) did in fact also contain the other group of rare particles, concentrated about equally with the lysosomes. It may be considered fortunate for the immediate subsequent developments that their search led to the identification of only one morphological entity, which turned out to be the right one. But the fact itself should be remembered as an illustration of the hazards that may attend electron microscope studies of inadequately purified fractions.

More recently, following the finding that particulate preparations practically free of mitochondria, but still containing the other two types of particles unresolved, can be separated by centrifuging in a sucrose-D_2O density gradient, new electron microscope examinations were made on such sediments in this laboratory. The presence of numerous pericanalicular dense bodies was noted, but, in addition, the preparations were found to contain many representatives of another group of particles, corresponding to the "microbodies" first described by Rouiller and Bernhard (26) and Gänsler and Rouiller (14). In view of the cytochemical evidence associating the lysosomes with the pericanalicular dense bodies, it was suggested in preliminary publications that the microbodies may represent the particles containing urate oxidase, catalase, and D-amino acid oxidase (7, 10). However, the morphological identification of the new particles obviously had to remain tentative until adequate separation procedures became available, especially since staining methods applicable to any of their three known enzymes have not yet been worked out.

Significant progress in the required direction has been achieved, thanks to the observation that lysosome-rich sediments, practically free of urate oxidase, catalase, and D-amino acid oxidase, though not of mitochondria, can be separated in glycogen gradients with 0.5 M sucrose as solvent (8), and to the finding that a preliminary treatment of the animals with Triton WR-1339 alters the density of lysosomes to such an extent as to eliminate them completely from the urate oxidase–rich sediments isolated by density equilibration in aqueous sucrose gradients (31). The present paper describes the appearance of such fractions in the electron microscope, together with the results obtained with other, less selective separation techniques. As already reported in a preliminary communication (3), the new data confirm the lysosomal nature of the pericanalicular dense bodies and demonstrate that the particles recognized biochemically by their content of urate oxidase, catalase, and D-amino acid oxidase are identical with the bodies described morphologically under the name "microbodies." They also throw some light on the structural and biochemical alterations which these bodies may suffer as a result of experimental manipulations.

METHODS

Biochemical

Female rats of a Wistar strain were used in all experiments, either as such or after injection of Triton WR-1339 (Rohm and Haas Company, Philadelphia, Pennsylvania) or of dextran (Dextran 150 Pharmacia, Stockholm, Sweden). A large granule fraction (fractions M + L[1] or fraction L of de Duve *et al.*, 12) was first isolated from the liver by differential centrifugation and, in most cases, further subfractionated by density equilibration in a density gradient. These procedures were carried out essentially as described by Beaufay *et al.* (6, 8) with a few minor modifications. In order to minimize contamination of the particulate preparations used as starting material, the nuclear fraction was separated at 14,000 g min. (instead of 10,000 and 6000) and the particles themselves were washed twice, care being taken to remove the fluffy layer as completely as possible.

When the subsequent density gradient step served to isolate a sediment (preparations 1 to 4), 0.6 ml of 64 per cent (w/w) aqueous sucrose was introduced at the bottom of the tube below the gradient to act as a cushion for the collection of the sediment. The density limits of the gradient were decided on the basis of previous results, the subfraction reserved for combined biochemical and morphological analysis being made up essentially of the material of density equal to or higher than the upper density limit of the gradient. It was collected by cutting a layer 3 mm thick cen-

[1] Abbreviations used in this paper: M, L, P, S, heavy mitochondrial, light mitochondrial, microsomal, and supernatant fractions of de Duve *et al.* (12); Prot., protein; A.Pase, acid phosphatase; A.DNase, acid deoxyribonuclease; Ur.Ox., urate oxidase; Catal., catalase; DAA.Ox., D-amino acid oxidase; Cyt.Ox., cytochrome oxidase; G-6-Pase, glucose 6-phosphatase; E, cytoplasmic extract; R.S.A,, relative specific activity: $\frac{\text{Percentage of total recovered activity}}{\text{Percentage of total recovered protein}}$.

TABLE I

Biochemical Data on Preparation 1

Washed M + L fraction equilibrated for 2½ hours at 39,000 RPM in gradient of glycogen extending initially in a linear fashion from 0 to 30 gm per 100 gm of water, with 0.5 M sucrose as solvent (gradient Gh(10) of Beaufay *et al.* (8)).

Purification step	Fraction	Prot.	A.Pase	A.DNase	Ur.Ox.	Catal.
Fractionation of cytoplasmic extract (E)	% of E: M + L fraction	27	72	105	61	51
	P + S supernatant	71	23	11.5	24.4	25
	Recovery	98	95	116.5	85.4	76
	R.S.A. of M + L fraction	1.00	2.75	3.26	2.6	2.43
Subfractionation of M + L fraction in density gradient Gh(10)	% of M + L: Sediment (prep.1)	4.3	25.0	19.6	7.0	7.6
	Other subfractions	96	73	56	88	124
	Recovery	100.3	98	75.6	95	131.6
	R.S.A. of prep. 1	1.00	16.2	19.2	4.4	3.6
	% of E: Final yield in prep. 1	1.16	18.0	20.6	4.3	3.9
	Over-all recovery	98	94	91	82	92

tered on the interface between the cushion and the gradient.

As a rule, two or three identical tubes were prepared and spun simultaneously; the three subfractions were combined and used partly for biochemical determination and partly for morphological examination. Samples of the cytoplasmic extract, of the complete particulate fraction, and of the remaining subfractions were also kept for biochemical analysis, to allow estimates of recovery and of purity. All assays were performed according to Beaufay *et al.* (6, 8).

Morphological

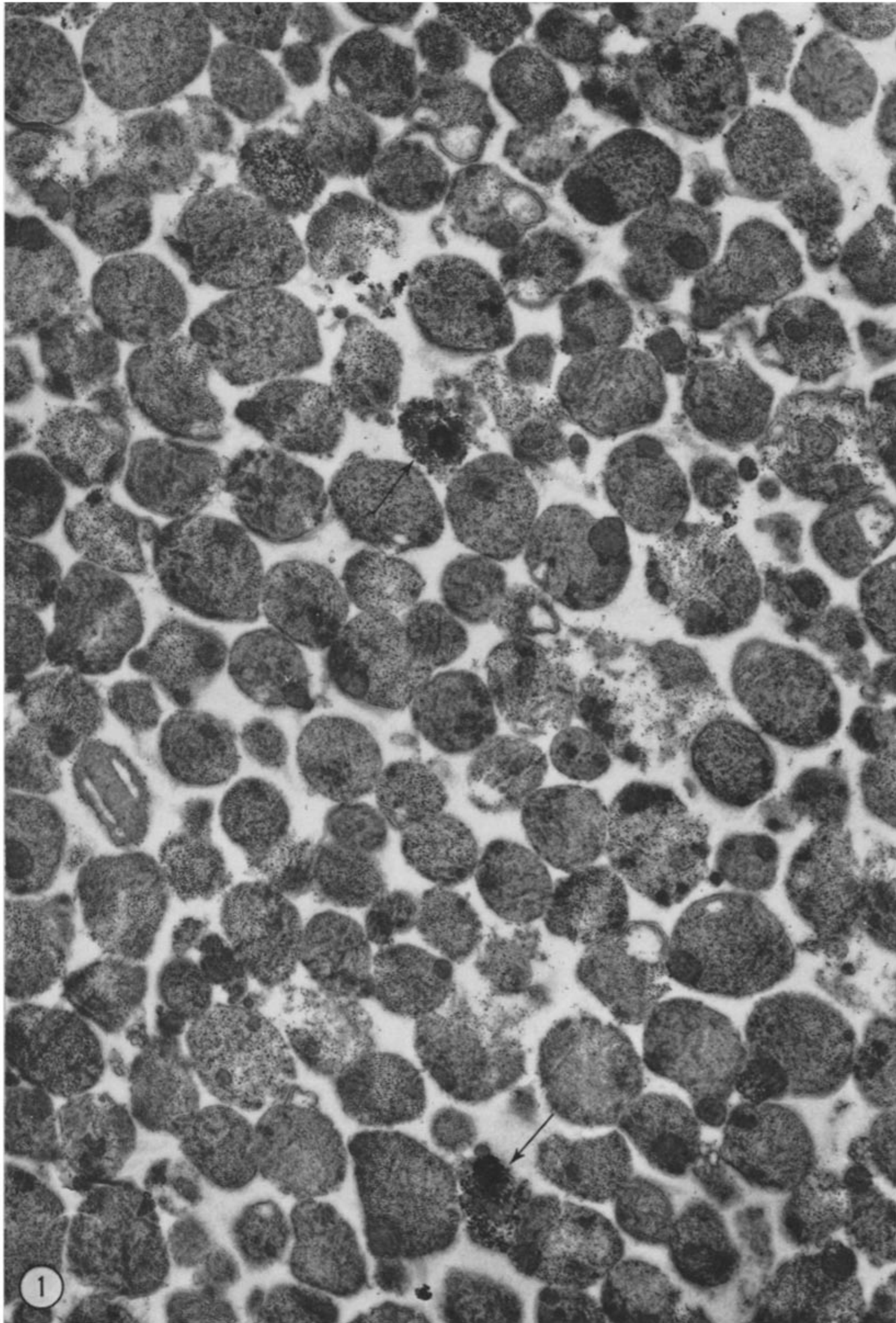
The samples of particle suspension reserved for morphological examination were mixed with an equal volume of a freshly prepared ice cold fixative solution, containing 2 per cent osmium tetroxide, 0.055 M Veronal–acetic acid buffer, pH 7.4 (preparations 1 to 4) or 0.1 M phosphate buffer, pH 7.6 (preparations 5 and 6), and either sucrose at a concentration equal to that of the suspension or 0.8 M sucrose when the sucrose concentration in the suspension exceeded this value. The latter procedure was chosen as a compromise between the desire to avoid osmotic shocks and the necessity for diluting the sucrose to allow the subsequent collection of the particles by centrifugation.

