THE SYNTHESIS AND TURNOVER
OF RAT LIVER PEROXISOMES

III. The Size Distribution of Peroxisomes
and the Incorporation of New Catalase

BRIAN POOLE, TOKUHIKO HIGASHI, and CHRISTIAN de DUVE

From The Rockefeller University, New York 10021. Dr. Higashi's present address is the
School of Pharmacy, Showa University, 1-5-8 Hatano-Dai, Shinawaga-Ku, Tokyo, Japan

ABSTRACT

Rat liver peroxisomes have been separated according to size by zonal sedimentation. A method is described for calculating the size of the particles from their final position in the gradient. Peroxisomes seem biochemically homogeneous throughout their size distribution. 3 hr after injection of tritiated leucine, the specific radioactivity of catalase is the same in peroxisomes of different sizes, and it remains so for up to 1 wk after administration of the precursor. This observation rules out the possibility that peroxisomes have an extended period of independent growth. If individual particles maintain an independent existence, they must be formed very rapidly. The other possible explanation is that peroxisomes exchange material within the liver cell.

INTRODUCTION

Previous papers in this series have dealt with the fractionation (13) and turnover (16) of peroxisome proteins. It was shown that these proteins lose radioactivity incorporated from labeled precursors in a simple exponential fashion, and also that all major protein components of the peroxisomes turn over with the same kinetics. These results suggest strongly that rat liver peroxisomes are destroyed as wholes by a random process.

In this paper we report the results of some studies bearing on the mechanism of biogenesis of peroxisomes. The only biochemical data on peroxisome formation come from the work of Higashi and Peters (10). These authors studied the specific radioactivity of catalase in fractions of rat liver separated by differential centrifugation at various times after the injection of labeled leucine. Their results seem to indicate that catalase is formed in the endoplasmic reticulum (ER)

and then transferred to the peroxisomes. This possibility receives support from numerous morphological observations that have shown the existence of connections between the endoplasmic reticulum and microbodies (for reviews see references 7 and 11). According to Novikoff and Shin (14), ER-like projections from the microbodies are seen so frequently as to suggest that the particles are always attached to the endoplasmic reticulum. If such is the case, one can picture the biogenesis of peroxisomes as taking place by a budding mechanism; newly synthesized proteins would move from rough to smooth elements of the endoplasmic reticulum and then accumulate in a growing appendage projecting from the smooth endoplasmic reticulum. According to most observers, the Golgi apparatus would not be involved in this process.

A model of this kind has been examined by de

Duve and Baudhuin (7), who assumed that peroxisomes grow linearly for a fixed length of time and then are destroyed. They showed that the kinetics of catalase recovery in animals treated with aminotriazole and of catalase loss in animals receiving allylisopropylacetamide, reported by Price et al. (17), were compatible with such a model, with a ripening time for the particles of the order of 4.4 days. However, the finding that radioactive precursors incorporated into peroxisome proteins decay exponentially (16) cannot be reconciled with this model, which predicts that label should be lost from peroxisomes at a linear rate for a period corresponding to the growth time of the particles, after which no label should remain in them. Of course, the interpretation of turnover data from radioisotope experiments is always complicated by the problem of precursor reutilization, but even with labeled δ-aminolevulinic acid, which should not be reutilized at all for catalase synthesis, the same exponential kinetics were observed. Moreover, it has been found by Poole (15), who has explored the kinetic properties of a simple reutilization model, that random destruction complicated by reutilization can give rise to a pseudoexponential decay curve of the incorporated label, whereas the same is not true for particles with a fixed lifetime. Consequently, we can take it as established that the probability of destruction of peroxisome proteins is independent of their age. Even if such is the case, the peroxisomes could still grow as buds off the endoplasmic reticulum, either with a continuous increase in size throughout their entire lifetime or for a certain period until they reach terminal size. The experiments described in this paper were designed as a test of this possibility. The argument on which they were based runs as follows.

