

THE NUCLEOIDS OF RAT LIVER CELL MICROBODIES

Fine Structure and Enzymes

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ABSTRACT

The nucleoids of microbodies of rat liver cells were isolated in a highly homogeneous and pure state, by treating the microbody-rich fraction, prepared from 10% polyvinylpyrrolidone-0.25 M sucrose homogenate, with Triton X-100. Three treatments with 0.1% detergent were enough to render the nucleoids free from contamination with mitochondria, microsomes, lysosomes, and intact microbodies. Electron microscopically, the nucleoids were found to consist of parallel bundles of highly dense hollow tubules, the outer and inner diameters of which are approximately 150 and 50 Å, respectively. Ten tubules are arranged around a longitudinal space 190×200 Å in width. The nucleoids thus show a honeycomb appearance in the cross-plane and a parallel-packed structure in the longitudinal plane. Biochemically, the nucleoids were found to bear only urate oxidase among probably microbody-enzymes, and they might be the only cytoplasmic particles of rat liver cells in which the enzyme locates. Urate oxidase activity, on a unit protein basis, of the nucleoid preparation is approximately 380 times as high as that of the whole homogenate, and is almost comparable with that of a commercial type I enzyme preparation. No enzymes of mitochondrial, microsomal, and lysosomal origins were detected in the nucleoids. The fine structure of the nucleoids is described in detail, and a probable schematic diagram is presented.

INTRODUCTION

On the basis of density gradient centrifugation of cytoplasmic particles of rat liver cells, it was reported that the particles bearing catalase, urate oxidase, and D-amino acid oxidase showed an equilibrium density different from that of mitochondria and lysosomes (7). Furthermore, the possibility was suggested by Baudhuin and Beaufay (3), Baudhuin, Beaufay, and de Duve (4), and Beaufay and Berthet (5) that these particles correspond to microbodies described by Rouiller and Bernhard (19) in rat liver cells.

Among these three enzymes, urate oxidase was found not to be released from the particles in any

suspending media commonly used in the fractionation of intracellular particles and even in those to which was added a detergent such as Triton X-100 (10, 21). On the other hand, a major portion of particle-bound catalase was readily solubilized in isotonic sucrose solution, and a considerable portion of bound D-amino acid oxidase was also released from the particles in the presence of the detergent (10, 21).

Because of the fact that urate oxidase is bound firmly to a particulate component, the present experiments were carried out to isolate and identify the particles involved, or their enzyme-bearing

portion, and also to clarify the fine structure of the particles themselves.

MATERIALS AND METHODS

Male rats of Mk/Wistar strain weighing 160 to 180 g were used. The animals were fed a compressed diet (Oriental, No. NMF) and received drinking water *ad libitum*. They were subjected to light anesthesia with ether, and their livers were perfused *in vivo* with 10 ml of cold saline solution prior to use.

FRACTIONATION OF CYTOPLASMIC PARTICLES: 10% homogenate was made with 10% polyvinylpyrrolidone-0.25 M sucrose solution (pH 7.6 with NaOH). This homogenizing medium was considered to be suitable not only for fractionation of cytoplasmic particles but also for isolation of the particles in a state wherein they are capable of retaining catalase and D-amino acid oxidase, which were reported as probably located in microbodies, in addition to urate oxidase (4-7, 10, 11, 21).

The fractionation procedure of the so called mitochondrial and microbody-rich fractions was previously described (10). In the present experiments, a cytoplasmic fraction sedimentable between 10,000 and 50,000 *g* for 15 min was also prepared. These fractions, each prepared from 1 g of liver tissues, were suspended in 10 ml of 0.1% Triton X-100-0.25 M sucrose solution (pH 7.2), stirred vigorously with the aid of a syringe equipped with a long needle, and then centrifuged at 50,000 *g* for 15 min. This Triton treatment was repeated three times. The resulting sediments, after one washing with 0.25 M sucrose solution, were used for electron microscopic examinations, or were further resuspended in distilled water to give appropriate concentrations for biochemical determinations. These operations were all done at 4°C.

ELECTRON MICROSCOPIC EXAMINATIONS: Small liver tissue fragments and the Triton-treated sediments were fixed in 2% osmium tetroxide in Veronal-acetate buffer (pH 7.4) for 60 min at 4°C, dehydrated through a graded series of cold ethanols, and then embedded in Epon 812. Sections were cut on a Porter-Blum microtome and stained with saturated uranyl acetate-75% ethanol for 30 min. Negatively stained specimens of the sediments treated with Triton were prepared by suspending the sediments in 0.4% sucrose solution, mixing with an equal volume of 2% tungstic acid-0.4% sucrose solution, and then dropping onto carbon-coated collodion grids. These preparations were examined with an Hitachi 11HA electron microscope.

BIOCHEMICAL DETERMINATIONS: Determination of urate oxidase activity was done manometrically according to Leon's method (15). The reaction was induced at 37°C and lasted for 20 min. Catalase activity was measured principally according

to Adams' method (1), except that the specimens were diluted with 0.25% Triton-1% arabic gum solution (pH 7.0 with NaOH). D-Amino acid oxidase activity was measured colorimetrically, according to the method of Endahl and Kochakian (8), in the presence of FAD (flavin-adenine dinucleotide) (10 μ g in 3 ml of reaction medium) and with a higher concentration of pyrophosphate buffer (0.1 M). Nobunaga's method (16), Potter's method (18), and Strecker's method (20) were used for determination of β -glucuronidase, succinoxidase, and esterase activities, respectively. Protein content was measured by Hagiwara's method (12) of the copper-Folin reaction, with crystalline bovine serum albumin as the standards.