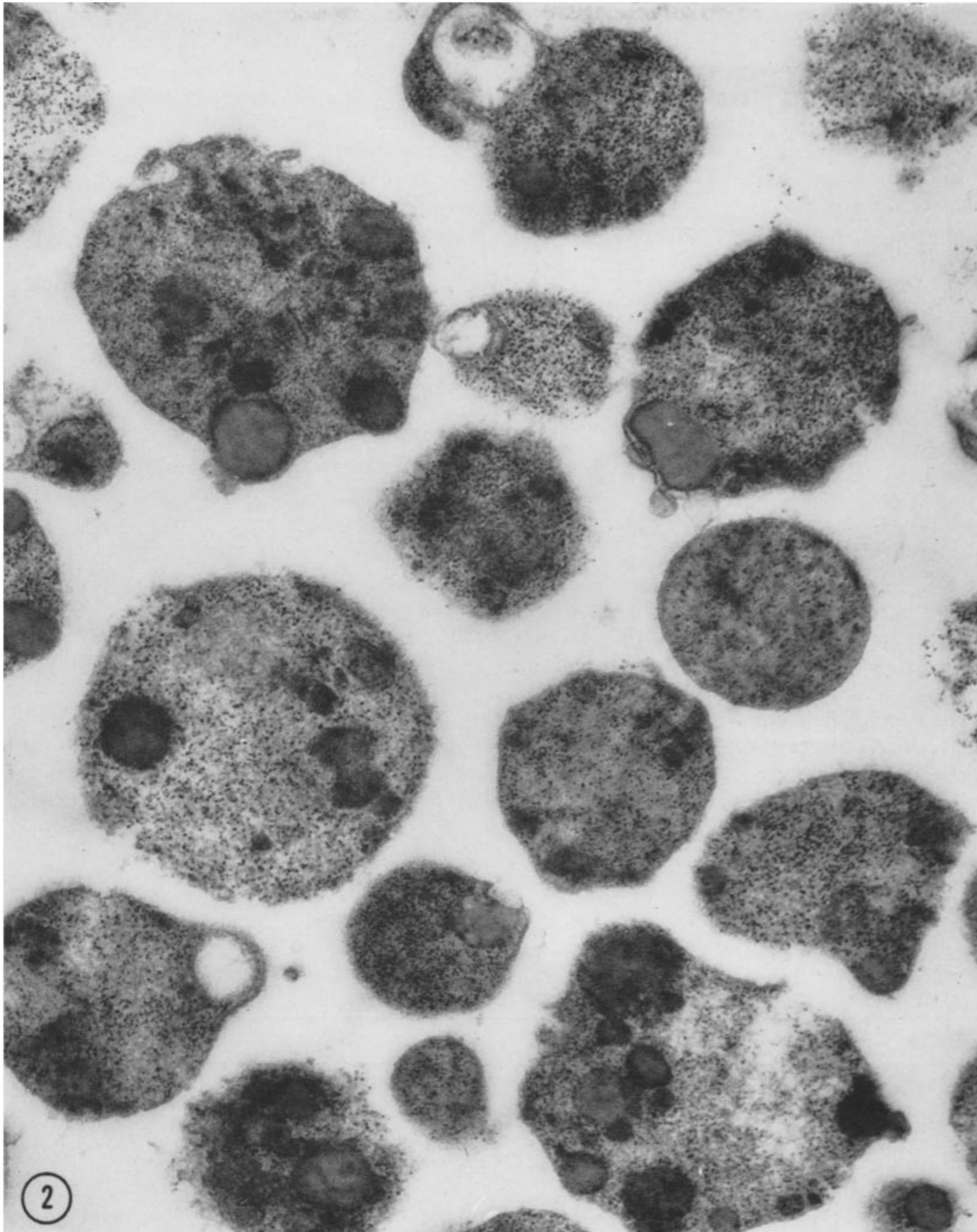
The fixed particles were packed by centrifuging for 30 minutes at top speed in the SW-39 rotor of the Spinco Model L preparative ultracentrifuge (approximately 3×10^6 *g* min.). For this purpose, special tubes were constructed by polymerizing Araldite (Ciba Company, Basel, Switzerland) in the manner described by Mercer and Birbeck (16) around a stainless steel shaft 7.5 mm in diameter and sharpened at the end to an 11° cone. The polymerized mass was then machined around the shaft to fit the rotor buckets and the shaft was removed. Centrifugation caused the particles to accumulate at the bottom of the tube as a small conical pellet which could easily be detached *in toto* by sectioning the bottom of the tube flush with the tip of the pellet and sucking a little suspension fluid through the tiny hole thus made in the tube. The pellet was then dehydrated and embedded in methacrylate (preparations 1 to 4) or in Epon (preparations 5 and 6) in the usual manner and sectioned with a Porter-Blum or LKB microtome. The conical shape of the pellet made it possible to orient the specimen with respect to the centrifugal field in which it was packed and to explore it at different levels. The sections were deposited on naked grids, stained with lead hydroxide (29) or citrate (24), eventually coated with a carbon film (preparations 1 to 4) and examined in a Siemens Elmiskop I under an accelerating voltage of 60 kv.

RESULTS

Preparation 1

BIOCHEMISTRY: This preparation was obtained in a glycogen gradient with 0.5 M sucrose as solvent. As shown by Beaufay *et al.* (8), lysosomes accumulate in the sediment in such a gradient, while the bodies containing urate oxidase, catalase, and D-amino acid oxidase are mostly concentrated in the top layers, the mitochondria occupying an intermediate position. The biochemical data





FIGURES 1 AND 2 Preparation 1, lower part of the sediment. The field shown in Fig. 1 is occupied exclusively by typical dense bodies in various states of preservation. Fig. 2 shows part of another field at a higher magnification. Note single outer membrane, empty membrane-lined vacuoles, ferritin granules, and round masses of dense material. In some bodies, these masses tend to form bleblike protrusions, believed to be signs of incipient osmotic damage. Final stage is shown in Fig. 6 and at arrows in Fig. 1. Fig. 1, × 24,000; Fig. 2, × 60,000.

listed in Table I indicate that the lysosomes, as represented by two marker acid hydrolases, were purified approximately 16-fold over the cytoplasmic extract, with a 20 per cent yield. The values obtained for urate oxidase and catalase indicate that the bodies containing these enzymes were eliminated from the preparation to a fairly large extent, though not completely. No mitochondrial marker enzyme was measured, but it may be inferred from the results of Beaufay *et al.* (8) and from the protein content of the preparation that less than 3 per cent of the total mitochondria of the liver could have been present. However, in terms of bulk, this could still represent a significant proportion of the total material.

MORPHOLOGY: Examination of this preparation showed it to consist essentially of mitochondria and of particles exhibiting the morphological characters of pericanalicular dense bodies. These two components were largely separated from each other during the centrifugal packing of the fixed material, so that the dense bodies were present in highly purified form in the lower layers of the sediment (Fig. 1), whereas the upper layers contained a mixture of mitochondria and of dense bodies. Despite extensive scanning throughout the sediment, very few microbodies were seen in this preparation.

In Fig. 2, a few dense bodies are shown at a higher magnification. Characteristic of these particles, as they occur in our animals, is the presence of numerous small granules of very high electron opacity, approximately 50 A in diameter. These granules had already been seen by Novikoff *et al.* (20), who suggested that they may represent ferritin micelles. It has indeed been found that lysosome-rich fractions contain an excess of easily detachable iron (5). However, Holt and Hicks (15) failed to identify ferritin granules in the pericanalicular dense bodies of their animals, and it is possible that ferritin is not a constant feature of rat liver lysosomes, its abundance depending on extraneous factors, such as, for instance, the diet.

The particles also contain many rounded masses of dense material of unknown nature, as well as occasional empty vacuoles. The latter appear to be lined by a membrane similar to the outer membrane of the particles and could be invaginations rather than true vacuoles.

Preparation 2

BIOCHEMISTRY: According to the data of Table II, this preparation, which was isolated in a gradient of aqueous sucrose, contained about 20 per cent of the total urate oxidase purified 34-fold, together with much lower amounts of catalase and D-amino acid oxidase. Lysosomal enzymes, especially acid deoxyribonuclease, were also present in relatively high quantities. These results are entirely consistent with those reported by Beaufay *et al.* (8).

MORPHOLOGY: Large areas of this preparation were occupied by densely packed particles showing the typical features of the microbodies (Figs. 3 and

TABLE II

Biochemical Data on Preparation 2

Washed M + L fraction equilibrated for 2½ hours at 39,000 RPM in linear gradient of sucrose extending from 59.7 to 117 gm per 100 gm of water, with H_2O as solvent (gradient Sh(30-58) of Beaufay *et al.* (8)).

Purification step	Fraction	Prot.	A.Pase	A.DNase	Ur.Ox.	Catal.	DAA.Ox.
Fractionation of cytoplasmic extract (E)	% of E: M + L fraction	24.3	67	79	56	58	50
	P + S supernatant	68	30.6	16	20	30	35
	Recovery	92.3	97.6	95	76	88	85
	R.S.A. of M + L fraction	1.00	2.6	3.16	2.8	2.5	2.24
Subfractionation of M + L fraction in density gradient Sh(30-58)	% of M + L: Sediment (prep. 2)	2.7	5.7	10.7	34	6.8	4.8
	Other subfractions	87	78	68	68	54	74
	Recovery	89.7	83.7	78.7	102.0	60.8	78.8
	R.S.A. of prep. 2	1.00	6.0	15	34	8.3	4.4
	% of E: Final yield in prep. 2	0.66	3.8	8.5	19	3.94	2.4
	Over-all recovery	90	87	78	77	65	74

TABLE III

Biochemical Data on Preparation 3

Same M + L fraction as for preparation 2, but washed twice with distilled water at 250,000 *g* min. and then equilibrated in gradient Sh(30-58), like preparation 2.

Purification step	Fraction	Prot.	A.Pase	A.DNase	Ur.Ox.	Catal.	DAA.Ox.
Fractionation of cytoplasmic extract (E)	% of E: M + L fraction	24.3	67	79	56	58	50
	P + S supernatant	68	30.6	16	20	30	35
	Recovery	92.3	97.6	95	76	88	85
	R.S.A. of M + L fraction	1.00	2.6	3.16	2.8	2.5	2.24
Treatment of M + L fraction with H_2O	% of M + L: Sediment	8.5	8.0	4.3	79	31.6	50
	Supernatant	88	99	95	29	20.4	33.5
	Recovery	96.5	107	99.3	108	52.0	83.5
	R.S.A. of H_2O-treated M + L fraction	1.00	2.3	1.6	24.3	13.4	14.4
Subfractionation of H_2O-treated M + L fraction in density gradient Sh(30-58)	% of H_2O-treated M + L:						
	Sediment (prep. 3)	5.6	2.8	3.3	9.0	13.2	6.0
	Other subfractions	87	91	117	52	110	77
	Recovery	92.6	93.8	120.3	61	123.2	83
	R.S.A. of prep. 3	1.00	1.13	0.89	49	29	16.0
	% of E: Final yield in prep. 3	0.12	0.15	0.11	4.0	2.4	1.5
	Over-all recovery	92	102	95	63	64	72