In a population of growing particles the ratio of the size increment in unit time to the size of the particles should be greater in the smaller than in the larger particles. This means that after a short period of incorporation of isotopically labeled protein into the population of growing particles, the specific radioactivity of this protein should be higher in the smaller than in the larger particles, since in the latter the new material will be diluted in a larger amount of material already present. If all particles grow at the same rate, their specific radioactivity immediately after a short pulse of labeled precursor should be inversely proportional to size. The dependence of specific radioactivity on size would be less marked if the larger particles grew faster than did the smaller ones, and it would fail to be observed if the growth rate of the particles were proportional to their size. However, even in such an event, which implies a rather improbable autocatalytic growth mechanism, an influence of particle size on specific radioactivity should manifest itself at later times after the injection of the labeled precursor. Since labeled particles would go on increasing in size while new unlabeled particles are formed, we should witness a progressive transfer of label from the smaller to the larger particles. This systematic change should take place whatever the kinetics of particle growth, unless the time needed for the full growth of a particle is very short.

In the present experiments we have used zonal sedimentation in a B-XIV rotor to separate rat liver peroxisomes according to size. The specific radioactivity of catalase was determined in different size classes at times ranging between 3 hr and 1 wk after the injection of tritiated leucine. We have found no evidence of an influence of size on the specific radioactivity of this peroxisomal protein over the whole experimental period.

METHODS

Previous papers have described our methods of enzyme assays (12), immunochemical separation of catalase (16), and isotope counting (16).

Zonal sedimentation was performed in a B-XIV rotor (2) operated by a Spinco L ultracentrifuge (Beckman Instrument, Inc., Spinco Div., Palo Alto, Calif.). Two slightly different procedures were used depending upon the aim of the experiment. In either case, the tissue preparation was layered over a shallow stabilizing gradient of sucrose concentration. All sucrose solutions contained 0.1% (v/v) of ethanol.

In those experiments in which the catalase was precipitated with antiserum, the gradient was formed by pumping into the rotor from the inside the following series of four layers of decreasing sucrose concentration: 130 ml of 0.7 M, 110 ml of 0.6 M, 90 ml of 0.5 M, and 80 ml of 0.4 M. These layers were left in the rotor for an hour or so to smooth out the sharp boundaries. A sample of 50 ml of a postnuclear supernatant (E fraction, [9]), 100 mg of liver per ml, was pumped into the rotor followed by an overlayer of 130 ml of 0.2 M sucrose. The rotor was spun at 10,000 rpm for 15 min and then 30-ml fractions were collected into tared Spinco 30 rotor tubes. Each tube was weighed, its contents were mixed, a small sample was removed for analysis, and the remainder

was spun at 30,000 rpm for 1 hr to sediment the particles. This sediment was resuspended in 8 ml of solution containing 0.25% (v/v) Triton X-100 and 0.025 M sodium phosphate buffer, pH 6.8. This suspension was spun in the Spinco 40 rotor for 30 min at 40,000 rpm. The supernatant was then used for catalase immunoprecipitation.

In experiments to determine the size distribution of particles, a 420 ml gradient of sucrose concentration between 0.4 M and 0.8 M was pumped into the rotor from the outside. The gradient was formed by adding 1.15 M sucrose to the gradient-forming device, described by Anderson and Rutenberg (1), containing 355 ml of 0.4 M sucrose. This gradient was then displaced to the inside of the rotor with 60% (w/w) sucrose and a 20 ml sample of E fraction was pumped in over it, followed by an overlayer of 160 ml of 0.2 M sucrose. After spinning for 15 min at the chosen speed, the rotor contents were pumped out and collected in 15-ml fractions. The initial sucrose concentration profile and the sucrose concentrations of the fractions are shown in Fig. 1.

The peroxisome radius corresponding to the boundary between adjacent fractions was calculated in the following way. Unless otherwise indicated all quantities will be expressed in cm-g-sec units. For a

small spherical particle sedimenting in a centrifugal field we can write:

$$\frac{v}{\omega^2 x} = \frac{2r^2(\rho_p - \rho_m)}{9\eta} \qquad (a)$$

where v is the particle velocity, ω is the angular velocity of the rotor, x is the radial distance of the particle from the rotor axis, r is the radius of the particle, η is the viscosity of the suspension medium, and ρ_p and ρ_m are the densities of the particle and the medium, respectively (8). Thus:

$$\frac{dx}{dt} = \frac{2r^2(\rho_p - \rho_m)x\omega^2}{9\eta} \qquad (b)$$

and

$$\frac{9\eta\ dx}{2r^2(\rho_p - \rho_m)x} = \omega^2\ dt \qquad (c)$$

In the equation η, ρ_p, and ρ_m are complicated functions of x and if the particles shown any response to osmotic pressure, then r will vary with x as well. Moreover, we cannot measure x directly but only V, the volume of fluid removed from the inside of the rotor. Consequently, we made an approximate stepwise integration, using a digital computer.