A commercial type I urate oxidase preparation from the Sigma Company was also examined for the enzyme activity and protein content so that it could be compared with the preparations obtained in these experiments.

RESULTS

I. ELECTRON MICROSCOPIC OBSERVATIONS: Typical microbodies in rat liver cells are single membrane-limited cytoplasmic particles in which a moderately dense granular matrix and a highly dense core, the nucleoid, are included, as described by Rouiller and Bernhard (19) and others. In thin sections of rat liver cells, the nucleoids were observed to contain highly dense lines running parallel to each other with almost equal spacing and passing through the nucleoids in one direction (Fig. 1). The density of the spaces between lines resembled that of the regions outside the nucleoid. The dense lines terminated in the matrix with no noticeable changes in the fine structure at the ends of the lines.

The microbody-rich fraction treated with Triton X-100 yielded pale brownish bluish sediments, which were identified electron microscopically as composed of the nucleoids, mentioned above, contaminated with a small number of glycogen particles. The fine structure of the nucleoids was noted more distinctly in the thin sections and far more obviously in the negatively stained dispersed preparations of these isolated particles than in the thin sections of the liver cells. Fig. 2 shows a section of the isolated nucleoids at low magnification; and Fig. 3 *a* and *b* are sections at higher magnification showing longitudinal and cross-sections, respectively. Figs. 4 and 5 show the negatively stained dispersed preparations of the isolated nucleoids. In the cross-sections (three nucleoids in Fig. 3 *b*) as well as in the surfaces

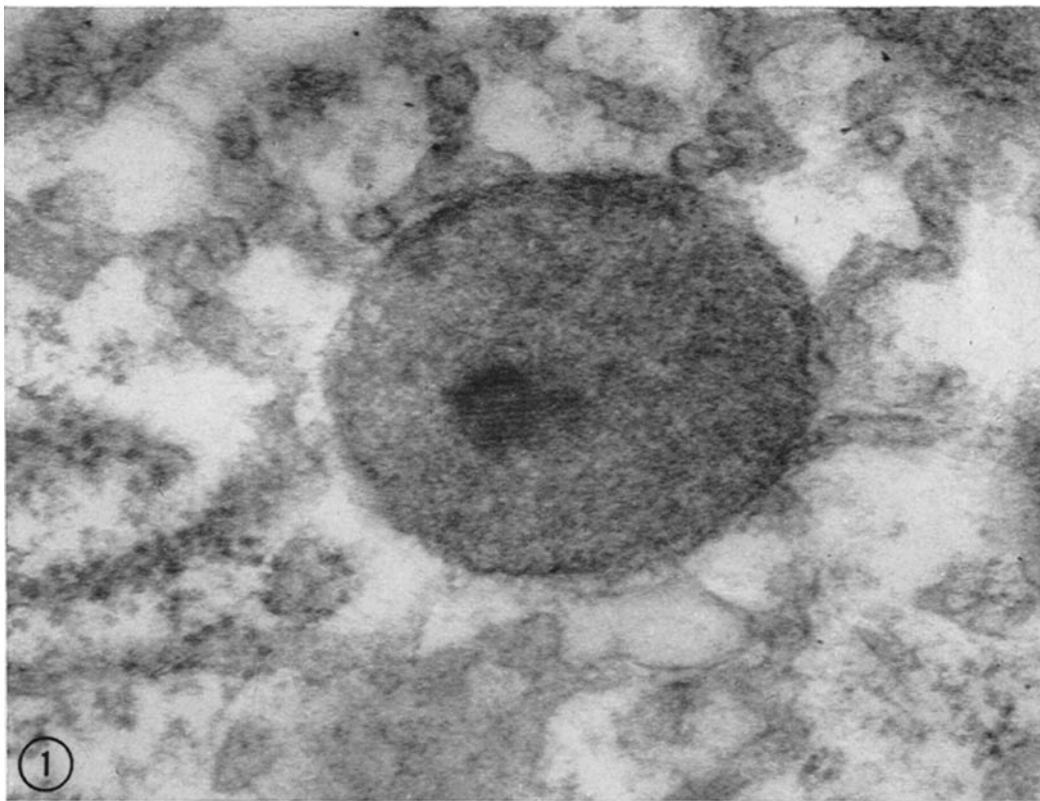


FIGURE 1 A typical microbody in a liver cell of a rat. The microbody, limited by a single-layered membrane, is seen containing a granular matrix and a somewhat eccentrically situated nucleoid. In the nucleoid, highly dense lines run parallel to each other. Neither the limiting membrane nor any special structures are found surrounding the nucleoid. $\times 100,000$.

which fell at right angles to the longitudinal axis of the dispersed nucleoids (one nucleoid at the center of Fig. 4 and that on the right side of Fig. 5), it was clearly noted that the nucleoids consist of orderly oriented circular structures. The outer diameter of the unit circles is approximately 150 A, and the inner diameter is 50 A. Ten circles, each of which shares its wall with adjacent circles, were found to be arranged forming a space of approximately 190×200 A in size at the center of the configuration. The configuration thus formed measured about 450×500 A in outer diameter and adjoined adjacent ones by sharing two circles in two directions and one circle in one direction. The nucleoids were, thereby, shown to be constructed as hexagonally packed honeycomb structures as a whole, when they were well preserved. At the marginal portion of the nucleoids, it was occasionally observed that the circles are

not arranged to form completely closed configurations and furthermore that the structures of the circles themselves are also more or less defective.