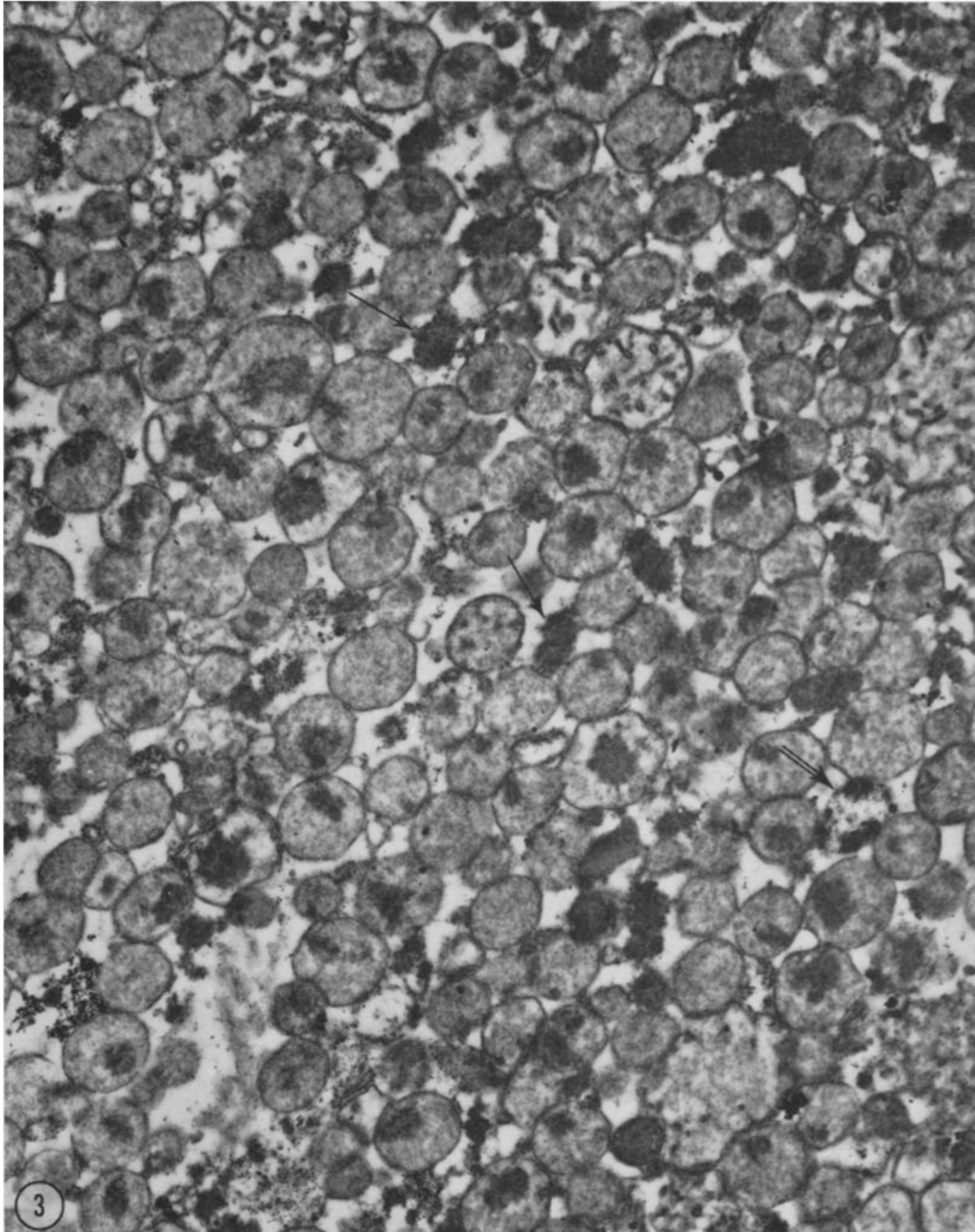
4 *a*). In addition to many whole microbodies, the preparation was also found to contain numerous isolated cores apparently derived from damaged particles (Figs. 3 and 4 *b*), a fair amount of dense bodies (Fig. 4 *b*), and a few mitochondria and microsomal elements (Fig. 3), as well as some unidentifiable debris. In general appearance, this preparation resembled very much the sediment isolated in a sucrose-D_2O gradient and described briefly by Beaufay and Berthet (7).

Characteristic of the microbodies are a well delineated single outer membrane, a finely granulated "sap," and an inner core of relatively high electron opacity which, upon higher magnification, is seen to possess a very regular honeycomb type of ultrastructure. In longitudinal section, the core shows parallel pairs of dense lines with an inner space of low electron opacity, while cross-sections appear as lacelike sheets with regularly spaced holes disposed according to a hexagonal lattice. Elements of this structure can be recognized in Fig. 4 *a* and in Figs. 6 to 9. The cross-sectional appearance of the core shows up in a particularly clear fashion in Fig. 5, a micrograph of a guinea pig liver microbody which we owe to the courtesy of Dr. G. E. Palade. Occasionally, a flattened crista-like vesicle is seen in isolated microbodies, close to their peripheral membrane (see Figs. 6 and 8). This may correspond to the hook-shaped attachment to the endoplasmic reticulum recently described for some particles by Novikoff and Shin (22).

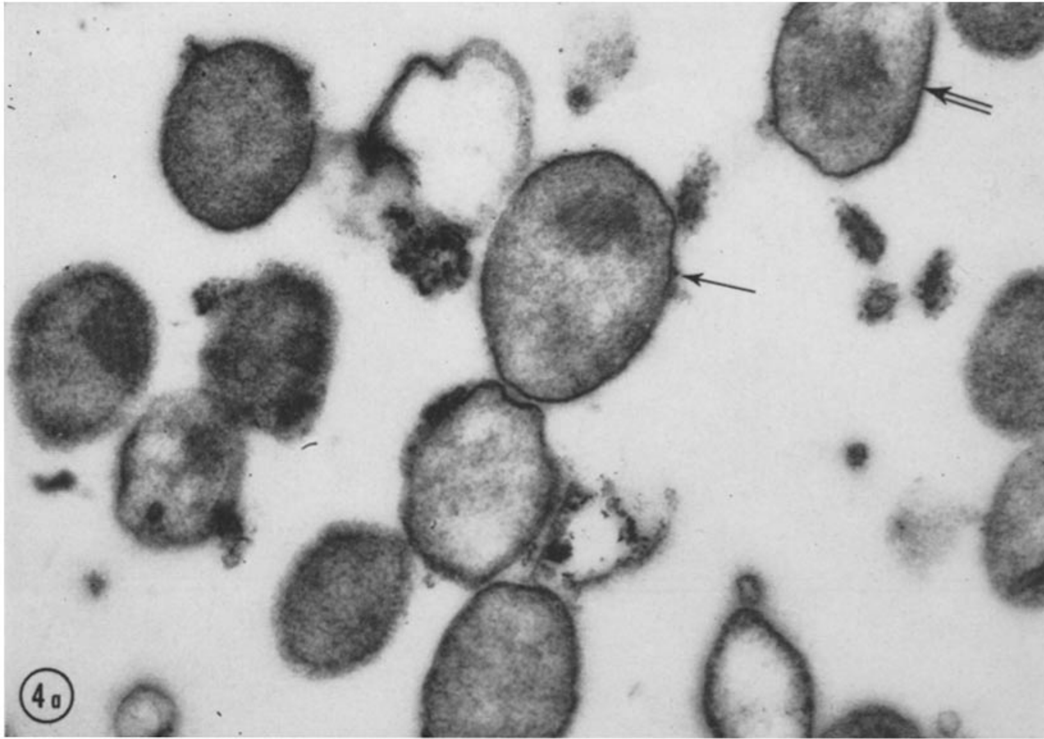
Preparation 3

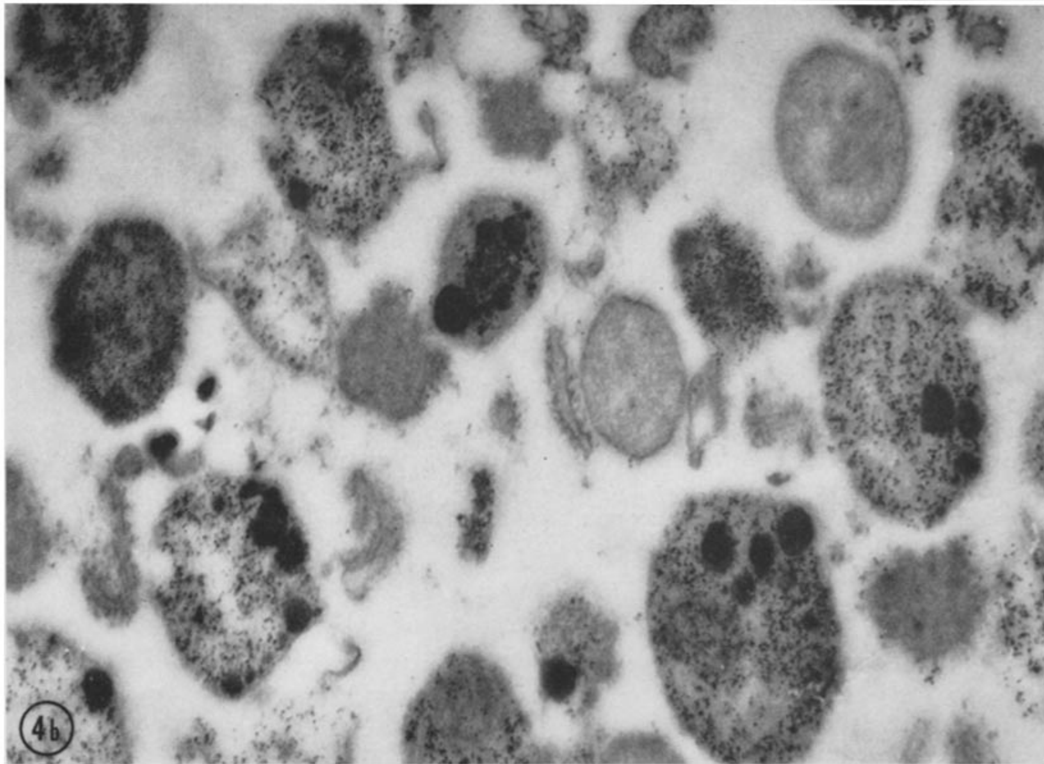
BIOCHEMISTRY: From equilibrium density measurements made in various concentrations of sucrose, Beaufay and Berthet (7) have come to the conclusion that the particles containing urate oxidase, catalase, and D-amino acid oxidase are essentially permeable to sucrose and should not be expected to respond osmotically to changes in sucrose concentration. Further support for this view has been obtained recently in experiments showing that catalase and D-amino acid oxidase are released to only a small extent from particles exposed to distilled water, although other treatments such as mechanical damage or exposure to surface-active agents readily liberate the enzymes in soluble form (2, 17). These observations suggested that treatment of a mitochondrial fraction with distilled water may lead to a rather selective preservation of the particles containing urate oxidase, catalase, and D-amino acid oxidase and thus allow them to be separated more easily from mitochondria and lysosomes. Table III shows the results of such an attempt, which was made on the same fraction as for preparation 2.

After distilled water treatment, centrifugation of the particles did indeed result in a considerable purification of urate oxidase, catalase, and D-amino acid oxidase with a relatively high yield.



