

# THE SYNTHESIS AND TURNOVER OF RAT LIVER PEROXISOMES

## II. Turnover of Peroxisome Proteins

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### ABSTRACT

After preliminary experiments had established that the injection of Triton WR-1339 necessary for the separation of lysosomes and peroxisomes did not affect the turnover rate of catalase, the decay of  $^3\text{H}$ -leucine incorporated into peroxisomes was studied in whole particles and in protein subfractions. It was shown that peroxisomes are destroyed in a completely random way, probably as wholes since the apparent half-life was the same for all subfractions, about  $3\frac{1}{2}$  days. In agreement with the results of Price et al. (11), the half-life of catalase derived from the rate of recovery from aminotriazole inhibition was about  $1\frac{1}{2}$  days, as was the apparent half-life of the heme prosthetic groups measured with  $^{14}\text{C}$ - $\delta$ -aminolevulinic acid. Guanidino-labeled arginine gave an apparent half-life of  $2\frac{1}{2}$  days with large statistical uncertainty. Either the leucine label was reutilized very extensively in our animals and the true half-life of peroxisomes is  $1\frac{1}{2}$  days, or the prosthetic groups of catalase turn over more rapidly than the protein part of the molecule.

### INTRODUCTION

Of the known enzymes contained within rat liver peroxisomes, catalase is the only one for which there is any information concerning the rate of turnover.

Theorell et al. (16) studied the uptake and decay of trace amounts of radioiron in the iron containing proteins of guinea pig liver. Their data are somewhat difficult to interpret because of uncertainty concerning the decay rate of the radioiron in the free iron pool, but it is clear from their results that the half-life of catalase in guinea pig is at least 5 days.

In rat liver, there is no direct evidence concerning catalase synthesis or destruction in the steady state. However, observations in three very different abnormal conditions suggest that catalase

there has a very short half-life. Greenstein (4) has observed that the depression of liver catalase activity brought about by the implantation of certain tumors is abolished within 2 days by the surgical removal of the tumors. The mechanism of catalase depression by tumors is still obscure and inhibitor substances may be involved. The data of Price et al. (11) are much more compelling. They studied the rate of recovery of catalase activity in rat liver after irreversible inhibition with 3-amino-1,2,4-triazole. By following the incorporation of radioiron, they verified that the recovery from aminotriazole was paralleled by catalase synthesis. They studied also the rate of decrease of catalase activity after the disruption of porphyrin metabolism with allylisopropylacetamide. In each case

their data indicated a half-life of  $1\frac{1}{2}$  days for catalase, provided that catalase is destroyed randomly. de Duve and Baudhuin (1) have pointed out that these data are equally consistent with a model of peroxisome ontogeny in which the particles grow continuously for a period of about 4 days, and then burst, releasing their contents into the cytoplasm where they are rapidly destroyed.

This paper will describe the results of some experiments that bear on the question of the turnover of catalase and of the other protein components of the peroxisome.

## MATERIALS AND METHODS

### *Animals*

Throughout the studies to be described, female Sprague-Dawley rats of about 200 g were used. These were supplied with Purina Laboratory Chow and water *ad libitum*, and maintained in a room with a light schedule of alternate 12-hr periods of light and darkness. For studies of isotope incorporation, the animals were kept in our animal room for at least a week before use. Rats of the CD1 strain were supplied initially by Cernac Laboratory Inc., (New York, N.Y.) and later by Charles River Breeding Laboratories (North Wilmington, Mass.).

The light regime was standardized in the hope of avoiding random variations in the rate of isotope incorporation as a consequence of phase differences in circadian cycles. In each experiment, labeled compounds were administered at the same time of day.

### *Radioisotopes*

Isotopically labeled compounds, all of which were purchased from New England Nuclear Corp. (Boston, Mass.), were injected intraperitoneally. Protein samples were precipitated in 10% trichloroacetic acid and collected on Whatman GF/C glass filter paper discs 2.4 cm in diameter. The filters were dried, then incubated in tightly closed scintillation vials at 55° with 1 ml of m hyamine hydroxide in methanol, for several hours with gentle shaking. The flasks were cooled and 0.1 ml of propionic acid was added, followed by 15 ml of 0.4% of BBOT (Packard) in toluene. This system gave about 5% counting efficiency for tritium. The samples were counted in a Packard 3375 scintillation counter on two channels with external standardization. The quenching correction was made by computer using a program now available in the Packard Program Library (No. 9672). Final specific activities are expressed as the "labeling index", defined as the ratio of the specific radioactivity of the proteins (in cpm per mg protein) to the total amount of radioactive precursor ad-

ministered per unit weight of animal (in cpm per mg whole rat).