In the longitudinal sections (five nucleoids in Fig. 3 a), parallel dense lines were found passing almost completely through the nucleoids. These dense lines observed in the sections were equivalent to the light lines seen in the negatively stained dispersed specimens (Figs. 4 to 6). At higher magnification (Fig. 6), the lines appeared to be composed of small granules in a beaded arrangement, suggesting that the walls of the unit circles mentioned above are comprised of these granules. As shown in Fig. 6, the light lines observed on the surfaces parallel to the longitudinal axis of the negatively stained nucleoids were classified into two varieties: lighter lines approximately 50 A in width and less light ones 20 to 25 A in width.

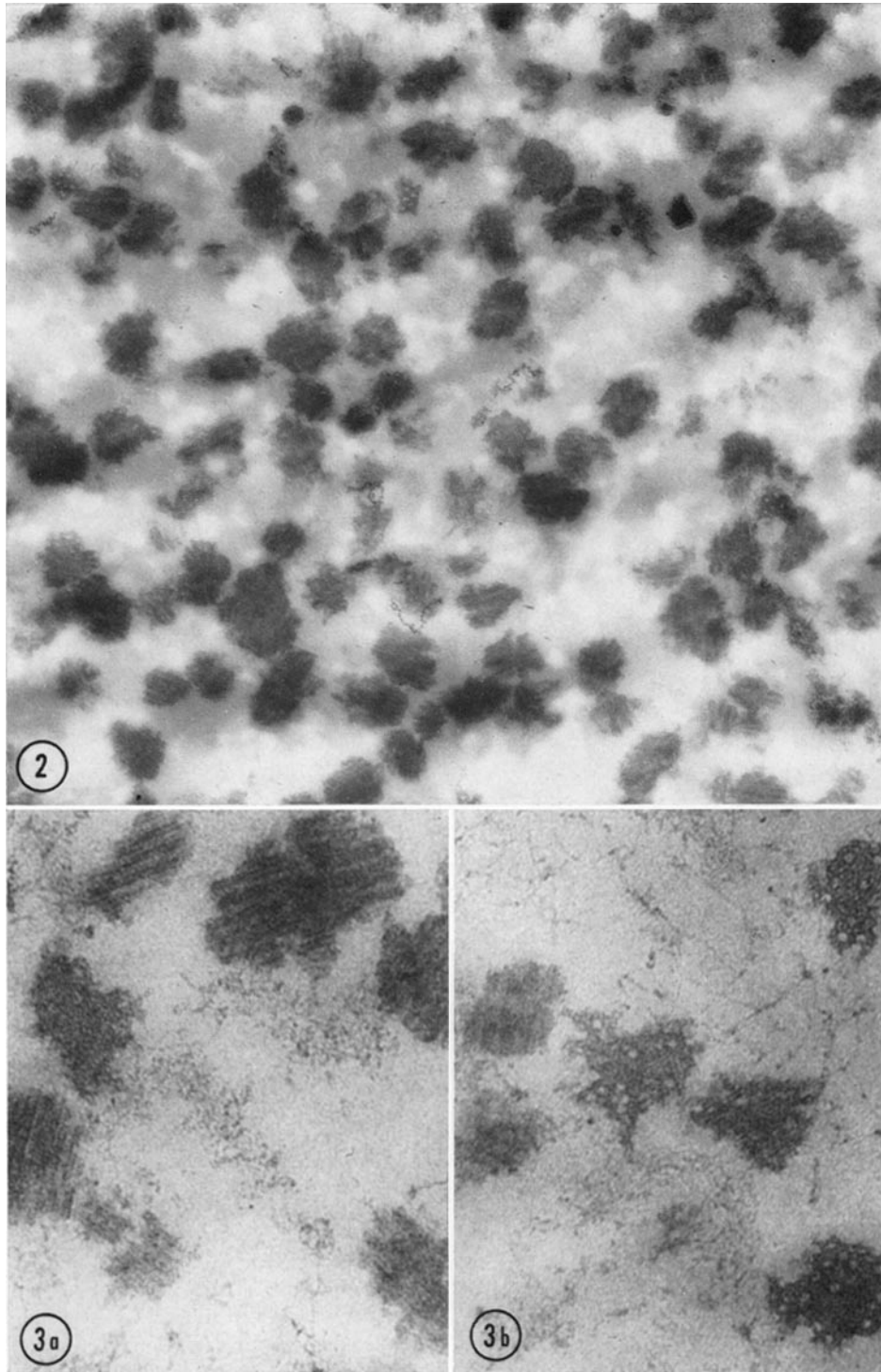


FIGURE 2 Thin section of isolated nucleoids obtained by treating the microbody-rich fraction with Triton X-100 repeatedly. A number of nucleoids are seen scattered. They are sectioned longitudinally, obliquely, and transversely. No contamination with mitochondria, lysosomes, microsomes, or intact microbodies is encountered. $\times 50,000$.