FIGURES 3, 4 *a*, AND 4 *b* Preparation 2. In the low power view of Fig. 3, microbodies outnumber mitochondria by at least ten to one. Numerous isolated cores (for examples see single arrows) and a few strands of granular endoplasmic reticulum are also seen. A badly damaged dense body (open arrow) can be recognized near the lower right hand corner. Fig. 4 *a* show as few microbodies at a higher magnification. Note the densely delineated membrane, the finely granular "sap," the polytubular structure of the core in the body shown by a single arrow, and the honeycomb structure in the body indicated by an open arrow. An area containing numerous dense bodies in addition to microbodies and isolated cores is shown in Fig. 4 *b*. Fig. 3, × 20,000; Fig. 4 *a*, × 56,000; Fig. 4 *b*, × 57,000.


4a


4b

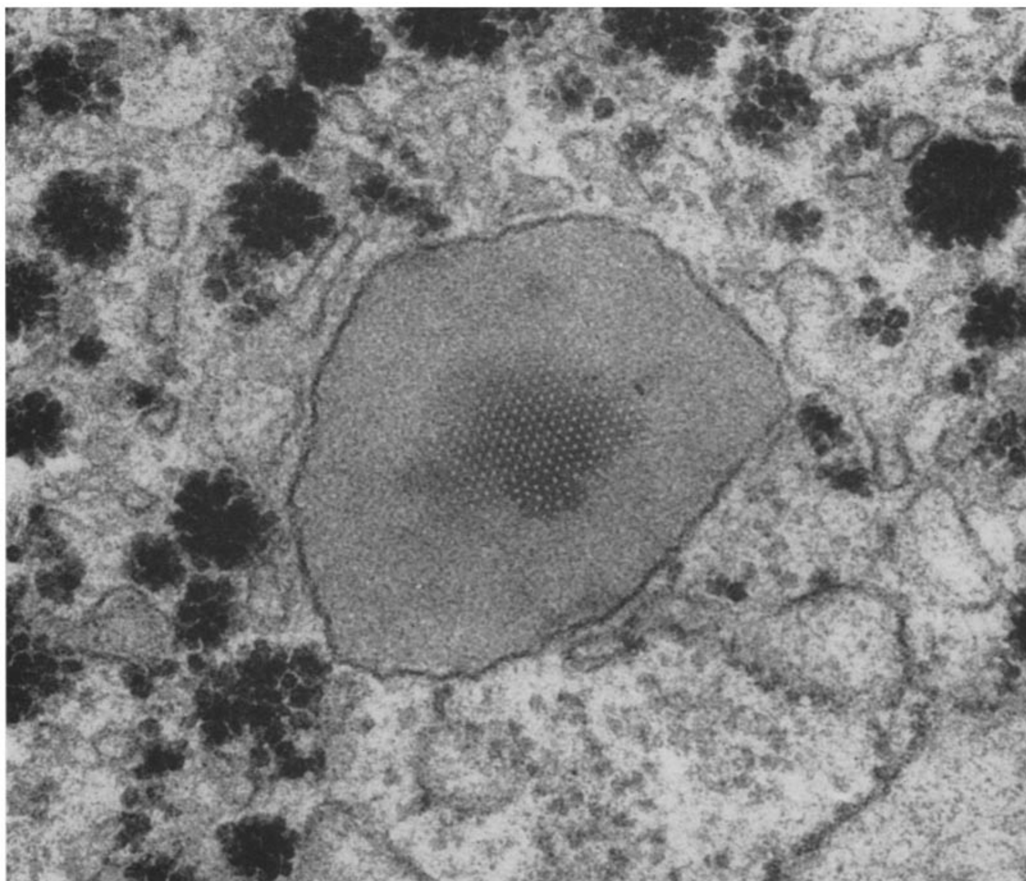


FIGURE 5 This picture of a microbody in a section of guinea pig liver shows very clearly the hexagonal lattice of the core when seen in cross-section. The spacing between the elements of the lattice is about 110 A. Courtesy of Dr. G. E. Palade. × 126,000.

Further subfractionation in a sucrose gradient gave a low yield but increased the purity further and lowered even more the contamination with lysosomal hydrolases.

MORPHOLOGY: Like preparation 2, this preparation was found to consist largely of typical microbodies and of isolated cores. In addition, it contained some microsomal elements, a few swollen mitochondria, and a fairly large proportion of unidentifiable debris presumably arising from disrupted mitochondria. Dense bodies were very rare and considerably altered in appearance.

Fig. 6 was selected to illustrate the structure of both types of particles; it is not representative of their relative frequency. It is seen that the microbodies have suffered only relatively minor changes as a result of exposure to distilled water. They appear slightly swollen and their interior is less dense; the regular structure of their inner core is relatively well preserved, though some change in the thickness and spacing of the parallel lines seen in longitudinal section may have occurred. The dense bodies are greatly damaged. They seem to have lost their membrane and show numerous blebs which may have originated from the rounded dense masses seen in the intact particles (Fig. 2). In some of them, the small ferritin granules seem to be replaced by larger dense aggregates.

Preparation 4

BIOCHEMISTRY: This preparation was isolated by the same method as preparation 2, but from the liver of a rat injected intravenously 4 days previously with 170 mg of Triton WR-1339. Com-

TABLE IV

Biochemical Data on Preparation 4

Rat injected intravenously 4 days previously with 170 mg of Triton WR-1339. Washed M + L fraction equilibrated in gradient Sh(30-58), like preparations 2 and 3

Purification step	Fraction	Prot.	Cyt.Ox.	A.Pase	A.DNase	Ur.Ox.	Catal.	DAA.Ox.	G-6-Pase
Fractionation of cytoplasmic extract (E)	% of E: M + L fraction	24.6	74*	52	60	78	55	53	11.3
	P + S supernatant	67	4.0	49	36	5.6	25	35.6	97
	Recovery	91.6	—	101	96	83.6	80	88.6	108.3
	R.S.A. of M + L fraction	1.00	3.5	1.92	2.33	3.5	2.56	2.2	0.39
Subfractionation of M + L fraction in density gradient Sh(30-58)	% of M + L: Top subfraction	5.7	0.4‡	69	58	0.3	4.7	2.0	33.5
	Sediment (prep. 4)	1.2	0	2.0	1.2	26	4.3	2.6	1.6
	Other subfractions	94	99.6‡	35	30	66	79	89	75
	Recovery	100.9	—	106.0	89.2	92.3	88.0	93.6	110.1
	R.S.A. of top subfraction	1.00	0.25	23	25.6	0.19	2.34	0.82	2.3
	R.S.A. of prep. 4	1.00	0	3.1	2.46	80	10	5	0.51
	% of E: Final yield in top subfraction	1.4	0.3	36	35	0.23	2.6	1.06	3.8
	Final yield in prep. 4	0.3	0	1.04	0.72	20.3	2.4	1.38	0.18
	Over-all recovery	92	78	104	90	78	73	85	109

* No direct determination available; value given represents sum of activities of subfractions.

‡ Expressed as percentage of sum of recovered activities.

TABLE V

Biochemical Data on Preparation 5

Rat injected intraperitoneally 4 days previously with 200 mg of dextran (mol. wt. 150,000). Washed L fraction.

Fraction		Prot.	Cyt.Ox.	A.Pase	A.DNase	Ur.Ox.	Catal.	DAA.Ox.
% of E:	M fraction	20.7	54	29.5	46	32.4	33	32
	L fraction (prep. 5)	4.4	8.3	11	8.5	37.5	41	29.5
	P + S supernatant	73	5.2	53.4	53	18.6	28.6	33.5
	Recovery	98.1	67.5	93.9	107.5	88.5	102.6	95.0
R.S.A. of L fraction		1.00	2.74	2.6	1.76	9.5	8.9	6.9

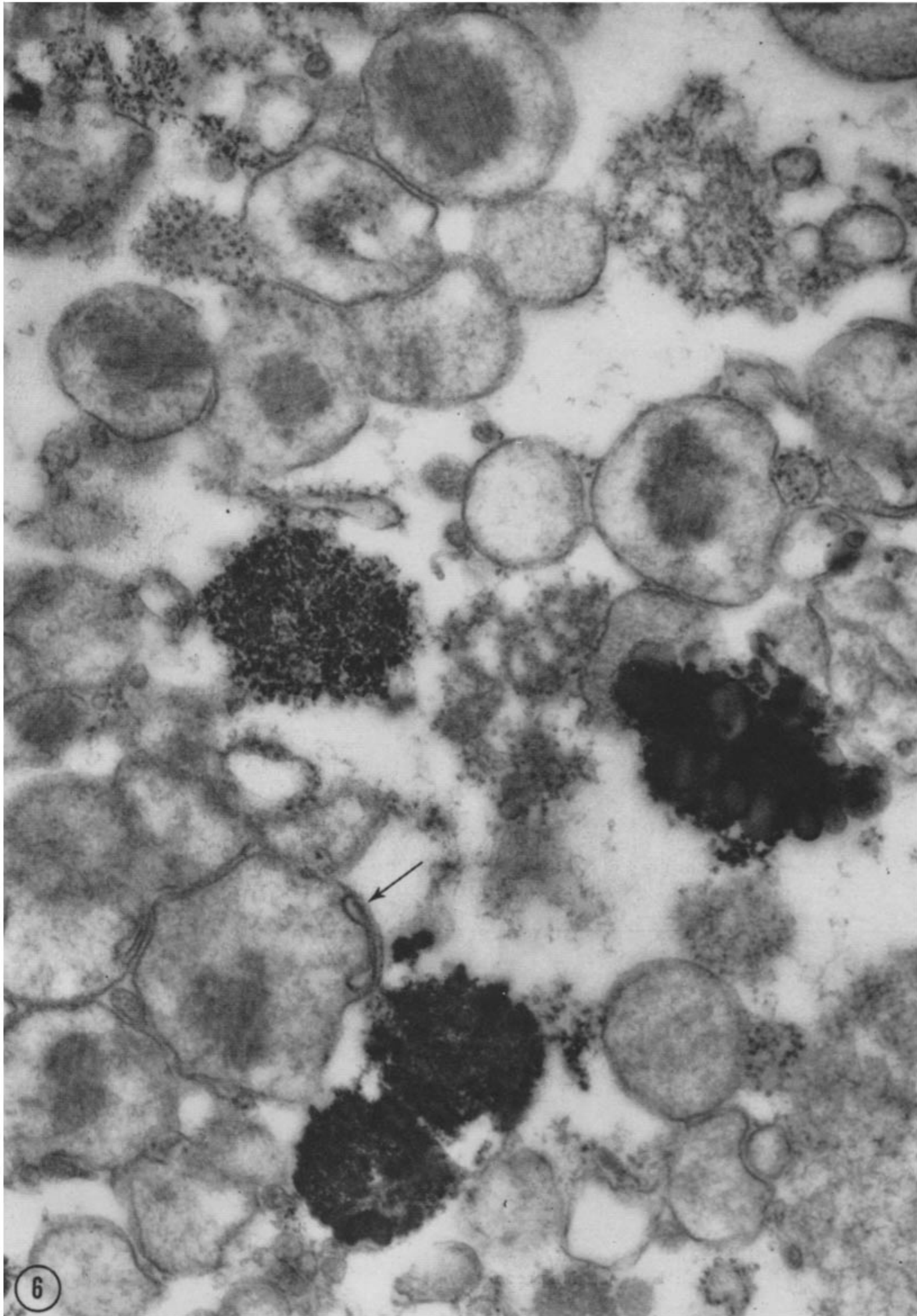
parison of the results of Table IV with those of Table II shows that the two preparations each contain about 20 per cent of the urate oxidase activity of the extract, but associated in preparation 4 with less than half the amount of protein present in preparation 2, resulting in an 80-fold purification of the enzyme. Preparation 4 is also characterized by very low levels of acid phosphatase and acid deoxyribonuclease. As shown by Wattiaux *et al.* (31), injection of Triton WR-1339 results in considerable decrease in the density of the lysosomes, which is due to the accumulation of the detergent within these particles and causes them to become concentrated largely in the top subfraction after centrifugation in the sucrose gradient employed. This phenomenon is illustrated by the data given in Table IV. The preparation also appears to be free of cytochrome oxidase activity, and thus of mitochondria, and to contain as principal contaminant some microsomal material, as indicated by its glucose 6-phosphatase activity. It has a particularly low content of catalase and D-amino acid oxidase activity.