To begin with, third-degree power series were calculated by the least squares method giving density, ρ_m, of sucrose solutions as a function of sucrose molarity, c, (data of Bates [3]), and giving the radial distance in the rotor, x (data of Anderson [personal communication]), and the sucrose molarity as a function of the volume, V, of fluid removed from the rotor. This last function was calculated from the measured density of the fractions removed, and the dotted line in Fig. 1 shows the curve used for a typical experiment. Our equations are:

$$\rho_m = 1.0 + 0.1365\ c - 2.022 \times 10^{-3}\ c^2$$
$$- 1.764 \times 10^{-4}\ c^3 \qquad (d)$$
$$x = 1.948 + 1.322 \times 10^{-2}V$$
$$- 1.647 \times 10^{-5}V^2 + 1.154 \times 10^{-8}V^3 \qquad (e)$$
$$c = C(V) \quad \text{(calculated for each experiment)} \qquad (f)$$

The viscosity poses a somewhat more difficult problem, since it depends upon temperature as well as upon sucrose concentration. However, over the temperature range from 0 to 15°C the logarithm of the viscosity at a particular sucrose concentration is linearly related to the inverse of the absolute temperature. We determined by the least squares method the linear parameters for temperature de-
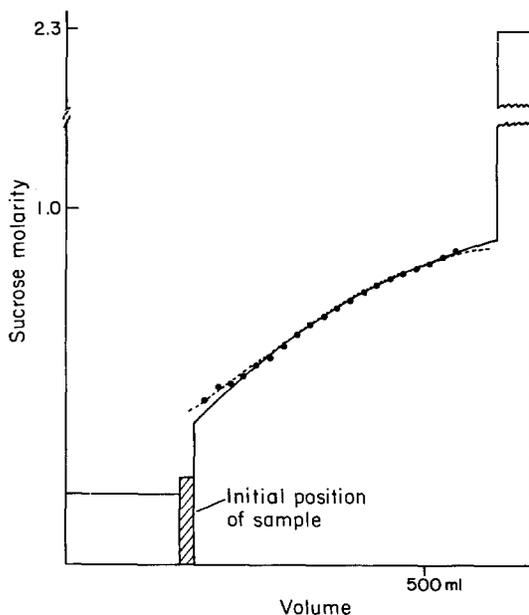

FIGURE 1 The initial and final sucrose concentration profiles in the B-XIV rotor. The solid line shows the theoretical initial curve and the points show measured final concentrations. The dotted line shows the cubic function fitted to the points by the least squares method and used in the mathematical analysis.

pendence of sucrose viscosity based on the measured values of Bates (3) at several sucrose concentrations. We then calculated third-degree power series relating these parameters to sucrose concentration. We found finally:

$$\log_e \eta' = -8.10 - 0.693c + 0.208c^2 - 0.572c^3$$
$$+ \frac{2369 + 481c - 27c^2 + 199c^3}{T} \quad (g)$$

where η' is in centipoises and T is in degrees Kelvin. This function gives viscosities that agree with Bates's measurements and interpolations within 1%.

Beaufay et al. (6) have shown that the density of peroxisomes in sucrose solutions containing no other low molecular weight solutes is given by:

$$\rho_p = \frac{1.251 + 2.55\rho_m}{3.55} \quad (h)$$

This equation is valid for the median of the density distribution of the peroxisome population and the numerical constants have been evaluated very precisely.

Integrating (c) over the path of the sedimenting particle we have:

$$\frac{9}{2r^2} \int_{x_0}^{x_i} \frac{\eta \, dx}{(\rho_p - \rho_m)x} = \int_0^t \omega^2 \, dt \quad (i)$$

Using equations (d)–(h) we can evaluate the left integrand in equation (i) for any value of x. We have integrated the expression using Simpson's rule and equal volume increments of 2 ml. The time integral of the squared angular velocity (right integral of equation $[i]$) was calculated for each run from a recording of the rotor velocity throughout the entire experiment including acceleration and deceleration. The speed recording system was calibrated with a stroboscope.