### *Isolation, Analysis, and Subfractionation of Peroxisomes*

These operations were performed as described previously (6, 7).

### *Immunochemical Procedures*

For the precipitation of catalase we used two preparations of rabbit anti-rat liver catalase. One was prepared in Japan by Dr. Tokuhiko Higashi, and the other in our laboratory with his assistance. Both antisera contained antibodies to liver constituents other than catalase, and it was necessary to purify partially the catalase, as described in the previous paper of this series, up to the stage of sodium sulfate precipitation (7). This material was only about 50% pure catalase, but, as shown in Fig. 1, it gave no precipitate with antiserum that had been absorbed with pure catalase. The sample of pure catalase used in these experiments had been prepared by the procedure described in the preceding paper (7). It had a higher specific activity than the samples used for immunizing the animals, and, in contrast with those samples, gave only a single precipitation line with the antisera in double diffusion Ouchterlony tests.

Samples of protein were incubated with antiserum for one hour at 37°, and then left overnight in a cold room. The precipitates were washed three times with cold 0.9% NaCl solution, and then dissolved in 0.01 M NaOH. Protein was determined as described previously (6).

## RESULTS

### *Turnover of Catalase Protein Measured with <sup>3</sup>H-Leucine*

We wished to study the turnover of peroxisomes using the isolation and fractionation methods previously worked out in our laboratory (6, 7). One possible source of trouble in such a study might be the injection of Triton WR-1339 into the animals before the fractionation of their livers. Novikoff and Essner (10) have reported increased autophagic activity in rat liver following such injections. This might mean that rats treated with Triton would have a higher rate of protein turnover than normal rats. In order to check possible effects of Triton on turnover and in order to check the catalase turnover in the livers of untreated rats, we used our anti-catalase serum to isolate the hepatic catalase at various times after the injection of labeled precursor.

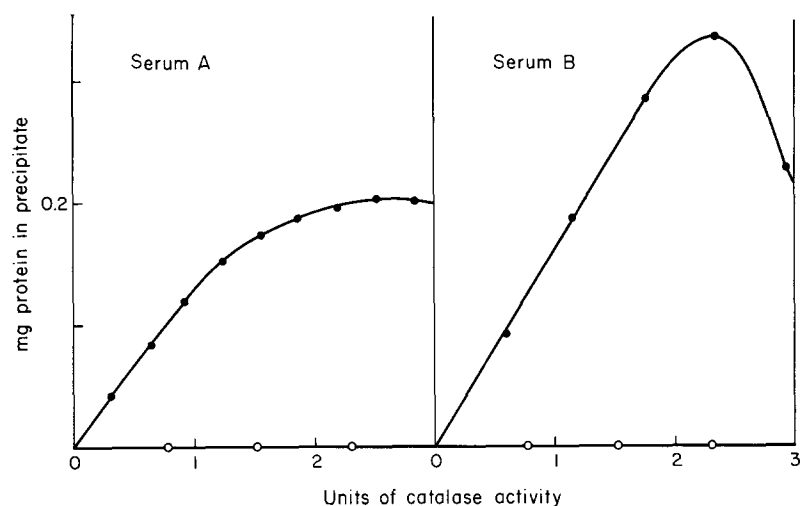


FIGURE 1 Precipitin curves with partially purified catalase for the two rabbit anti-rat liver catalase sera used to separate labeled catalase. ●: Precipitin curve of serum. ○: Precipitin curve of serum absorbed with an equivalent amount of pure catalase.

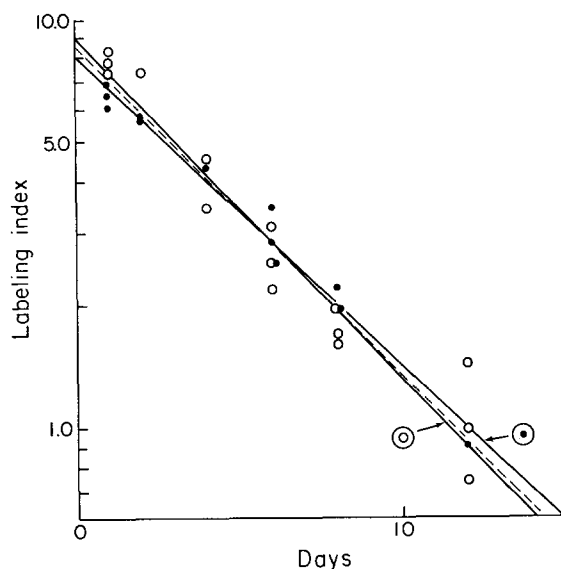


FIGURE 2 Decay of radioactivity incorporated into catalase protein. ●: Animals receiving standard injection of Triton WR-1339 12 hr before labeled leucine. ○: Control animals receiving only leucine.