MORPHOLOGY: Except for the presence of a fairly large amount of unidentified material and of some elements of the endoplasmic reticulum, preparation 4 was found to contain mostly microbodies in various states of structural preservation (Fig. 7 *a*). Some have a relatively normal appearance, but many others seem to have lost part of their inner contents. In addition, free cores occur in exceptionally large amounts in this preparation, large areas being occupied almost exclusively by these bodies (Fig. 7 *b*). In many of them, the polytubular structure is clearly recognizable.

Preparation 5

BIOCHEMISTRY: It has been shown by Daems (9) that dextran when injected parenterally to mice accumulates in the hepatic lysosomes. In recent unpublished experiments, Jacques has found that liver lysosomes from rats injected with dextran show a higher equilibrium density in sucrose gradients than do those from normal animals. This increase in density is attributed to the loading of the particles with the dense polysaccharide, like the reverse change observed by Wattiaux *et al.* (31) in the particles filled with Triton WR-1339, a compound of low density. Theoretically, dextran-filled lysosomes, being both

FIGURE 6 Preparation 3. Area selected for its content of dense bodies; these were very rare in the preparation. Note absence of peripheral membrane, numerous blebs, and dense aggregates in osmotically disrupted dense bodies. Most of the microbodies have an intact peripheral membrane, but appear somewhat dilated and less electron opaque than in preparations not treated with H_2O. The cores also seem augmented in size; the striation is clearly visible in several, but may be somewhat altered in its spacing. One particle, indicated by an arrow, shows an internal membrane-lined vacuole flattened against the peripheral membrane. No more free cores were present in this preparation than in a preparation not exposed to distilled water. × 61,000.


6

larger and denser than normal lysosomes, should show an accelerated sedimentation rate in a centrifugal field. It occurred to us that advantage could be taken of this property to prepare an L fraction relatively poor in lysosomes. Accordingly, preparation 5 was isolated by simple differential centrifugation according to de Duve *et al.* (12) from the liver of a rat injected intraperitoneally 4 days previously with 200 mg of dextran (mol. wt. 150,000). Further purification was not attempted (see, however, preparation 6) since it seemed advisable to examine also preparations containing a larger proportion of the particles bearing urate oxidase, catalase, and D-amino acid oxidase, even though in lower state of purity.

As shown in Table V, preparation 5, though containing about the same amounts of protein, cytochrome oxidase, urate oxidase, catalase, and D-amino acid oxidase as an L fraction from a normal animal, does indeed exhibit much lower acid phosphatase and acid deoxyribonuclease activities. In a normal preparation, these would be of the same order of magnitude as the urate oxidase activity; in preparation 5, they are reduced to one-fourth this value. As predicted, the M fraction is correspondingly richer in acid hydrolases, but not sufficiently so to account for the whole loss suffered by the L fraction. Presumably, the larger dextran-loaded lysosomes are disrupted during homogenization and release their enzymes in soluble form, thus explaining the high hydrolase content of the P + S supernatant. As indicated in Table V, the lysosome-poor preparation yielded by this procedure concentrates urate oxidase, catalase, and D-amino acid oxidase with a roughly comparable yield of 30 to 40 per cent, but in a relatively low degree of purity. It is heavily contaminated with mitochondria, as shown by the presence of 8.3 per cent of the total cytochrome oxidase activity.

MORPHOLOGY: Preparation 5 was found to be made up largely of mitochondria and of easily recognizable microbodies (Fig. 8). Very few isolated cores were seen. Dense bodies were distinctly rarer than microbodies and were generally small. Images of dextran-filled vacuoles, comparable to those described by Daems (9) in the intact tissue and shown by him to stain positively for acid phosphatase, were not seen in this preparation, probably because they either were disrupted by the homogenization procedure or came down with the heavier M fraction.

Preparation 6

BIOCHEMISTRY: In this experiment, an L fraction from a rat injected with dextran was further subfractionated in a glycogen–0.5 M sucrose gra-

TABLE VI

Biochemical Data on Preparation 6

Rat injected intraperitoneally 4 days previously with 200 mg of dextran (mol. wt. 150,000). Washed L fraction equilibrated for 2½ hours at 39,000 RPM in the same glycogen gradient as preparation 1 (gradient Gh(10) of Beaufay *et al.* (8)).

Purification step	Fraction	Prot.	Cyt.Ox.	A.Pase	A.DNase	Ur.Ox.	DAA.Ox.
Fractionation of cytoplasmic extract (E)	% of E: M fraction	24.7	76	50	43	36	41
	L fraction	3.5	7.8	11.5	8.3	23.3	18.4
	P + S supernatant	69	4.5	20.7	44	25	40
	Recovery	97.2	88.3	82.2	95.3	84.3	99.4
	R.S.A. of L fraction	1.00	2.46	3.9	2.4	7.7	5.2
Subfractionation of L fraction in density gradient Gh(10)	% of L: Top fraction (prep. 6)	36.6	35.4	13	7.9	46	58
	Other subfractions	51	45	75	112	45	31
	Recovery	87.6	80.4	88	119.9	91	89
	R.S.A. of prep. 6	1.00	2.4	1.4	0.52	9.9	8.4
	% of E: Final yield in prep. 6	1.28	2.76	1.5	0.66	10.7	10.7
	Over-all recovery	97	87	81	97	82	97

dient. The top fraction, which was expected to be particularly rich in urate oxidase, catalase, and D-amino acid oxidase (8), was separated and constituted preparation 6.

As shown in Table VI, the L fraction had the same low content of acid hydrolases as preparation 5, but was not as rich in urate oxidase and D-amino acid oxidase. The gradient step was only partly successful and did not give the degree of resolution obtained by Beaufay *et al.* (8) on a normal M + L fraction. It led to a further elimination of lysosomes and probably also of microsomal material, but it did not decrease the relative mitochondrial contamination. Consequently, except for its distinctly lower content of lysosomal enzymes, preparation 6 is not superior in purity to preparation 5 and shows a distinctly poorer yield. Like preparation 5, it contains urate oxidase and D-amino acid oxidase in comparable amounts. Catalase was not measured in this experiment, but its concentration may be expected to be of the same order of magnitude as that of D-amino acid oxidase (8).

MORPHOLOGY: As illustrated in Fig. 9, preparation 6 closely resembles preparation 5. Mitochondria and well preserved microbodies are very abundant. Isolated cores are rare. Dense bodies occur infrequently and show a normal aspect. Dextran-filled vacuoles appear to be absent.

DISCUSSION

Lysosomes and Dense Bodies

As illustrated by the summary in Table VII, there is a good correlation in all six preparations between the concentration of lysosomes, as estimated from the relative specific activities of acid phosphatase and acid deoxyribonuclease, and the observed frequency of dense bodies. This correlation is hardly surprising since the lysosomal nature of the pericanalicular dense bodies is now a well established fact, supported both by biochemical data (20, 31), and by cytochemical staining for acid phosphatase at the electron microscope level (9, 13, 15, 28). Our results also show that the microbodies cannot be lysosomes, since they were very abundant in some of the preparations with the lowest lysosomal enzyme content. This point is of some importance in view of an early claim that microbodies stain positively for acid phosphatase, at least in animals injected with bilirubin (13, 21). However, this claim has since been withdrawn (22), and other workers have stressed the fact that the microbodies show an entirely negative reaction for acid phosphatase (15, 28).

The proposed identification of the lysosomes with the pericanalicular dense bodies applies only

TABLE VII

Correlation between Lysosomes and Dense Bodies

Preparation no.	Purification data	Biochemistry (R.S.A.) A.Pase	Biochemistry (R.S.A.) A.DNase	Morphology: Dense bodies
1	Normal rat, M + L, glycogen–0.5 M sucrose gradient	16.2	19.2	Very numerous
2	Normal rat, M + L, sucrose-H_2O gradient	6.0	15	Numerous
3	Same as 2, but M + L washed twice with H_2O	1.17 (lysosomes largely disrupted)	0.94	Very rare and considerably damaged
4	Rat injected with Triton WR-1339, procedure as for 2	3.1	2.46	Not seen
5	Rat injected with dextran, L fraction	2.6	1.76	Very rare
6	Same as 5, glycogen–0.5 M sucrose gradient	1.4	0.52	Very rare

to the parenchymatous cells of the liver, and this raises the problem of the fate of the Kupffer cell lysosomes when liver is homogenized and fractionated. At present, it is not easy to assess quantitatively the contribution of these cells to the total lysosome population in liver preparations. In sections incubated for acid phosphatase activity, Kupffer cells stain much more heavily than parenchymatous cells (18, 19, 21). On the other hand, iron-loaded Kupffer cells separated from the parenchymatous cells by means of a magnet were found to be about twice as rich, on a nitrogen basis, in some lysosomal enzymes, but not in acid phosphatase, which showed the same specific activity in the two cell fractions (30). Since both experimental approaches are exposed to artifacts, a decisive answer to the question raised will have to await further evidence. In any case, it must be pointed out that the particles which give a positive reaction for acid phosphatase in Kupffer cells have, except for their larger size, many structural features in common with the pericanalicular dense bodies, and would not be easily distinguished from the latter in our preparations.

