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The Biogenesis of Peroxisomal Catalase in Rat Liver

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THE BIOGENESIS OF PEROXISOMAL CATALASE IN RAT LIVER

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by

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PREFACE

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ABSTRACT

Rat liver peroxisomal catalase was purified to homogeneity. The starting material was purified peroxisomes. The purity of the final preparation was judged by Sephadex G-200 chromatography, SDS gel electrophoresis, spectral analysis, and immunochemical means.

Two sera containing anticatalase antibodies were prepared. The first, a rabbit serum, contained a minor impurity. The anticatalase was purified forty times by affinity chromatography and the contaminating antibodies were thereby removed. The second serum, prepared in a goat, was proved to be monospecific by immunodiffusion and immunoelectrophoresis.

The standard methods of immunoprecipitation were modified to allow the specific precipitation of a minor component (catalase) out of a crude cell homogenate and fractions thereof. The use of siliconized, glass, conical tubes and the omission of a 37° incubation were found to be essential. Immunochemical and biochemical controls were employed.

The incorporation of ^{3}H-leucine into catalase was determined in rats receiving the isotope by intraportal injection, in order to effect a nearly pulse-chase situation. Under these conditions about 10% of the injected isotope is incorporated into TCA-insoluble material in the liver within minutes (the apparent half-life of the liver's free leucine pool is 2 minutes).

The kinetics of incorporation of ^{3}H-leucine into material precipitated from the liver by anticatalase was identical to the kinetics of labeling of total protein just described. However, when newly synthesized, this material does not accompany catalase through chemical purification. Its migration on SDS polyacrylamide gels is identical to that of catalase. It is quantitatively converted into purifiable, authentic catalase in two hours or more. We interpret these results as the synthesis of a biosynthetic intermediate of catalase.

Analogous experiments were carried out using ^{3}H-δ-aminolevulinic acid (ALA) to label the heme moiety of catalase. Pulse-labeling was not achieved using this precursor. Nevertheless it was possible to identify a catalase biosynthetic intermediate with this label as well. Detailed analysis of the incorporation kinetics revealed that the intermediates identified with leucine and with ALA were not the same and in fact there are two leucine-containing intermediates: one without heme, representing about 2% of the total liver catalase, and one containing heme, representing about 0.5% of the liver catalase.

A method was designed for fractionating postnuclear supernatants on sucrose density gradients, taking advantage of both differences in density and differences in size of the various cell organelles. By means of this procedure a reasonable separation of most of the cell organelles can be effected within two hours. This technique may be applied to localizing enzymes within liver cells and can be adapted to swinging-bucket rotors.

The locations of labeled catalase and its biosynthetic intermediates within the liver cells were investigated by applying the above fractionation method to the livers of rats double-labeled with ^{3}H-leucine and ^{14}C-ALA. The overall labeling kinetics in these double-labeled rats confirmed the existence of two biosynthetic intermediates: one without, and one containing heme. The leucine-containing intermediate, still lacking its heme, was found to be transported rapidly to the peroxisomes, with a transport half-time of approximately 12 minutes. Inside the peroxisomes it was equipped with heme and converted to authentic, purifiable catalase. In addition, some heme appeared to be added to apocatalase outside of the peroxisomes, and some of this extra-peroxisomal catalase even appeared to become purifiable. The significance of these latter findings was difficult to assess, and the possibility of an artifact could not be excluded.

The labeled catalase intermediates which had not yet reached the peroxisomes were found partly in the microsome-rich fractions and

largely at the top of the gradients where the layer was applied. Many experiments were performed to clarify the early events in catalase biosynthesis and transport, but they did not succeed in completely solving the problem. A participation of the rough endoplasmic reticulum is strongly implicated. The very considerable label found at the top of the gradients is considered to arise, either from the synthetic activity of free polyribosomes, or from a homogenization artifact whereby a fragile particle is broken open.