These calculations are affected by three types of uncertainties. One is the temperature of the solution through which the particles sediment. It is not possible to control the temperature of the B-XIV rotor, and we find a difference of as much as a few degrees between the fluid pumped into it and that pumped out. We suppose that most of this heating occurs during passage through the rotating seal on top of the rotor but there is no way to be certain of this and no way to measure the temperature of the rotor itself while it is spinning. In practice we take the mean of the input and output temperatures. This possible error of one or two degrees in the sedimentation temperature would make an error of 2 or 3% in the calculated radius.

Another uncertainty arises from the fact that all the particles do not sediment from the same starting

position owing to the finite thickness of the original layer. This point will be discussed below.

Finally, it could be that there is some correlation between the density of the particles and their size, in which case there would be a systematic error in our calculated radii at least for particles of some sizes. This question will also be considered in the discussion of the results.

RESULTS

Size Distribution of Peroxisomes

Under Materials and Methods we have described a method for the calculation of the radius of peroxisomes from the effluent volume from the rotor in which they are present after centrifugation. As a check on this method we performed zonal sedimentation experiments on liver homogenates from three different rats. Each liver preparation was centrifuged at two different speeds. The sedimentation time was held constant at 15 min. Catalase activity was assayed in the subfractions from the rotor. The data from each of these six experiments were analyzed to give a size distribution for the peroxisomes. We found that the median radius calculated for the particle population of a given rat liver was essentially the same whether centrifugation was performed at
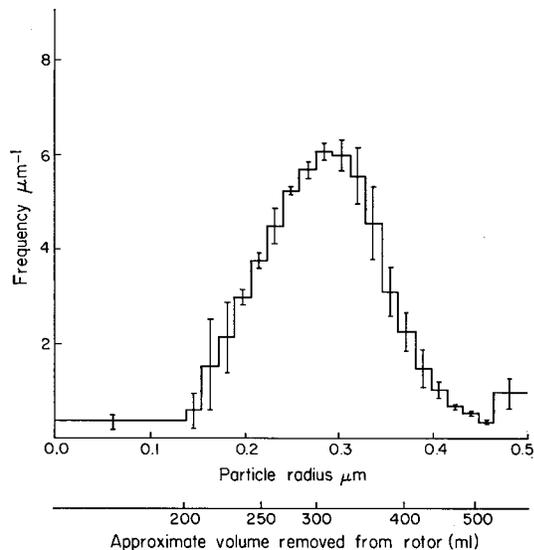

FIGURE 2 Apparent size distribution of rat liver peroxisomes after spinning 15 min at 7500 rpm. The histogram was calculated from the distribution of catalase activity after zonal sedimentation. Average of three experiments. Vertical bars are standard deviations.

the higher or at the lower speed. But in each case the calculated distribution histogram was somewhat flatter and wider with the lower centrifugation speed. This point will be commented upon below. Small but reproducible differences in the position of the mode were found among the three rats, suggesting that there may be individual variations in the size distribution of peroxisomes.

The three distributions obtained at 7500 rpm were averaged by the method of Leighton et al. (12) and are shown in Fig. 2. The distributions obtained at 10,500 rpm are represented in a similar fashion in Fig. 3. Below these distributions we have indicated the approximate effluent volume corresponding to the scale of calculated particle radius.