Two groups of rats were used. One group received a standard injection of Triton WR-1339 (85 mg per 100 g body weight) 12 hr before injection of 1 mc of L-leucine-4,5- $^3\text{H}$  5 c per mmole). The other group received only the leucine injection. The rats were killed at various times after the injection of label and the catalase of their liver was partially purified and then precipitated with the antiserum.

The specific radioactivities observed in these precipitates at various times after injection of label are plotted in Fig. 2. There was no significant difference between the decay curves calculated for each of the two experimental groups. However, the decay we observed, with a half-life of 3.76 days (95% confidence interval 3.32-4.29), is much slower than that calculated by Price et al. (11) from indirect data.

One important conclusion we can draw from these data is that the breakdown of catalase protein follows first order kinetics. The significance of this observation will be examined in the discussion. In addition, these results validate our application of the Triton WR-1339 method of peroxisome isolation to studies of peroxisome turnover. We have obtained no evidence of any disturbance of the steady state between catalase synthesis and breakdown after injection of this substance. The decay curves of incorporated leucine with and without Triton injection have the same slope, showing that the degradation of catalase is not affected by such an injection. Since the two curves extrapolate to the same point at zero time, we can conclude also that the rate of incorporation of the label into catalase is not affected either, at least 12 hr after Triton injection.

#### *Turnover of Peroxisomes and Peroxisome Components Measured with $^3\text{H}$ -Leucine*

We had a method for the separation in workable quantities of highly purified peroxisomes from rat liver, utilizing the effect of Triton WR-1339 upon the density of the hepatic lysosomes (6). We had shown that the injection of this substance did not affect the turnover rate of the catalase contained in these particles. And we had developed methods to subfractionate the purified peroxisomes (7).

Thus we were ready to investigate the turnover of whole peroxisomes and peroxisome subfractions.

In each experimental group, 14 rats were injected with 1 mc each of L-leucine-4,5- $^3\text{H}$  5 c per mmole). At various times after this injection, the rats were killed, their hepatic peroxisomes were isolated and this peroxisomal fraction was subfractionated. The leucine injections were made within 1 hr of 1 p.m., and the rats were killed within 1 hr of 5 a.m. All had received an injection of Triton WR-1339 3½ days before death in order to permit the purification of their peroxisomes.

In Fig. 3, we have plotted the specific activities of the total protein of these peroxisomes (corrected for the initial dilution of the precursor in the rat). We can see that the decay of the incorporated label is strictly exponential, which indicates that if differences in decay rate among the component proteins of the peroxisomes exist, then either these differences are very small, or the components in question contribute very little radioactivity to the whole peroxisome. It should be noted also that the three first points of Fig. 3 belong to rats that received Triton WR-1339 at times ranging between 68 and 20 hr *before* injection of label. The other points are from animals that were injected with Triton *after* they had been given labeled leucine. There is no indication that the initial incorporation of label into peroxisome proteins was affected

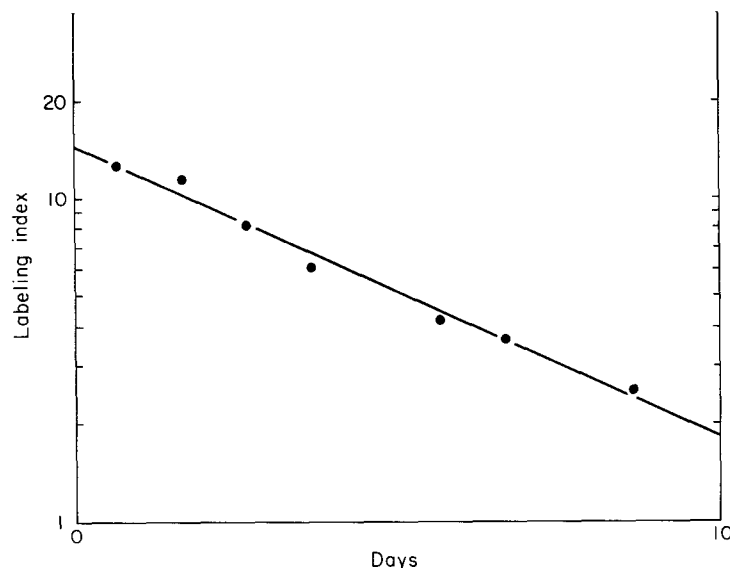


FIGURE 3 Decay of radioactivity incorporated into total peroxisome proteins from tritiated leucine injected.