FIGURE 3 *a* Thin section of isolated nucleoids. Seven nucleoids are seen in this figure, five of which are sectioned longitudinally. $\times 100,000$.

FIGURE 3 *b* Thin section of isolated nucleoids. Three of six nucleoids in this figure are sectioned transversely. $\times 100,000$.

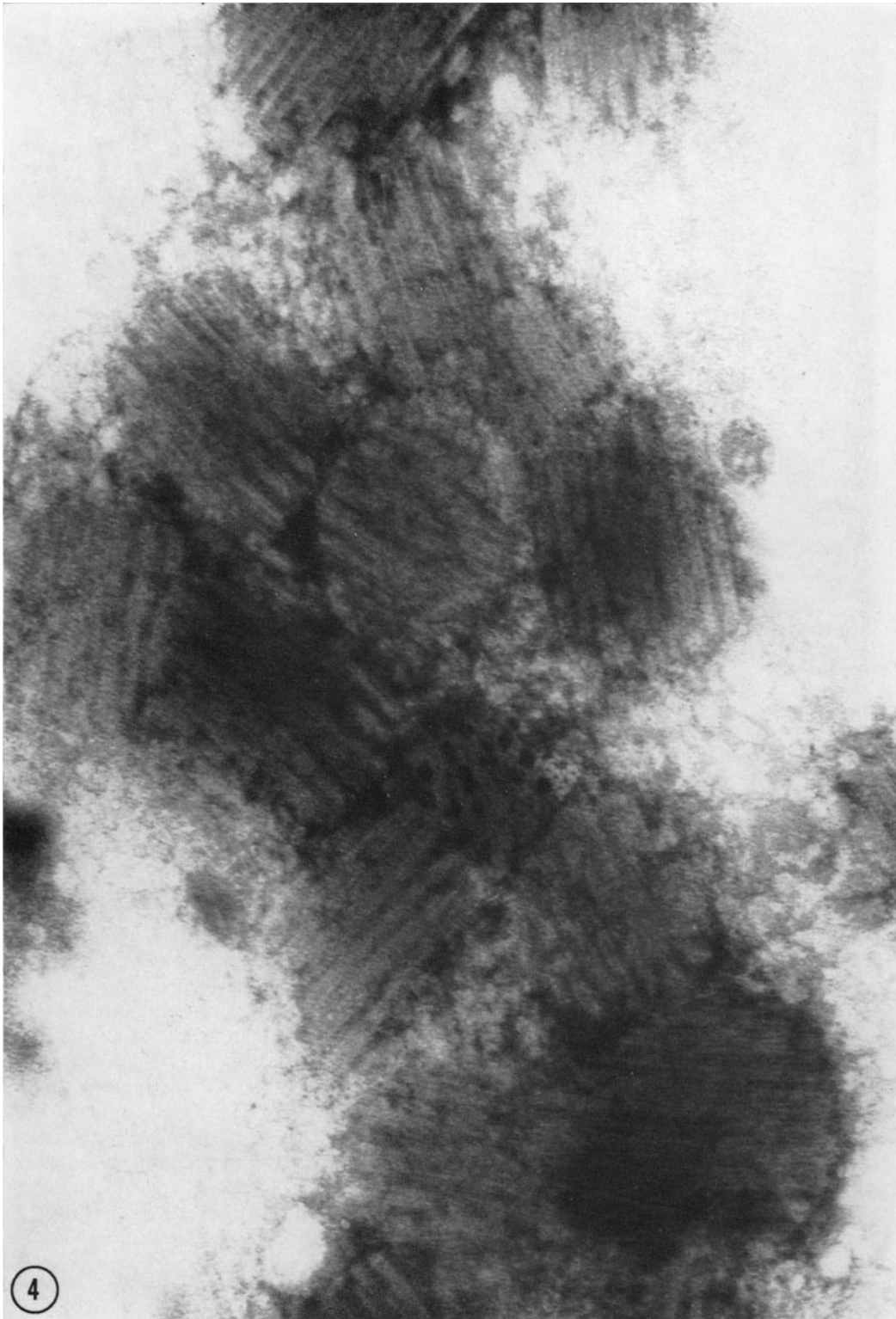


FIGURE 4 Negatively stained dispersed preparations of isolated nucleoids. Most of the nucleoids are seen to be arranged parallel to the longitudinal axis of the nucleoid. At the center of this figure is a nucleoid whose surface falls at right angles to the longitudinal axis. Glycogen particles are seen surrounding the aggregate of the nucleoids. $\times 150,000$.