Microbodies as Sites of Urate Oxidase, Catalase, and D-*Amino Acid Oxidase*

According to the biochemical data which have led to their discovery, the particles containing urate oxidase, catalase, and D-amino acid oxidase should have an average diameter of a little over 0.5 μ, comparable to that of the lysosomes (11). It is already significant in this respect that an extensive search through all purified preparations that have been isolated has revealed the existence of only one type of particle of the predicted size, in addition to mitochondria and pericanalicular dense bodies, namely the microbodies. This is even true of intact liver, since particles of the required size that cannot be identified as mitochondria, pericanalicular dense bodies, or microbodies are encountered only very infrequently in tissue sections. Such considerations led, by a simple exclusion process, to the conclusion that the microbodies are probably the bearers of urate oxidase, catalase, and D-amino acid oxidase (7, 10).

Though this qualitative agreement between the biochemical predictions and morphological observations is suggestive, it is not in itself decisive. For instance, we have to consider the possibility that the postulated particles may be extremely rare and have escaped detection in even the most purified preparations. In point of fact, this objection cannot be valid in the particular case of catalase. It can be calculated from the activities reported by Baudhuin *et al.* (4) and from the data of Price *et al.* (23) that normal rat liver contains about 1.2 mg of catalase per gm wet weight. At least 80 per cent of this amount is present within particles with an average dry weight of 2.4×10^{-11} mg (11). Even if these particles were made up exclusively of catalase, they would number 4×10^{10} per gm of liver. This figure may be compared with the number of mitochondria, which has been variously estimated at between 12×10^{10} (27) and 33×10^{10} (1) per gm of liver. Obviously, there must be, at the very least, one catalase-containing body for every eight mitochondria;[2] the detection of these particles cannot be a problem. However, there still remains the possibility that the postulated particles simply represent a special population of mitochondria. There is as yet no compelling evidence that all hepatic mitochondria are alike biochemically and characterized by the same enzyme complement, even though they may have the same general morphological features. Con-

[2] This proportion is probably underestimated by at least 50 per cent, since it is based on the highest value recorded for the number of mitochondria and on an evaluation of the number of microbodies which neglects any loss of catalase suffered by these particles upon homogenization as well as the fact that they contain other proteins besides catalase in significant amounts. For these reasons, the true proportion is probably closer to one microbody for every four or five mitochondria.

This ratio is undoubtedly higher than the ratio between microbody profiles and mitochondrial profiles in ultrathin sections. However, it must be remembered that, owing to its smaller size, a single microbody contributes fewer profiles than a single mitochondrion. In first approximation, this factor will decrease the ratio of the two profiles by a factor of 2 (assuming perfectly spherical particles and a mitochondrial diameter twice that of a microbody). In addition, since microbodies are best identified by their cores, the size of the core rather than that of the whole microbody may determine the ratio unless very careful examinations are made. Assuming the core to be half the size of the microbody, we arrive at a ratio, in an ultrathin section, of one microbody core for every sixteen to twenty mitochondria. This does not appear unreasonable, especially if it is remembered that microbodies may occur in clusters (22) and may therefore be present in many sections with a lower than average frequency.

versely, there is no *a priori* reason why enzymatic dissimilarities should necessarily be reflected in obvious morphological differences.

More positive correlations are therefore needed before the microbodies can be definitely established as the particles containing urate oxidase, catalase, and D-amino acid oxidase, and the present work was undertaken largely with the aim of obtaining sufficient data for this purpose. The data are summarized in Table VIII.

Two major difficulties complicate the interpretation of the results listed in Table VIII. On the morphological side, we are faced with the difficulty of putting the results of electron microscope examinations in quantitative terms, especially with pellets that have been organized in a non-random fashion by centrifugal packing. In our opinion, our sampling cannot be considered sufficiently random to validate any quantitative counting procedure. However, we consider our examinations sufficiently exhaustive and the preparations sufficiently different in composition to allow comparative semiquantitative estimates of the various structural forms present. It may be added that, except in the water-treated preparation 3, little material was encountered that could not be identified as one of the objects listed in Tables VII and VIII. This material consisted largely of ergastoplasmic fragments and some amorphous debris that do not deserve serious consideration as possible sites of location of the enzymes under study.

On the biochemical side, there is the problem that some of the most highly purified preparations, as judged by their content of urate oxidase, are relatively deficient in catalase and D-amino acid oxidase. According to Beaufay *et al.* (8), this heterogeneity is characteristic of dense sediments separated in aqueous sucrose gradients and must be considered an artifact. The particles containing the three enzymes are believed to be essentially homogeneous when intact, but to be exposed to selective losses of their soluble catalase and D-amino acid oxidase with retention of their insoluble urate oxidase, as a result of accidental damage to their membrane. In line with this interpretation, the ratios of catalase and D-amino acid oxidase to urate oxidase have been listed in Table VIII as representing a measure of the integrity of the particles.

It is interesting that unmistakable morphological evidence of damage to the microbodies has indeed been found, in several preparations, in the form of empty-looking particles and, especially, of isolated cores. The latter are easily identified and undoubtedly originate each from a single disrupted microbody. It is thus possible to compare the frequency of isolated cores with the total frequency of both intact and damaged microbodies, and to arrive in this manner at some kind of morphological integrity index for the microbodies. For the reasons explained above, this cannot be expressed in accurate quantitative terms, but it will be obvious from the data given in Table VIII that there exists a significant correlation between the biochemical and morphological results. The frequency of total microbodies, both intact and injured, appears directly related to the relative specific activity of urate oxidase, while the proportion of isolated cores is inversely related to the biochemically determined integrity index. In preparations 5 and 6, which show a very high biochemical integrity index, the microbodies are very well preserved and the number of isolated cores is small. On the other hand, the proportion of isolated cores is exceptionally high in preparation 4, in which the biochemical evidence indicates that some 90 per cent of the particles must have been severely damaged. Preparations 2 and 3 are intermediate between these two extremes, both biochemically and morphologically. We have, it is true, seen no clearcut evidence of a greater proportion of injured microbodies in preparation 2 than in preparation 3, in spite of the observed difference in their biochemical integrity. However, the expected morphological difference is one that imperfect sampling could easily have blurred. These findings not only confirm the identity of the microbodies with the postulated particles; they also provide direct morphological support to the contention, deduced from biochemical results, that deficiencies in catalase and D-amino acid oxidase relative to urate oxidase are an artifact caused by the selective loss of soluble enzymes from the damaged particles (8).

The identification of the microbodies as the bearers of urate oxidase, catalase, and D-amino acid oxidase receives particularly strong confirmation from the observations made on the water-treated preparation 3. It has been inferred from biochemical results that the particles containing the three enzymes are permeable to sucrose (7) and are not severely damaged by exposure to distilled water, as witnessed by the fact that this

treatment does not release catalase or D-amino acid oxidase in appreciable quantities (2, 17). This treatment has indeed allowed the separation of a purified fraction with a relatively high biochemical integrity index, and it is certainly striking that the microbodies, which are very numerous in this preparation, show relatively minor structural alterations. This is in contrast to the gross changes, both morphological and biochemical, which exposure to distilled water causes in the lysosomes and in the mitochondria. Another point of interest concerning this preparation is that it has a very low content of morphologically recognizable mitochondria, while exhibiting the highest value observed so far in any preparation for the relative specific activity of catalase. This definitely rules out the possibility, already rendered very unlikely by all the other evidence discussed, that catalase may be associated with a special type of mitochondria.

For all the above reasons, the conclusion appears to us inescapable that the microbodies are the specific bearers of urate oxidase, catalase, and D-amino acid oxidase. We may state further that catalase and D-amino acid oxidase are not part of the internal core of the particles and must be situated in the structureless "sap" which seems to leach out easily from damaged particles. Indeed, catalase must be a major constituent of this sap, since the total number of microbodies can be no more than a few multiples of the absolute minimum number calculated above as being necessary to house all the particulate catalase of the liver to the exclusion of any other constituent. The core itself is a likely site for the location of urate oxidase, but this is by no means established, since this enzyme could also be attached to the outer membrane of the particles, which would be very difficult to recognize in injured preparations.

It is unfortunate that the new particles should be known under so unspecific a name as "microbody," especially since this term has also been applied to other tissues, such as kidney, to designate particles which may or may not bear a relationship to the hepatic microbodies. However, it is felt that the nomenclature can gain nothing from the addition of a new name unless it reflects the functional significance of the particles. Too little is known of their enzyme complement and of their role in the physiology of the liver cells to substantiate a proposal at the present time.

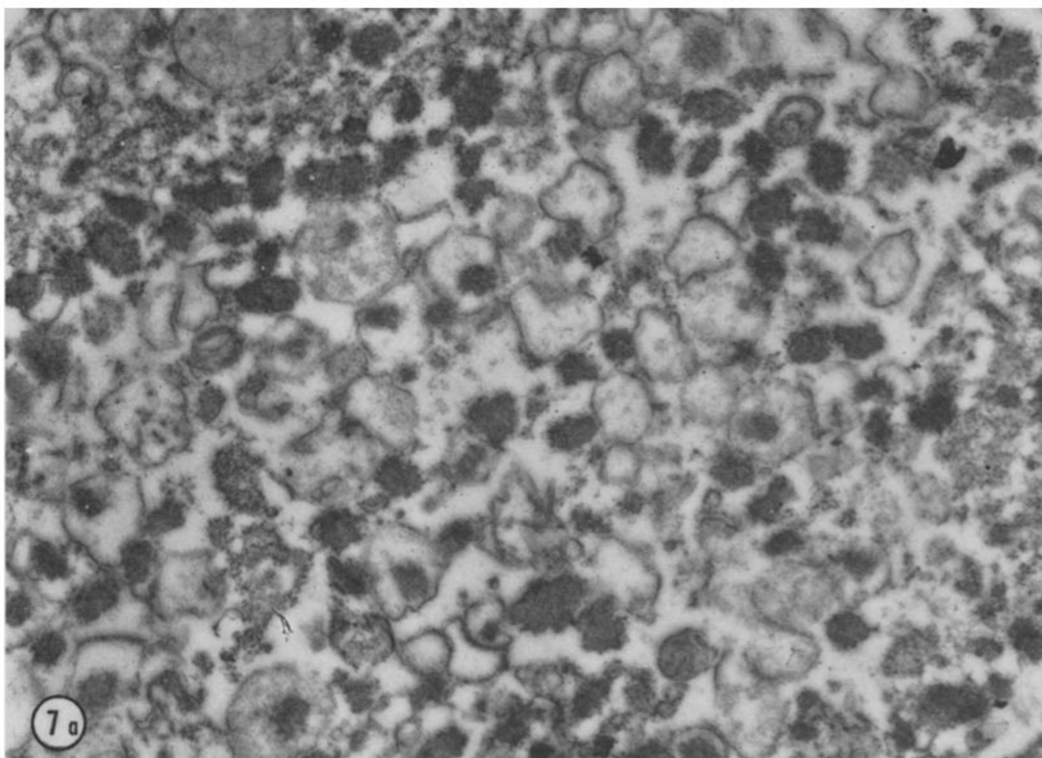
For References, see p. 241.