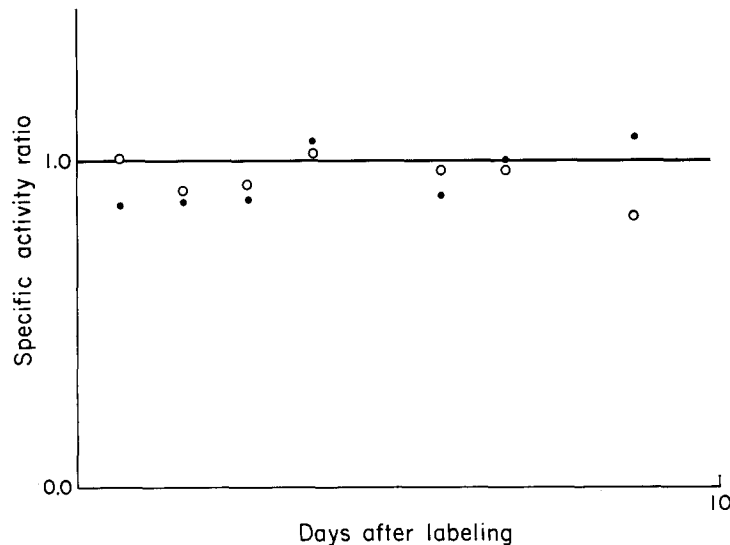


FIGURE 4 Specific activity ratios of peroxisomal subfractions to total protein.  $\circ$ :  $S_2$  (soluble protein).  $\bullet$ :  $P_4$  (core fraction).

by the treatment of the animals with the detergent. This confirms, and extends to longer times after Triton injection, the conclusion reached from Fig. 2 with respect to the incorporation of tritiated leucine into catalase 12 hr after treatment with Triton WR-1339. The decay rate indicated by the data of Fig. 3 corresponds to a half-life of 3.34 days (95% confidence interval 2.81–3.80). The difference between this value and that calculated from the data of Fig. 2 on the turnover of catalase is not statistically significant.

These peroxisomal preparations were subfractionated according to the scheme outlined in the previous paper (7) into a soluble protein subfraction  $S_2$ , and the insoluble fraction  $P_4$ . In Fig. 4, we have plotted the ratios of the specific activities of these two subfractions to the specific activity of the total peroxisome protein. There is clearly no systematic change in these ratios with time. This shows that there is no detectable difference between the turnover rate of the peroxisome core and that of the matrix proteins.

The matrix protein,  $S_2$ , was then fractionated further by Sephadex G-200 chromatography. In Fig. 5, we have plotted the radioactivity of the protein fractions separated from the column against their protein content. In these graphs, the height of each bar gives the relative specific radioactivity of the corresponding fraction, i.e. the ratio of the cpm per mg protein in the fraction to

the cpm per mg protein in the original  $S_2$  fraction. The width of the bar is a measure of the proportion of the total  $S_2$  proteins found in the fraction. The fractions are represented along the abscissa scale, from left to right, in the order in which they are eluted from the column. The seven individual diagrams have been averaged by the computerized procedure described by Leighton et al. (6), to yield the mean plot shown in the upper left hand corner of Fig. 5, in which the vertical bars represent standard deviations. The mean elution diagram of catalase in the seven experiments is represented in the same manner, but in terms of relative specific enzymic activity, in the diagram shown in the upper right hand corner of Fig. 5.

If we had separated on the column some protein species with a turnover rate different from that of the other proteins, then we should see some systematic change with time in some part of the graphs of Fig. 5. As we can see from the small standard deviations indicated on the mean specific radioactivity plot, there is very little variation from one time point to another. The curves all have the same general shape. There is a large amount of noise in the specific activities of the early fractions, as we should expect since these fractions contain very little protein or radioactivity. These early fractions are followed by a series of fractions corresponding to the peak of specific catalase activity (plot in upper right hand corner), whose relative