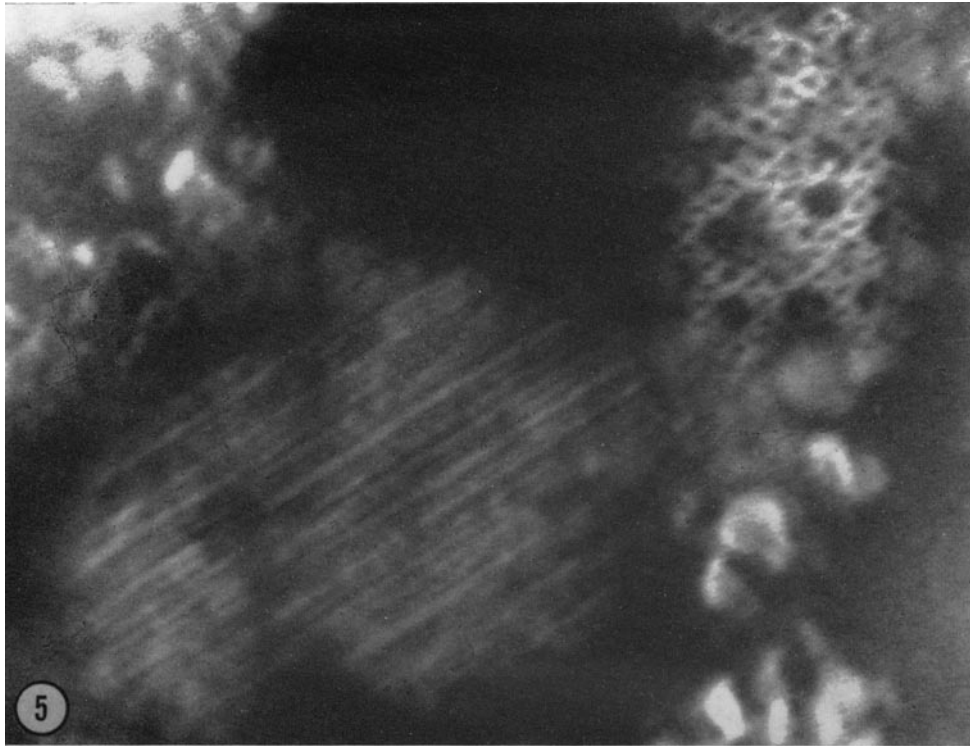


FIGURE 5 Negatively stained dispersed preparations of isolated nucleoids. One nucleoid at the left side is seen on its longitudinal surface, and the other one on the right side is seen on its end-on surface. On the end-on surface, it is clearly noted that the nucleoid consists of orderly oriented small circles. Ten circles, each of which shares its wall with adjacent circles, are seen arranged around a wider space. The unit configuration which consists of 10 circles is seen also sharing its circles with adjacent unit configurations. Amorphous deposits seen in this figure seem to be dried sucrose. $\times 300,000$.

The former lines were found to run parallel with spaces of either 150 or 50 A in between. In the wider spaces, there are two less light lines, while in the narrower spaces there is one less light line. These varieties in the width of the lines and spaces might be due to the variation in the direction in which they are observed. Neither a single circle nor a unit configuration composed of 10 circles was found scattered in microbodies in situ or in the Triton-treated preparations. The size of the nucleoids calculated from 150 particles on the negatively stained dispersed preparations ranged between $180 \times 200 \text{ m}\mu$ and $320 \times 360 \text{ m}\mu$, the mean size being $250 \times 270 \text{ m}\mu$.

The fine structure of the isolated nucleoids, thus, might be schematically expressed as shown in Figs. 7 and 8. Fig. 7 represents the cross-section or the surface which falls at right angles to the longitudinal axis of the nucleoids, and Fig. 8

shows a three-dimensional view of a nucleoid as a whole.

The particles obtained from the detergent-treated mitochondrial fraction were also examined in the negatively stained specimens, revealing that the particles involved showed the same structure of the nucleoids as that mentioned above.

2. BIOCHEMICAL RESULTS: In the previous investigations (10, 21), it was reported that a cytoplasmic fraction sedimented between 3,500 and 10,000 g (electron microscopically, the microbody-rich fraction) showed much higher activities of urate oxidase, catalase, and D-amino acid oxidase on an unit protein basis as compared with those in the mitochondrial fraction, whereas the enzyme activities themselves were only less than one-fifth of those in the whole homogenate.

In the present experiments, distribution of urate oxidase activity among the mitochondrial