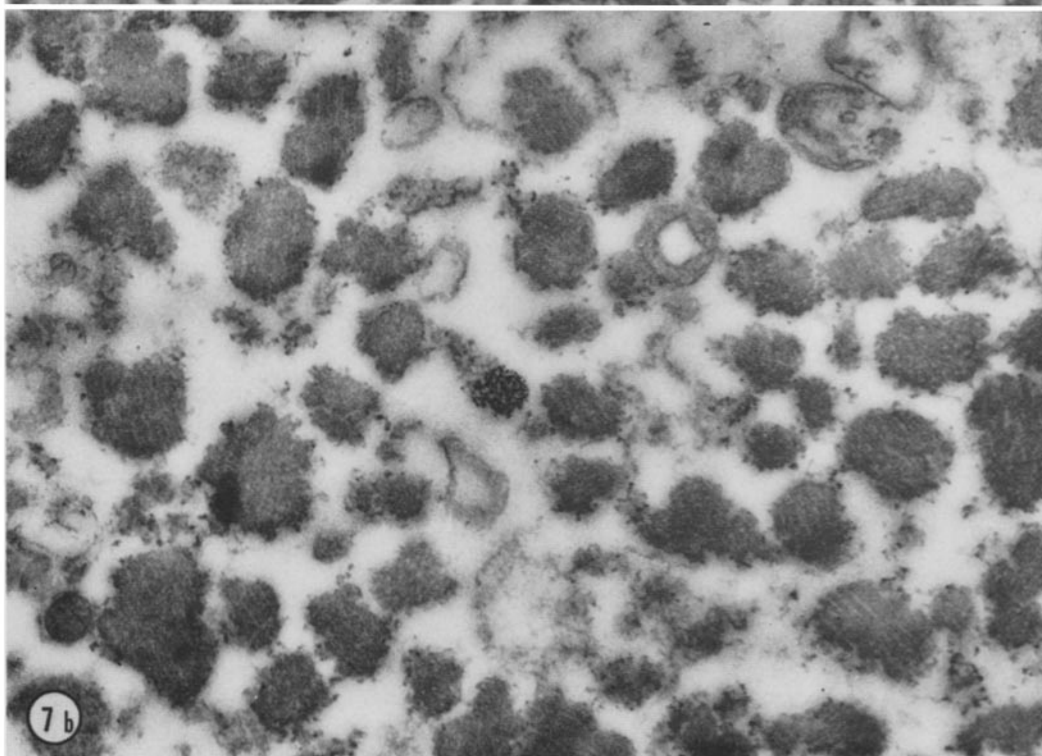
FIGURES 7 *a* AND 7 *b* Preparation 4. Most of the microbodies seen in Fig. 7 *a* show various signs of damage. Isolated cores are very numerous. Fig. 7 *b* shows, at a higher magnification, an area occupied almost exclusively by isolated cores. All intermediates between longitudinal striation and cross-sectional honeycomb structure can be recognized. Fig. 7 *a*, × 22,000; Fig. 7 *b*, × 46,000.

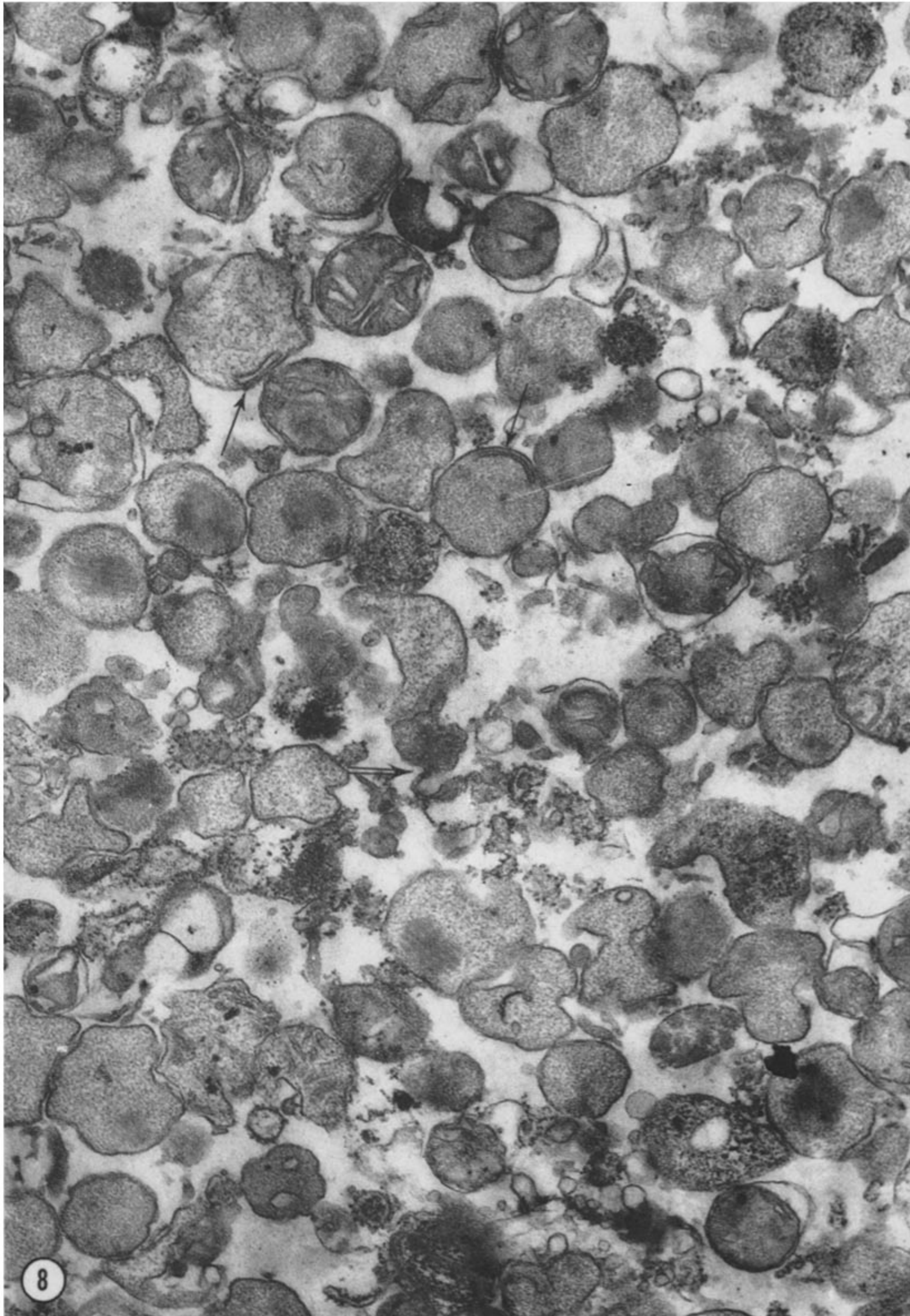
FIGURE 8 Preparation 5. This field contains numerous microbodies; several mitochondria and dense bodies, as well as clumps of granular endoplasmic reticulum, are also present. Most microbodies are in a good state of preservation; isolated cores are present, but in small number. Several microbodies show an internal vacuole flattened against the peripheral membrane (single arrows). One (open arrow) shows a looplike projection, similar to the attachment to the endoplasmic reticulum described by Novikoff and Shin (22). × 33,000.

FIGURE 9 Preparation 6. This field contains mostly mitochondria and microbodies. The mitochondria show all degrees of structural preservation, from a practically normal appearance to completely unfolded internal membranes. In some, with slightly swollen cristae, the infoldings of the inner membrane are particularly evident. Most microbodies are well preserved; there are very few, if any, isolated cores. A single damaged dense body is seen in the upper part of the figure (arrow). × 24,000.

For Figs. 8 and 9, see following pages.


7a


7b



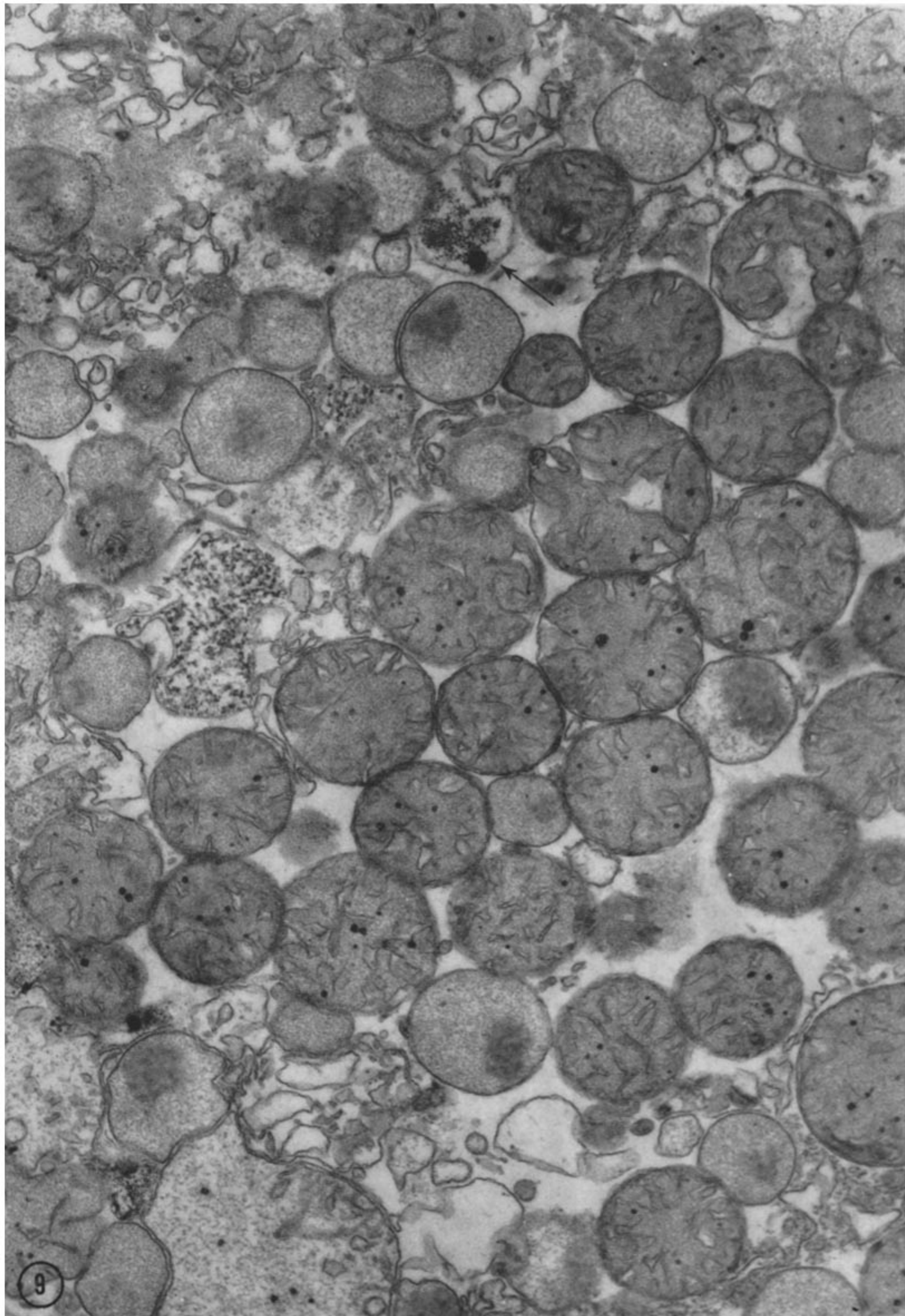


TABLE VIII

Correlation between Urate Oxidase, Catalase, and D-Amino Acid Oxidase and Microbodies