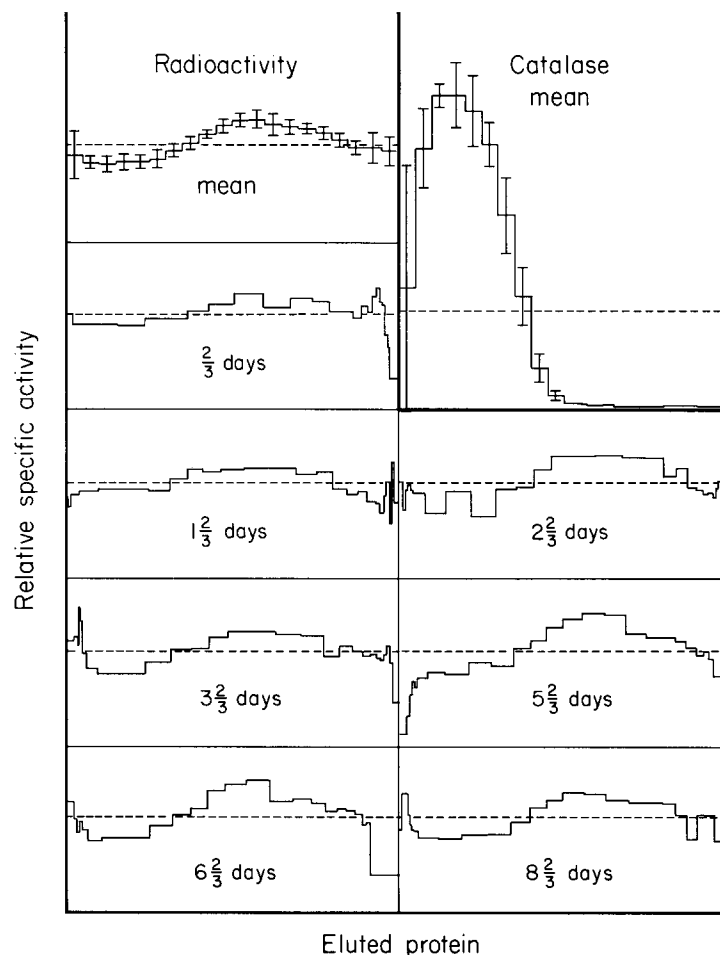


FIGURE 5 Fractionation of labeled soluble peroxisomal proteins on Sephadex G-200. Ordinate for all plots except that of catalase represents relative specific radioactivity, i.e. ratio of specific activity (cpm per mg protein) of fraction to specific radioactivity of starting material; ordinate for catalase plot represents relative specific enzyme activity, i.e. ratio of specific activity (units per mg protein) of fraction to specific activity of starting material. Abscissa represents protein content of fractions, cumulatively from left to right, in the order in which they are eluted from column. In each plot, the horizontal dotted line indicates the level of unity, corresponding to the specific activity of the starting material. Two upper plots labeled "mean" are averages of the seven experiments computed as described by Leighton et al. (6). The vertical bars on these plots are standard deviations.

specific radioactivity is less than unity. Then we have a transition to a region where this ratio is slightly greater than unity, followed by a region of noise again representing the last traces of protein to leave the column.

If we look closely at these curves, we can see that the curves for  $\frac{2}{3}$  day and for  $1\frac{2}{3}$  days are slightly different from the others. These two early curves are flatter than the others, with less difference between the minimum and the maximum specific ac-

tivities in the earlier and the later parts of the curves respectively. This could be explained by the presence, in the early part of the column eluate, of some protein species with a very rapid turnover. However, this species would have to represent only a tiny fraction of the protein there, since we would expect such a component with a rapid turnover to be labeled much more heavily than was the rest of the protein, which, as we have seen, has an apparent half-life of almost 4 days. It is also possible

that the injection of Triton WR-1339 received some time before leucine injection subtly affected the proportions of the various protein species synthesized. In either case, this very small effect noted in the curves does not prevent us from drawing the general conclusion that the vast bulk of the proteins of the peroxisome matrix turn over at the same rate. There may be a trace component with a much more rapid turnover but we cannot exclude the possibility that this represents some contaminant in our original peroxisomal preparation.

It will be noticed that the specific activity of the proteins varies as much as 1.5-fold from one region of the chromatographic eluate to another. The simplest explanation of this variation is that it reflects differences in the number of leucine residues per milligram protein as determined by the automated Lowry procedure (6) with bovine serum albumin as standard. We can see from the amino acid analyses presented in the previous paper (7), that the leucine content of the protein in the first peak of the eluate is indeed lower than it is in the second. However, this difference in itself cannot explain the entire difference observed in specific radioactivity. In addition, the protein in the second peak must give somewhat less color per milligram dry weight than does that in the first peak. As mentioned above, there are indeed indications that the

non-catalase protein in the first peak has a high reactivity in the Lowry procedure relative to its absorbance at 280 nm.

#### *The Recovery of Catalase Activity after Inhibition with Aminotriazole*

Thus far we have shown that catalase protein and at least the bulk of the other peroxisomal proteins turn over at the same rate with an apparent half-life of about 3.8 days. However, Price et al. (11), in experiments mentioned in the introduction, measured indirectly the half-life of catalase and found it to be about 1.5 days. We wondered whether this discrepancy could not arise, at least in part, from a difference in experimental animals used. Consequently, we repeated the experiment of Price et al. (11) in which they studied the recovery of liver catalase activity after this activity had been irreversibly inhibited *in vivo* by the injection of 3-amino-1,2,4-triazole.