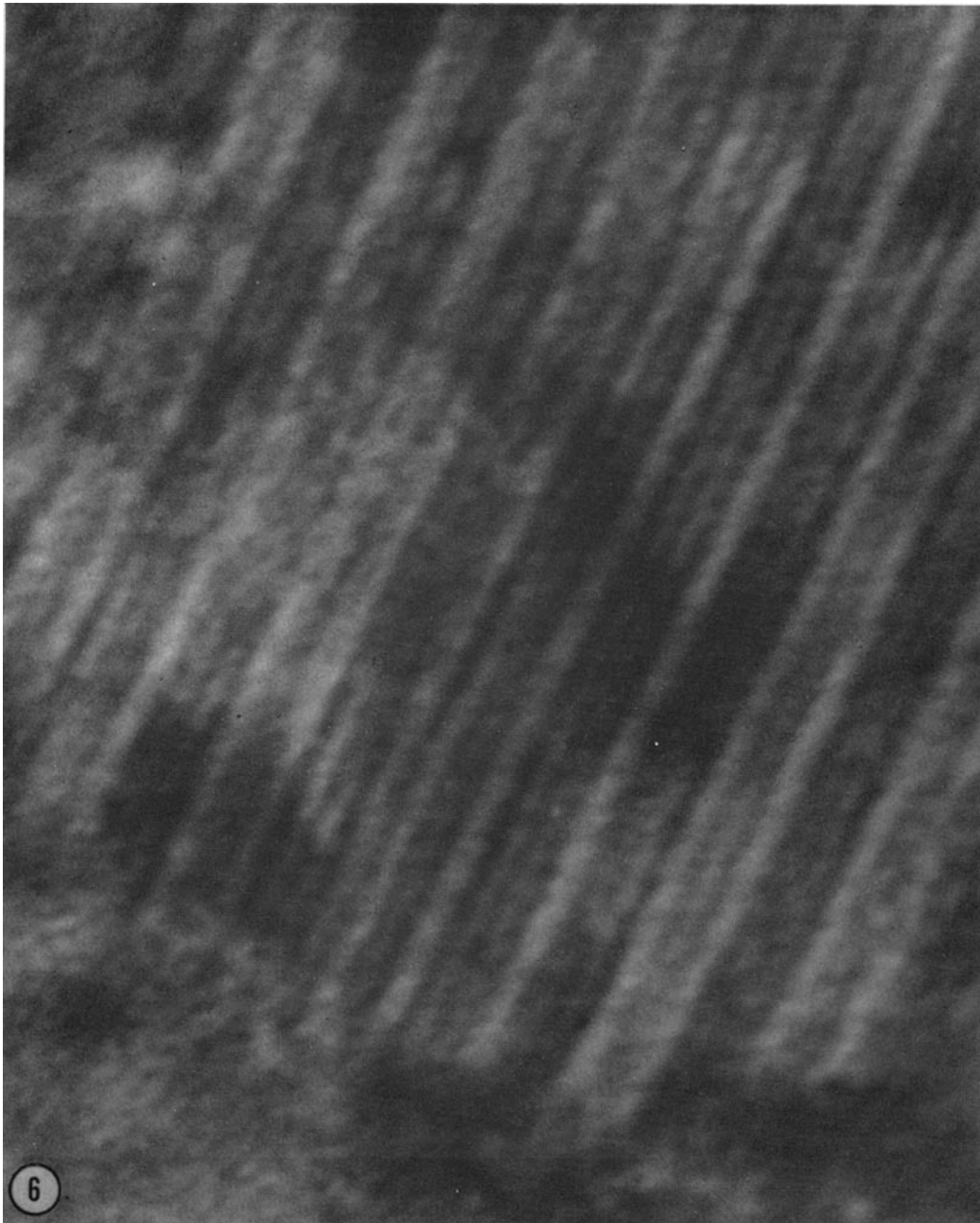
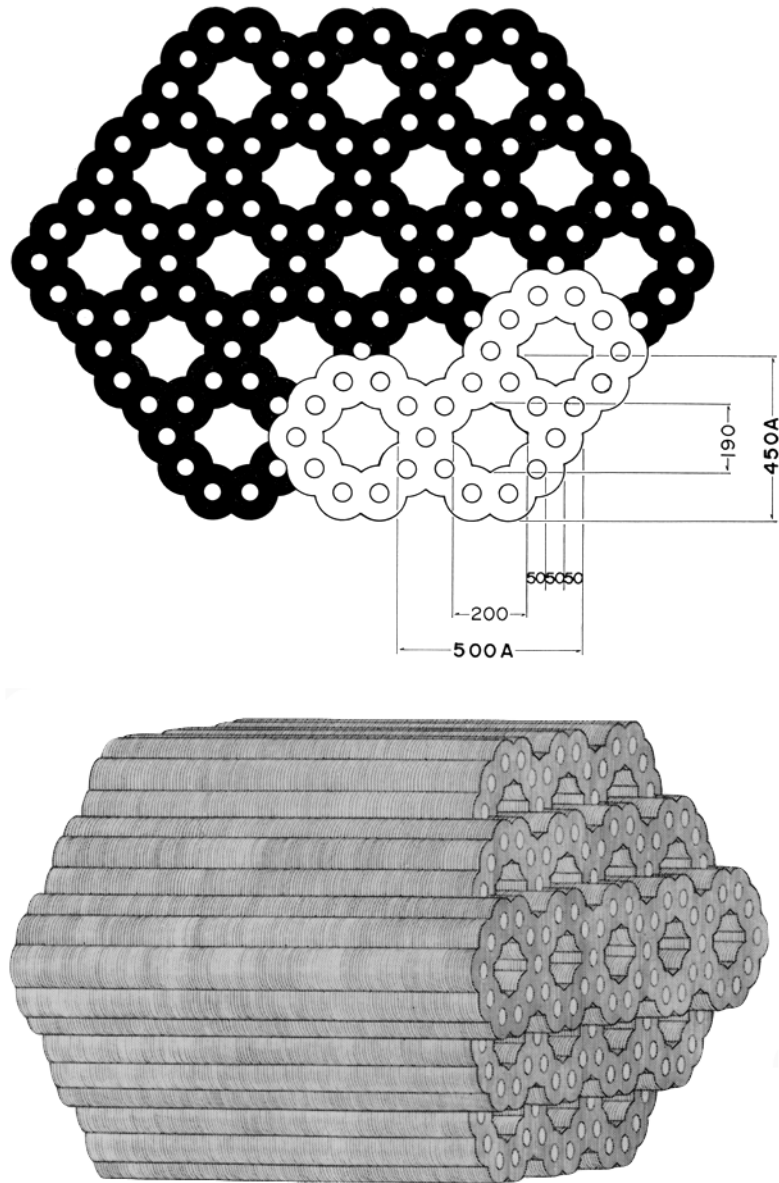


FIGURE 6 Longitudinal surface of the negatively stained nucleoid. Two varieties of light lines are seen running parallel to each other: lighter and thicker lines, and less light and thinner ones. Between two lighter lines there are either one or two less light lines. These lines are seen consisting of a beaded arrangement of small granules. $\times 800,000$.