We see that while these two distributions are centered around the same median their shapes are significantly different. The distribution calculated after centrifugation at the lower speed is considerably broader than the other one where the particles were driven more deeply into the gradient. The origin of this discrepancy lies in the finite thickness of the original layer of sample in the rotor which causes the computed size distribution of particles to be broader than it is in reality. The error is less as the particles move further down the gradient. Thus, the distribution of Fig. 3 is more accurate than that of Fig. 2, although its dispersion may still be somewhat overestimated.
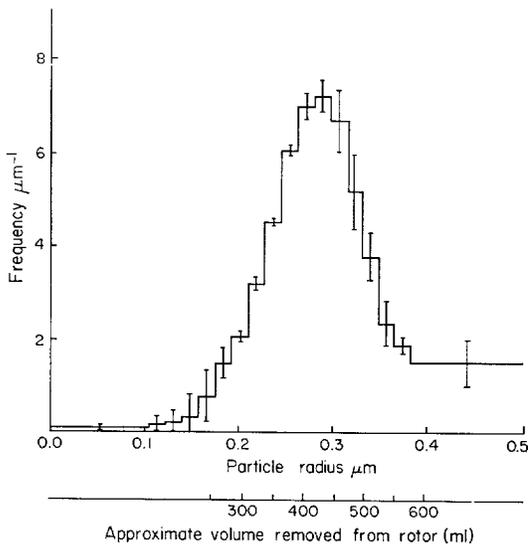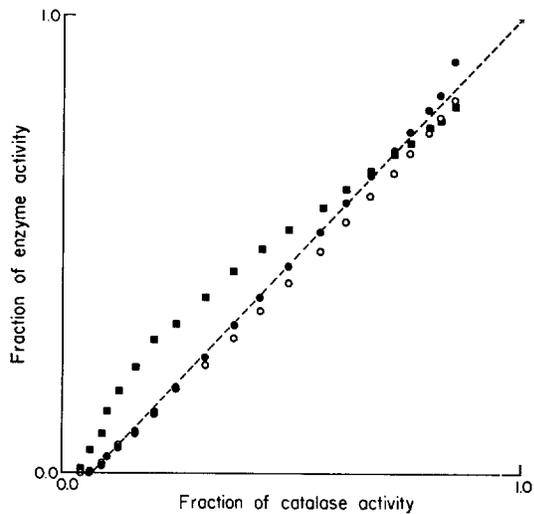

FIGURE 4 Comparison of frequency distribution of catalase with L-α-hydroxy acid oxidase, ●; D-amino acid oxidase, O; and urate oxidase, ■. Cumulative distribution from smaller to larger particles.

In Fig. 4 we have plotted the cumulative activity of three other peroxisomal enzymes against the activity of catalase, taking subfractions from zonal sedimentation in order of increasing sedimentation rate. L-α-hydroxy acid oxidase and D-amino acid oxidase activities correlate very well with the activity of catalase, indicating that the peroxisomes are highly homogeneous in these three enzymes. Urate oxidase shows significant deviations but this is undoubtedly due to the presence in our preparations of free cores arising from ruptured particles. All the urate oxidase activity of the peroxisome is carried by the core (5).

Distribution of Newly Formed Catalase According to Particle Size

Rats were given 1 mCi of L-leucine-4,5-[3]H (New England Nuclear Corp., Boston, Mass., 5 Ci per mmole) intraperitoneally and killed in groups of five at various times after injection. A sample of the pooled liver homogenates from each group was subjected to zonal differential sedimentation as in the previous experiments. The catalase in each subfraction was separated immunochemically and its specific radioactivity was determined. The small amounts of catalase present did not allow the preliminary purification of the catalase that was performed in our previous studies (16)

FIGURE 3 The same as Fig. 2 except centrifugation was performed for 15 min at 10,500 rpm.

to remove impurities that are carried down with the immunoprecipitate in each subfraction. However, we hoped that these impurities might be separated largely by zonal centrifugation. To check this we measured the ratio of catalase activity to immunoprecipitate in each subfraction. These data are plotted in Fig. 5. We see that there is a range of particle radius between about 0.2 and 0.3 μm where the contamination

of the immunoprecipitate is very small. This was the region studied in the incorporation experiments.

The results obtained are plotted in Fig. 6. At no time between 3 hr and 1 wk after injection do we detect any significant variation of the specific radioactivity of catalase with particle size, in spite of an almost fourfold difference in peroxisome volume from one end of the distribution to the other.
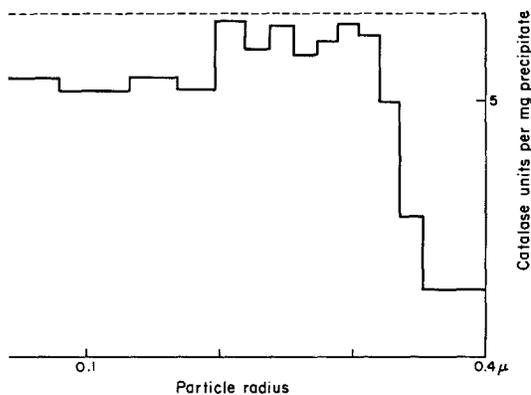

FIGURE 5   Ratios of catalase activity in extracts of particle fractions to immunoprecipitate formed after incubation of the extracts with antiserum. The horizontal dotted line indicates the ratio measured with catalase purified according to reference 13.