Rats were injected intraperitoneally with a dose of 1 g per kg (5) of 3-amino-1,2,4-triazole (K & K Laboratories Inc., Plainview, N.Y.) in 5% (w/v) aqueous solution, and killed at various times after injection. The catalase activity in the livers of these animals was measured and compared with that of control animals which had received no injection. The data from this experiment are

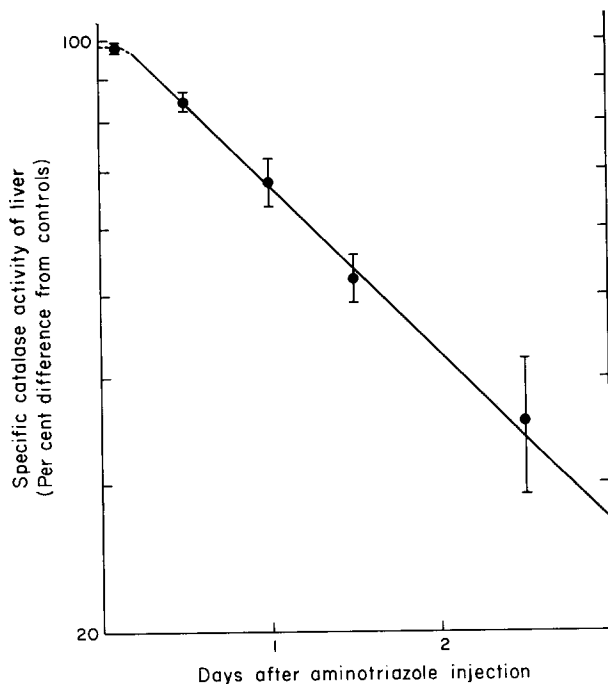


FIGURE 6 Catalase activity in the livers of animals recovering from catalase inhibition by 3-amino-1,2,4-triazole. The points represent the means of measurements made on five rats and the vertical bars are standard deviations.

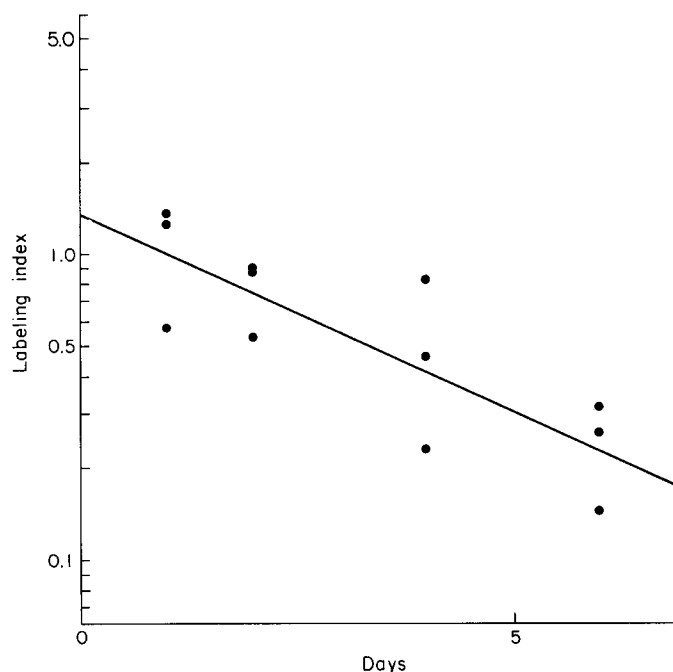


FIGURE 7 Decay of radioactivity incorporated into catalase from guanidino- $^{14}\text{C}$ -arginine.

plotted in Fig. 6. We confirm completely the finding of Price et al. (11), that the half-time for catalase recovery from aminotriazole inhibition (equal to the half-life if the animals are in a steady state) is about 1.5 days.

#### *Turnover of Catalase Measured with Guanidino- $^{14}\text{C}$ -Arginine*

Studies have shown that essential amino acids like leucine are significantly reincorporated into proteins, resulting in longer apparent half-lives (14). This difficulty is not encountered to as great an extent when arginine labeled in the guanidino group is used as precursor. The use of this labeled compound would have been prohibitively expensive in the experiments concerning the turnover of whole peroxisomes, but we decided to use it to measure the turnover of catalase. In this experiment, we used the same procedures we did with  $^3\text{H}$ -leucine. Each rat received an injection of  $80\ \mu\text{c}$  of guanidino- $^{14}\text{C}$ -arginine  $2\ \text{mc}/\text{mmole}$ ).