FIGURES 7 and 8 Schematic diagram of the nucleoids. Fig. 7 presents the surface which falls at right angles to the longitudinal axis of the nucleoid, and Fig. 8 is three-dimensional view of the nucleoid as a whole.

fraction (sedimentable between 700 and 3,500 *g*), the microbody-rich fraction (sedimentable between 3,500 and 10,000 *g*), and the fraction sedimentable between 10,000 and 50,000 *g* was examined after these fractions were treated with Triton X-100. Except for urate oxidase, succinoxidase (mitochondrial origin), β -glucuronidase

(lysosomal origin), and catalase and D-amino acid oxidase (probably microbody origin) were not measurable in any fractions treated with the detergent. The activities of these enzymes in the whole homogenate were 300 (μ l O₂-uptake/20 min/80 mg wet tissues) for succinoxidase, 1.190 (Δ E at 400 m μ /30 min/25 mg wet tissues) for

β -glucuronidase, 0.806 (ΔE at 328 $m\mu/5$ min/12.5 mg wet tissues) for esterase, 0.58188 (catalase-k/1 min/2 mg wet tissues) for catalase, and 0.888 (ΔE at 520 $m\mu/20$ min/100 mg wet tissues) for D-amino acid oxidase. Therefore, using the same amount of the tissues, it was calculated that these enzyme activities in the detergent-treated fractions might be, at most, less than $\frac{1}{300}$, $\frac{1}{9520}$, $\frac{1}{12896}$, $\frac{1}{58188}$, and $\frac{1}{1776}$, respectively, as compared with the activities in the whole homogenate.

Table I shows the distribution of urate oxidase activity and protein amount in the cytoplasmic fractions mentioned above. It was noted that the

microbody-rich fraction. The activity per unit protein amount in the treated fraction sedimented between 10,000 and 50,000 g was also evidently lower than that in the microbody-rich fraction. In Table I, it also can be noted that the enzyme activity per unit protein amount in a commercial type I urate oxidase preparation (Sigma Company) is almost comparable to that of the nucleoids obtained from the microbody-rich fraction.

DISCUSSION

In our previous investigations (10, 21), it was found that a cytoplasmic fraction sedimentable

TABLE I
Distribution of Urate Oxidase Activity and Protein Amount among Various Cytoplasmic Fractions

	Homogenate	Cytoplasm	Triton-treated fractions			A type I urate oxidase preparation from Sigma Company
			700 to 3,500 g	3,500 to 10,000 g	10,000 to 50,000 g	
Urate oxidase activity (μ l O ₂ -uptake/100 mg wet tissues)	90.3 $\pm 2.71^*$	82.3 ± 0.98	41.1 ± 1.17	16.3 ± 1.64	9.7 ± 1.52	81.9
Protein (mg/100 mg wet tissues)	21.77 ± 0.625	20.43 ± 0.226	0.064 ± 0.0018	0.0104 ± 0.00203	0.0109 ± 0.00240	0.0448
Enzyme activity/Protein	4.1	4.1	640	1570	890	1830

* Standard error of the mean.

total urate oxidase activity in these fractions accounted for approximately 80% of the activity in the whole cytoplasm. The residual 20% might exist in particles of lesser densities. About 50% of cytoplasmic urate oxidase activity was recovered from the mitochondrial fraction, although the detergent-treatment of the fraction gave a sediment which is shown to consist of the nucleoids of microbodies when electron microscopically examined. Almost 20% of the activity was found in the microbody-rich fraction, and about 10% was in the fraction sedimented between 10,000 and 50,000 g .

In a comparison of the treated mitochondrial and microbody-rich fractions, a relative richness in protein amount was noted in the mitochondrial fraction. Thus, it might be said that the enzyme activity on a unit protein basis was much lower in the treated mitochondrial fraction than in the

between 3,500 and 10,000 g from polyvinylpyrrolidone-sucrose homogenates of rat liver is shown to comprise a considerable number of microbodies together with fewer mitochondria and other cytoplasmic organelles when electron microscopically examined. The fraction was thus designated as the microbody-rich fraction. Biochemical examination revealed that significantly high activities of catalase, urate oxidase, and D-amino acid oxidase on a unit protein basis were present in this fraction, supporting the observations of the Belgian researchers, who investigated the intracellular distribution of various enzyme activities using rat liver sucrose-homogenate (3-7).

On the other hand, Novikoff and Essner (9, 17) asserted that most, if not all, of the hepatic microbodies were lysosomes, although Holt and Hicks (13) were not able to demonstrate lysosomal

enzymes in microbodies in their cytochemical electron microscopic investigations. Furthermore, many researchers who investigated the intracellular localization of liver catalase reported that the enzyme might be located in mitochondria. Yet, a possibility of transformation from microbodies to mitochondria was suggested by Rouiller and Bernhard (19), who reported that a series of membranous structures resembling cristae mitochondriales were seen in microbodies in rat liver cells.

These discrepant assumptions concerning the morphological nature and functional entity of microbodies are considered to be ascribed to the difficulty in isolation of microbodies in a pure state as well as in a state wherein they are capable of retaining the enzymes involved.