Prepara- tion no.	Purification data	Biochemistry					Morphology			
		Relative specific activity			Integrity index*		Microbodies			Mitochondria
		Ur.Ox.	Catal.	DAA.Ox.			Total	Free cores	Integrity index	
1	Normal rat, M +L, glycogen–0.5 M sucrose gradient	4.4	3.6	—	0.82	—	Extremely rare	Extremely rare	—	Numerous
2	Normal rat, M + L, sucrose–H_2O gradient	34	8.3	4.4	0.24	0.13	Very numerous	Numerous	Low	Rare
3	Same as 2, but M + L washed twice with H_2O	50	30	16.4	0.6	0.33	Very numerous	Numerous	Low	Rare
4	Rat injected with Triton WR-1339, procedure as for 2	80	10	5	0.13	0.06	Extremely numerous	Very numerous	Extremely low	Extremely rare
5	Rat injected with dextran, L fraction	9.5	8.9	6.9	0.94	0.73	Moderately numerous	Very rare	Very high	Very numerous
6	Same as 5, glycogen–0.5 M sucrose gradient	9.9	—	8.4	—	0.85	Moderately numerous	Very rare	Very high	Very numerous

* The first figure represents the ratio Catal./Ur.Ox., the second the ratio DAA.Ox./Ur.Ox.

The work reported in this paper was supported by grants from the Fonds National de la Recherche Scientifique and the Fonds de la Recherche Scientifique Fondamentale Collective, of Belgium; and from the Rockefeller Foundation and the United States Public Health Service (Research Grant GM-08705 from the Division of General Medical Sciences). Dr. Baudhuin is Chargé de Recherches du Fonds National de la Recherche Scientifique and Dr. Beaufay is Associé du Fonds National de la Recherche Scientifique.

Received for publication, October 28, 1964.

REFERENCES

1. Allard, C., Mathieu, R., de Lamirande, G., and Cantero, A., Mitochondrial population in mammalian cells. I. Description of a counting technique and preliminary results on rat liver in different physiological and pathological conditions, *Cancer Research*, 1952, **12**, 407.
2. Baudhuin, P., Propriétés de la catalase, de la D-aminoacide oxydase et de l'urate oxydase liées aux microbodies du foie de rat, *in* Abstracts of the 6th International Congress of Biochemistry, New York, 1964, 296.
3. Baudhuin, P., and Beaufay, H., Examen au microscope électronique de fractions purifiées d'organites cytoplasmiques de foie de rat, *Arch. internat. physiol. et biochim.*, 1963, **71**, 119.
4. Baudhuin, P., Beaufay, H., Rahman-Li, Y., Sellinger, O. Z., Wattiaux, R., Jacques, P., and de Duve, C., Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue, *Biochem. J.*, 1964, **92**, 179.
5. Beaufay, H., Bendall, D. S., Baudhuin, P., and de Duve, C., Tissue fractionation studies. 12. Intracellular distribution of some dehydrogenases, alkaline deoxyribonuclease and iron in rat-liver tissue, *Biochem. J.*, 1959, **73**, 623.
6. Beaufay, H., Bendall, D. S., Baudhuin, P., Wattiaux, R., and de Duve, C., Tissue fractionation studies. 13. Analysis of mitochondrial fractions of rat liver by density-gradient centrifuging, *Biochem. J.*, 1959, **73**, 628.
7. Beaufay, H., and Berthet, J., Medium composition and equilibrium density of subcellular particles from rat liver, *in* Biochemical Society Symposia, No. 23, Methods of Separation of subcellular Structural Components, (J. K. Grant, editor), Cambridge, England, Cambridge University Press, 1963, 66.
8. Beaufay, H., Jacques, P., Baudhuin, P., Sellinger, O. Z., Berthet, J., and de Duve, C., Tissue fractionation studies. 18. Resolution of mitochondrial fractions from rat liver into three distinct populations of cytoplasmic particles by means of density equilibration in various gradients, *Biochem. J.*, 1964, **92**, 184.
9. Daems, W. Th., Mouse Liver Lysosomes and Storage, Leiden, Netherlands, Drukkerij "Luctor et Emergo," 1962.
10. de Duve, C., Intracellular localization of enzymes, *Nature*, 1960, **187**, 836.
11. de Duve, C., The separation and characterization of subcellular particles, *Harvey Lectures, Series 59 (1963–1964)*, 49.
12. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F., Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue, *Biochem. J.*, 1955, **60**, 604.
13. Essner, E., and Novikoff, A. B., Localization of acid phosphatase activity in hepatic lysosomes by means of electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 773.
14. Gänsler, H., and Rouiller, C., Modifications physiologiques et pathologiques du chondriome, *Schweiz. Z. allgem. Path. u. Bacteriol.*, 1956, **19**, 217.
15. Holt, S. J., and Hicks, R. M., The localization of acid phosphatase in rat liver cells as revealed by combined cytochemical staining and electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 47.
16. Mercer, E. M., and Birbeck, M. S. C., Electron Microscopy, Oxford, England, Blackwell Scientific Publ., 1961, 59.
17. Miller, L. A., Studies on the localization of intracellular catalase of mouse liver, *Exp. Cell Research*, 1964, **34**, 283.
18. Novikoff, A. B., Lysosomes and related particles, *in* The Cell, (J. Brachet and A. E. Mirsky, editors), New York, Academic Press, 1961, **2**, 423.
19. Novikoff, A. B., Lysosomes and possible roles in the reticulo-endothelial system, *in* Rôle du système réticulo-endothélial dans l'immunité antibactérienne et antitumorale, Paris, Editions du Centre National de la Recherche Scientifique, 1963, 67.
20. Novikoff, A. B., Beaufay, H., and de Duve, C., Electron microscopy of lysosome-rich fractions from rat liver, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 179.

21. Novikoff, A. B., and Essner, E., The liver cell. Some new approaches to its study, *Am. J. Med.*, 1960, **29**, 102.
22. Novikoff, A. B., and Shin, W.-Y., The endoplasmic reticulum in the Golgi zone and its relations to microbodies, Golgi apparatus and autophagic vacuoles in rat liver cells, *J. Micr.*, 1964, **3**, 187.
23. Price, V. E., Sterling, W. R., Tarantola, V. A., Hartley, R. W., Jr., and Rechcigl, M., Jr., The kinetics of catalase synthesis and destruction *in vivo*, *J. Biol. Chem.*, 1962, **237**, 3468.
24. Reynolds, E. S., The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, *J. Cell Biol.*, 1963, **17**, 208.
25. Rouiller, C., Les canalicules biliaires. Etude au microscope électronique, *Compt. rend. Soc. biol.*, 1954, **148**, 2008.
26. Rouiller, C., and Bernhard, W., "Microbodies" and the problem of mitochondrial regeneration in liver cells, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 355.
27. Striebich, M. J., Shelton, E., and Schneider, W. C., Quantitative morphological studies on the livers and liver homogenates of rats fed 2-methyl or 3-methyl-4-dimethylaminoazobenzene, *Cancer Research*, 1953, **13**, 279.
28. Trump, B. E., and Ericsson, J. L., Electron microscopic observations on the localization of acid phosphatase in mouse hepatic parenchymal cells, *Exp. Cell Research*, 1964, **33**, 598.
29. Watson, M. L., Staining of tissue sections for electron microscopy with heavy metals. II. Application of solutions containing lead and barium, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 727.
30. Wattiaux, R., Baudhuin, P., Berleur, A. M., and de Duve, C., Tissue fractionation studies. 8. Cellular localization of bound enzymes, *Biochem. J.*, 1956, **63**, 608.
31. Wattiaux, R., Wibo, M., and Baudhuin, P., Influence of the injection of Triton WR-1339 on the properties of rat-liver lysosomes, *in* Lysosomes, Ciba Foundation Symposium, (A. V. S. de Reuck and M. P. Cameron, editors), Boston, Little, Brown and Co., 1963, 176.

NOTE ADDED IN PROOF

In a recent paper, Hruban and Swift (33) have reported findings indicating that urate oxidase is localized in the core of the microbodies. This conclusion is based on similarities in crystalline pattern between the core as it is seen in tissue sections and structures found in a commercial preparation of urate oxidase which the authors describe as crystalline. Highly purified, though non-crystalline, preparations of urate oxidase have been isolated by Mahler *et al.* (34). As shown in Table IX, the specific activity of this preparation is about ten times higher than that of the Sigma type I preparation, which is the one examined in the electron microscope by the authors (Hruban, personal communication), whereas the latter has about the same specific activity as our preparation 4, in which isolated cores were very abundant. Since the Sigma preparation is isolated from hog liver (Sigma Company, personal communication), it appears to us likely that the structures found in it are preformed cores concentrated by the purification procedure rather than enzyme crystals. Thus the findings of Hruban and Swift corroborate the correlation underlined in this paper between the abundance of cores and the specific activity of urate oxidase. They strengthen the conclusion that urate oxidase is probably associated with the cores. Although they do not allow us to exclude entirely the possibility of a localization in some other structure, for instance the microbody membrane, they make this possibility much less likely, since the technique followed in the purification of urate oxidase for commercial purposes is bound to be very different from those used to obtain our preparations.

TABLE IX

Specific Activity of Urate Oxidase in Various Preparations

Activities reported by other workers have been corrected to our assay conditions (pH 7.4, 37°C) by means of the data of Baum *et al.* (32).

Enzyme preparation	Specific activity	Reference
	units per mg protein	
Whole rat liver	0.017 ± 0.005 (S.D.)	4
Preparation 4	1.36	This paper
Sigma type I	0.9–1.7	Sigma catalogue
Sigma type I, lot 54B-175	2.0	Our measurement
Highly purified	20	33

Additional References

32. Baum, H., Hübscher, G., and Mahler, H. R., Studies on uricase. II. The enzyme-substrate complex, *Biochim. et Biophysica Acta*, 1956, **22**, 514.

33. Hruban, A., and Swift, H., Uricase: Localization in microbodies, *Science*, 1964, **146**, 1316.

34. Mahler, H. R., Hübscher, G., and Baum, H., Studies on uricase. I. Preparation, purification and properties of a cuproprotein, *J. Biol. Chem.*, 1955, **216**, 625.