DISCUSSION

In Methods we discussed briefly the uncertainties inherent in our calculation of peroxisome size from zonal sedimentation behavior. The most serious of these is the assumption that the density properties of all particles irrespective of size can be approximated by the median density properties of the whole population. We have shown the apparent homogeneity of enzyme distribution in particles of different sizes, and the homogeneity in particles of different density has been established previously (12). This suggests that the matrix of the particles may be very uniform throughout the population. However, it is evident that the ratio of membrane area to contents must be larger in smaller particles than it is in larger ones. A particle with proportionally more membrane would be expected to be less dense than one with less membrane. If this were true, it would mean that small particles would sediment more slowly than our equations predict. Large particles would sediment more quickly. The effect of this would be to widen the apparent size distribution of the particles. We have seen above that the finite thickness of the starting layer also contributes to make the computed size distribution broader than the true distribution. There are thus two reasons to believe that rat liver peroxisomes vary less in size than is indicated by Fig. 3. Their computed median radius should not be affected significantly by either cause of error and probably approximates the true value fairly closely. The size distribution of peroxisomes has also been studied by Baudhuin (4) on particulate fractions from rat liver by means of both biochemical and morphological methods. His biochemical results, which were derived from the analysis of the shape of the sedimentation boundary of particulate catalase in a sucrose gradient, are in fair agreement with ours. On the other hand, his morphological measurements differ signifi-
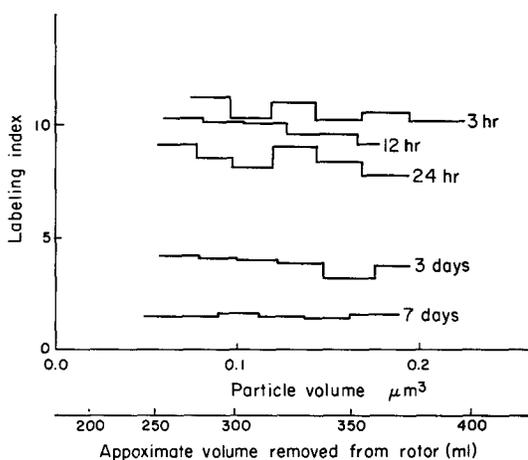

FIGURE 6   Specific radioactivity of catalase in rat liver peroxisomes in subfractions from zonal sedimentation at various times after injection of tritiated leucine. Labeling index (16) is ratio of the specific radioactivity of catalase (in cpm per mg catalase) to the total amount of radioactive leucine administered per unit weight of animal (in cpm per milligram whole rat).

cantly from the biochemical estimations; the distribution is narrower and is centered around a smaller median radius. Particle contraction as a result of fixation and embedding and lack of recognition of a number of smaller profiles are believed by Baudhuin (4) to affect the morphological measurements, which he considers less reliable than the values derived from the sedimentation properties of the particles.

Fortunately, it is not essential for the purpose of our study that the size distribution of the particles be known with great precision. What is important is that the separation achieved by zonal sedimentation should depend mainly on size, and there can be little doubt that such is indeed the case. As we have explained in the Introduction, a population of growing particles, whatever the kinetics of growth, should show some change with time in the distribution of labeled material incorporated into the particles during a period short in relation to the period during which a particle undergoes growth. We have seen no indication of such temporal changes of label distribution. There could be two very different explanations for this observation. We may assume either that the peroxisomes within a liver cell exist as individuals, each with a life history independent of the others, or that in fact the contents of the peroxisomes form a single pool within which there is rapid exchange. In the former case, considering the peroxisomes as individuals (the intuitive concept arising from static electron microscopic images), we can conclude that the period of formation of a peroxisome is short relative to the time scale of our experiments. The earliest time point plotted in Fig. 6 is 3 hr. However, we have made less complete observations at 1 hr

without detecting any size dependence of the catalase specific radioactivity. So we must conclude that the growing period of peroxisomes (if they exist as individuals) is less than 1 hr or less than one-fiftieth their average lifetime.