The results of this experiment are shown in Fig. 7. We have here much more individual variation between rats than we had in any of the experiments described previously. This variation is probably due to variations in the size of the arginine pool. The rapid turnover of the guanidino

group, which makes this amino acid ideal as a precursor for proteins in turnover studies, may also result in a greater sensitivity of pool size to small differences in the metabolic, nutritional, or sexual state of individual rats. The average half-life obtained by this method for catalase was 2.48 days (95% confidence interval 1.60–4.65).

#### *Turnover of Catalase Measured with $^{14}\text{C}$ - $\delta$ -Aminolevulinic Acid*

Another way we can label the catalase molecule is to incorporate labeled  $\delta$ -aminolevulinic acid into the heme prosthetic groups. This will give us a means of measuring the turnover of the prosthetic groups, which is probably a good measure of the protein turnover as well, unless catalase is involved in some very unusual repair mechanism. Moreover, the known pathways of heme degradation seem to preclude the possibility of reutilization of this label.

Rats were injected with  $40\ \mu\text{c}$  of  $\delta$ -amino-levulinic-4- $^{14}\text{C}$  acid ( $3\ \text{mc}/\text{mmole}$ ), and at various times their hepatic catalase was partially purified and precipitated with antiserum. The results of this experiment are shown in Fig. 8. We find very much less noise in the data than we did in the experiment with guanidino-labeled arginine. The



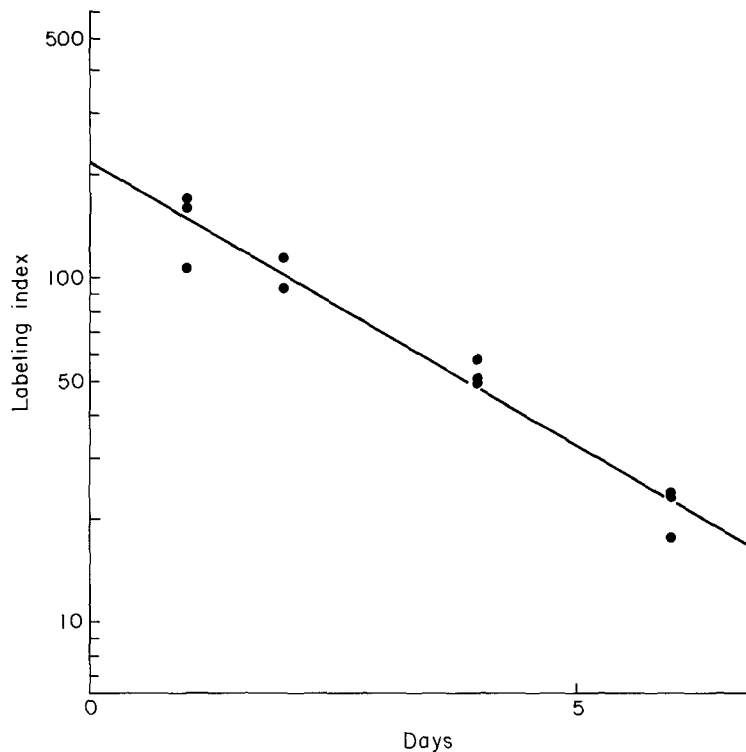


FIGURE 8 Decay of radioactivity incorporated into catalase from  $\delta$ -aminolevulinic-4- $^{14}\text{C}$  acid.

apparent half-life for catalase is 1.8 days (95% confidence interval 1.6–2.2), in good agreement with the half-life indicated by the kinetics of recovery from inhibition by aminotriazole.

#### DISCUSSION

The three isotopically labeled catalase precursors we have used in these studies gave very different levels of incorporation. We can expect that the efficiency of incorporation will vary according to the amount of labeled compound injected, but the labeling index extrapolated to zero time should give us a rough estimate of the relative fraction of the precursor pool that is consumed in the synthesis of catalase. These data are summarized in Table I.  $\delta$ -Aminolevulinic acid is by far the most efficient precursor, whereas leucine is only about seven times more efficient than the guanidino group of arginine.

With regard to the turnover of peroxisomes we can draw two important conclusions from the results of our experiments. The decay of label incorporated into whole particles or into the catalase contained within them follows an essentially ex-

ponential course, suggesting that peroxisomes are destroyed within the liver cell in a random way. In particular, it would appear that an old particle has no more likelihood of being destroyed in a particular interval of time than does a young one. This observation argues against the theory of growth and ripening proposed by de Duve and Baudhuin (1) as a possible alternative explanation for the data of Price et al. (11). This point will be elaborated further in the next paper of this series.