The presence of characteristic inclusions is undoubtedly a unique morphological feature of microbodies of rat liver cells, being helpful for the identification of the organelle. The inclusions were described as multimembranous or crystalloid structures by Beaufay and Berthet (5), double-membranous structures by Rouiller and Bernhard (19), and as lamellar nucleoids by Holt and Hicks (13). However, it might be appropriate to term these inclusions simply "nucleoids" or "crystalloid nucleoids," since the present observations conflict with the "membranous" or "lamellar" nature of the inclusions.

Among the probable enzymes of microbodies, urate oxidase was found to be firmly bound to the particles and not to be solubilized even by exposing the particles to a detergent such as Triton X-100, while catalase and D-amino acid oxidase were readily released from the particles by treatment with the detergent (10, 21). With respect to this fact and also the fact that the detergent did not inhibit the enzyme activity, the isolation of the urate oxidase-containing portion of the particles in a highly pure state may be expected. With a conventional differential centrifugation of the homogenate, the microbody-rich fraction showed slight contamination with mitochondria, lysosomes, and microsomes, electron microscopically as well as biochemically; however, the detergent treatment resulted in a disappearance of these contaminations and also of intact microbodies. The only particles seen in this treated fraction were the nucleoids of microbodies, except for a scanty contamination with glycogen particles. This was confirmed not only electron micro-

scopically, but also in biochemical examinations which revealed practically a complete absence of succinoxidase, β -glucuronidase, esterase, catalase, and D-amino acid oxidase activities.

During the preparation of the present paper, a report of Baudhuin, Beaufay, and de Duve (4) was published, stating that the nucleoids were also prepared by fractionation of rat liver homogenate after intravenous injection of Triton WR-1339. In their preparation 4, a great number of the free nucleoids were seen; however, the nucleoids were still contaminated with a considerable number of microsomal contours. Biochemical data on this preparation showed that enzymes which locate in lysosomes and microsomes more or less remained.

The nucleoids obtained from microbodies in the present experiments showed a characteristic structure which was practically the same as that observed in thin sections of liver cells, *i.e.* a crystalloid structure, as described in detail in the experimental results. Figs. 7 and 8 represent the proposed schematic diagram of this structure. During the present experiments, a brief paper of Hruban and Swift (14) was published reporting a suggested structure of the nucleoids, based on a comparison of the structures electron microscopically observed in thin sections of rat liver microbodies and in a commercial urate oxidase preparation. The structures of the isolated nucleoids emphasized in the present observations on the thin sections and the negatively stained dispersed preparations are considered to be similar to those suggested by Hruban and Swift in outline; however, in the detailed description there might be some difference between our proposal and their suggestion. The nucleoids may not contain cylinders of two different sizes, but might consist of one species of tubules having one and the same size which are merely arranged to form longitudinal spaces of a greater size at the center of their configuration. Furthermore, in the scheme of Hruban and Swift, the tubules might not be so arranged as to form a hexagonally packed honeycomb structure in the cross-section. To produce a complete structure of the nucleoids, the unit configuration consisting of ten tubules might be arranged in such a manner as to share two tubules in two directions and one tubule in one direction with the adjacent unit configuration, as shown in Fig. 7. The structure of the nucleoid as a whole might thus be represented as shown in the three-

dimensional view in Fig. 8. This analysis of fine structure was made far more precisely on the isolated particles than on the particles in the liver cells *in situ*; yet, it was done more minutely on the negatively stained dispersed preparations than on the thin sections of the isolated particles. Defective arrangement of the tubules occasionally found at the marginal portion of the nucleoids may have resulted from the growth of the nucleoids which may occur at this portion by appositional crystallization of the protein associated with the enzyme, although some destruction could occur during the preparation.

With conventional differential centrifugation of rat liver homogenate, a considerably high urate oxidase activity was also recovered from the mitochondrial fraction as well as from fractions sedimentable at gravities greater than that needed for sedimentation of the microbody-rich fraction. Electron microscopic examinations of the detergent-treated specimens of these fractions would indicate that the urate oxidase activities involved are due merely to the fact that these fractions are contaminated with microbodies. As was described in the experimental results, the enzyme activity on an unit protein basis was much lower in these treated fractions than in the treated microbody-rich fraction. The question may arise whether the treated mitochondrial fraction is not so pure as the treated microbody-rich fraction with respect to urate oxidase. The difference in the fine structures of the nucleoids between the mitochondrial and microbody-rich fractions may be beyond the

resolution of electron microscopy. Yet, no contamination with cytoplasmic organelles other than the nucleoids and glycogen particles was found in the treated mitochondrial fraction. Thus, the relative richness of protein amount in the treated mitochondrial fraction might not simply be ascribed to contaminations with inert proteins derived from other cytoplasmic components. The relative richness of protein may depend on the richness of protein in the nucleoid skeleton with which urate oxidase is associated.

From a comparison run between the nucleoids prepared in the present experiments and the commercial type I urate oxidase (Sigma Company) which was examined by Hruban and Swift, it is suggested that the purity of the nucleoids with respect to the enzyme activity, at least of those nucleoids prepared from the microbody-rich fraction, is almost comparable to that of the urate oxidase purified by other biochemical means.

Under investigation now is the problem of whether the characteristic structure of the nucleoids is obligatory on urate oxidase activity or whether the enzyme of higher specific activity is further extractable from the nucleoids.

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