The other possibility is that peroxisomes do not exist as independent individuals but continuously or intermittently exchange material with one another. This could take place by direct fusion and fission of particles or by means of an intermediate system, such as the apparent attachments to the endoplasmic reticulum which we mentioned in the Introduction.

In further studies we should be able to distinguish between these two possibilities. If the peroxisomes exist as individuals that form rapidly, then after a short period of labeling only a small number of particles will be labeled and they should remain the only particles carrying the label. If, on the other hand, the peroxisomes exchange material, then we would expect that whatever the initial fraction of labeled particles, after a time the label would be distributed homogeneously throughout the population. Electron microscopic radioautography of isolated peroxisomes should settle this question.

REFERENCES

1. ANDERSON, N. G., and E. RUTENBERG. 1967. Analytical techniques for cell fractions. VII. A simple gradient-forming apparatus. *Anal. Biochem.* 21:259.
2. ANDERSON, N. G., D. A. WATERS, W. D. FISHER, G. B. CLINE, C. E. NUTLEY, L. H. ELROD, and C. T. RANKIN, JR. 1967. Analytical techniques for cell fractions. V. Characteristics of the B-XIV and B-XV zonal centrifuge rotors. *Anal. Biochem.* 21:235.
3. BATES, J. F., and Associates. 1942. Polarimetry, saccharimetry and the sugars. *Nat. Bur. Std. (U.S.), Circ.* C440.
4. BAUDHUIN, P. 1968. L'analyse morphologique

quantitative de fractions subcellulaires. Vander, Louvain.
5. BAUDHUIN, P., H. BEAUFAY, and C. DE DUVE. 1965. Combined biochemical and morphological study of particulate fractions from rat liver. *J. Cell Biol.* 26:219.
6. BEAUFAY, H., P. JACQUES, P. BAUDHUIN, O. Z. SELLINGER, J. BERTHET, and C. DE DUVE. 1964. 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.* 92:184.
7. DE DUVE, C., and P. BAUDHUIN. 1966. Peroxi-

somes (Microbodies and related particles). *Physiol. Rev.* **46**:323.

8. DE DUVE, C., J. BERTHET, and H. BEAUFAY. 1959. Gradient centrifugation of cell particles. Theory and applications. *Progr. Biophys. Biophys. Chem.* **9**:325.

9 DE DUVE, C., B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX, and F. APPELMANS. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.* **60**:604.

10. HIGASHI, T., and T. PETERS. 1963. Studies on rat liver catalase. II. Incorporation of ^{14}C-leucine into catalase of liver cell fractions *in vitro*. *J. Biol. Chem.* **238**:3952.

11. HRUBAN, Z., and M. RECHCIGL, JR. 1969. Microbodies and Related Particles. Academic Press Inc., New York.

12. LEIGHTON, F., B. POOLE, H. BEAUFAY, P. BAUDHUIN, J. W. COFFEY, S. D. FOWLER, and C. DE DUVE. 1968. The large-scale separation of peroxisomes, mitochondria, and lysosomes

from the livers of rats injected with Triton WR-1339. *J. Cell Biol.* **37**:482.

13 LEIGHTON, F., B. POOLE, P. B. LAZAROW, and C. DE DUVE. 1969. The synthesis and turnover of rat liver peroxisomes. I. Fractionation of peroxisome proteins. *J. Cell Biol.* **41**:521.

14. NOVIKOFF, A. B., and W. Y. SHIN. 1964. The endoplasmic reticulum in the Golgi zone and its relations to microbodies, Golgi apparatus and autophagic vacuoles in rat liver cells. *J. Microsc.* **3**:187.

15. POOLE, B. 1969. Biogenesis and turnover of rat liver peroxisomes. *Ann. N.Y. Acad. Sci.* **68**:229.

16. POOLE, B., F. LEIGHTON, and C. DE DUVE. 1969. The synthesis and turnover of rat liver peroxisomes. II. Turnover of peroxisome proteins. *J. Cell Biol.* **41**:536.

17. PRICE, V. E., W. R. STERLING, V. A. TARANTOLA, R. W. HARTLEY, JR., and M. RECHCIGL, JR. 1962. The kinetics of catalase synthesis and destruction *in vivo*. *J. Biol. Chem.* **237**:3468.