Secondly we have shown that except for a trace component in our preparations, which has a very rapid rate of turnover but which may or may not be a true peroxisomal protein, all the major protein components of the peroxisome have the same rate of turnover. This suggests very strongly that these particles are destroyed as wholes, whether by autophagy (2) or by some other process. This means that we can take the half-life of catalase protein as the half-life of whole peroxisomes.

However, our experiments on the turnover of catalase and those of Price et al. (11) have given somewhat different apparent half-life values depending on the method used. In Table II we have

TABLE I  
Efficiency of Incorporation of Labeled Precursors  
into Rat Liver Catalase

Precursor	Dose	Labeling index extrapolated to zero time
	<i>μmoles</i>	
δ-Aminolevulinic acid	13	210
Leucine	0.2	8.5
Arginine (guanidino group)	40	1.3

TABLE II  
Summary of Half-Lives Measured for Catalase

Method	Most probable half-life	95% Confidence interval
	<i>days</i>	
<sup>3</sup> H-leucine incorporated into catalase	3.74	3.32-4.29
<sup>3</sup> H-leucine incorporated into whole peroxisomes	3.34	2.81-3.80
Guanidino- <sup>14</sup> C-arginine incorporated into cata- lase	2.48	1.60-4.65
<sup>14</sup> C-δ-aminolevulinic acid incorporated into cata- lase	1.84	1.59-2.17
Recovery from aminotri- azole inhibition	1.73 1.34*	1.37-2.36 1.16-1.57*
Inhibition of catalase syn- thesis with allylisopro- pylacetamide	1.07*	0.93-1.50*

\* Calculated from the data of Price et al. (11).

summarized these results. We see that the apparent half-lives of catalase measured by the various methods fall into three groups. From the decay of radioactivity incorporated from leucine the apparent half-life is about 3.5 days. At the other extreme, we have the half-life measured with δ-aminolevulinic acid as a precursor for the heme prosthetic groups of catalase, and the half-lives measured less directly with aminotriazole and with allylisopropylacetamide. These measurements agree fairly well with each other and indicate a

half-life of about 1.5 days. Lying between these two extreme sets of measurements, which are very significantly different statistically, we have the results of turnover measurement using guanidino-labeled arginine. This experiment gave a half-life value of about 2.5 days, although the statistical uncertainty in this value is so great that the data are compatible with the long half-life measured with leucine, and just on the edge of significant difference from the short half-lives measured by various means.

The simplest explanation of all these data is that catalase, as well as the other peroxisomal proteins, turns over with a half-life of about 1.5 days, and that the longer half-life measured with leucine was simply the result of reutilization of the labeled leucine. Indeed, a number of workers have found that the use of radioactive leucine in turnover studies leads to an overestimation of the half-life of proteins, and that the error made in this manner may be considerable (see, for instance, references 12, 14, and 15). Since leucine is an essential amino acid, the extent to which it is reutilized may be expected to vary within relatively large limits with the composition of the diet and with the metabolic state of the animal. With due consideration of this fact and of the comparable data of other workers, it appears that reutilization of label could alone account for the differences in half-life observed in this work. As indicated by Swick et al. (15), some degree of reutilization of label occurs even with guanidino-labeled arginine.

There is another, although less likely, explanation for our results. The only direct measurement of a short half-life for catalase comes from the incorporation of labeled δ-aminolevulinic acid into the prosthetic groups of catalase. It could possibly be that the turnover rate of these prosthetic groups is faster than that of the protein part of the molecule. Allylisopropylacetamide is known to induce an elevation in chick embryo liver cells of δ-aminolevulinic acid synthetase (3), but this effect will not explain the fall in catalase activity attendant on the injection of this drug. If, as has been suggested (13), the drug also blocks the conversion of porphyrins into heme proteins, then this method of half-life measurement would measure prosthetic group turnover. It is more difficult to explain the recovery of catalase activity after aminotriazole inhibition in terms of heme turnover because Margoliash et al. (8) have demonstrated in vitro that <sup>14</sup>C-labeled aminotriazole remains attached to the protein after the heme has been removed from in-

hibited catalase. However, we do not know anything about the mechanism of aminotriazole inhibition of catalase, and it may well be that the primary change in the enzyme, which is responsible for the loss in activity, is a change in the prosthetic group. The attachment of the aminotriazole to the protein could be a secondary effect, not in itself inhibitory, or it could be an artifact produced during the chemical dissociation of the heme from the protein. It might be advantageous for an organism to have a turnover of the prosthetic groups of catalase more rapid than that of the protein part of the molecule. These prosthetic groups are known

to undergo many different types of rapid reactions leading to a loss of catalase activity (9).

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