LIST OF SYNONYMS AND ABBREVIATIONS

Solutions

Saline	0.15 M NaCl
PBS	Phosphate buffered saline (10 mM potassium phosphate), pH 7.0
PBSLA	PBS containing 0.1 mM L-leucine and 0.01 mM δ-amino-levulinic acid

Cell fractions - prepared according to de Duve *et al.* (1955)

Extract	postnuclear supernatant
M	mitochondrial fraction
L	light mitochondrial fraction

Chemicals

ALA	δ-aminolevulinic acid
BSA	bovine serum albumin
DAB	3,3'-diaminobenzidine
TCA	trichloroacetic acid

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I. INTRODUCTION

The peroxisome is a subcellular organelle which is characterized by the presence of catalase and one or more H_2O_2-producing oxidases. It was discovered and characterized by de Duve and his collaborators first in rat liver (de Duve, 1960; de Duve et al., 1960; de Duve et al., 1963; Baudhuin et al., 1964; Beaufay et al., 1964) and subsequently also in rat kidney and Tetrahymena pyriformis (Baudhuin et al., 1965). Since that time it has been found in a wide variety of other cell types including Acanthamoeba sp. (Müller and Møller, 1967, 1969), germinating castor-oil bean and other fatty seedlings (Breidenbach and Beevers, 1967; Breidenbach et al., 1968), spinach leaves (Tolbert et al., 1968) and yeast (Avers and Federman, 1968).

The rat liver peroxisomes contain catalase, urate oxidase and D-amino acid oxidase (de Duve, 1960; de Duve et al., 1960, 1963) as well as L-α-hydroxyacid oxidase (Baudhuin et al., 1965b). Evidently they are centers for the metabolism of hydrogen peroxide (whence their name). The possible physiological functions of the peroxisome have been discussed by de Duve and Baudhuin (1966) in their review, and it has been pointed out that catalase may function mainly in its peroxidatic capacity, with the catalatic activity acting as a safety valve in case of excess H_2O_2 production.

More recently, the liver peroxisomes have been shown to contain small amounts of NADP-linked isocitrate dehydrogenase (Leighton et al., 1968). Peroxisomes from other tissues (with catalase and at least one oxidase) have been found to contain a variety of additional enzymes. The Tetrahymena pyriformis particles contain the two key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthetase, but not the rest of the cycle enzymes, which are, however, present in the mitochondria (Müller and Hogg, 1967; Müller, Hogg and de Duve, 1968). Therefore, in Tetrahymena pyriformis the operation of the glyoxylate cycle requires the collaboration of two cell organelles. In germinating castor beans all of the glyoxylate cycle enzymes are present in the

peroxisomes (termed glyoxysomes; Breidenbach and Beevers, 1967; Breidenbach et al., 1968). In peroxisomes of spinach leaves, enzymes involved in photorespiration have been found to be peroxisomal (Tolbert et al., 1968). It is clear, therefore, that the peroxisomes may serve a wide variety of different functions in the different cell types in which they are found. These more recent developments have been reviewed by de Duve (1969a) and the possible relationships between the various types of peroxisomes have been discussed at a recent symposium (Hogg, 1969).

It should be emphasized that the peroxisome is a biochemical entity, defined operationally by the cosedimentation of catalase and oxidases when cell homogenates (or fractions thereof) are subjected to a centrifugal field. The rat liver peroxisomes have been shown by Baudhuin et al. (1965a) to be identical with the morphological entities, microbodies, first described in rat liver by Rouiller and Bernhard (1956). These structures contain a single limiting membrane, a finely granular matrix, and a dense core with a regular polytubular structure. Microbodies have since been described in a wide variety of cell types (Hruban and Rechcigl, 1969) but the dense core is not always present. The rat liver microbodies have been studied by many morphologists and we will have more to say about their observations in the Discussion (Chapter X).

The rat liver peroxisomes have been investigated in great detail by de Duve and his collaborators in New York, and the results of their studies form the foundation of information on which the present investigation is based. Leighton et al. (1968) devised a method for the large-scale isolation of peroxisomes from rat liver in a high state of purity (95%) and with good yield. The isolation procedure takes advantage of the observation of Wattiaux et al. (1963) that treatment of rats with Triton WR-1339 causes a selective decrease in the equilibrium density of lysosomes. The methods of fractionation, biochemical analysis and computation and display of the results were largely automated, making this isolation method practicable on a routine basis. Quantitative

electron microscopic examinations of the gradient fractions obtained in this way confirmed conclusively the identity of peroxisomes with microbodies, previously shown by Baudhuin et al. (1965a).

The proteins of the isolated peroxisomes were fractionated by Leighton et al. (1969), and the known enzymes found to contribute no more than 56% of the total peroxisomal protein. The urate oxidase and 10% of the protein were found in the insoluble core fraction (see also Tsukada et al., 1966). The balance of the protein and the other known enzymes were found in the soluble fraction of the peroxisomes.

The turnover of catalase and other peroxisomal proteins was studied by Poole et al. (1969). Peroxisomes were shown to be destroyed in a completely random way, probably as wholes, since the apparent half-life with leucine labeling of all subfractions was the same, about 3.5 days. In agreement with the results of Price et al. (1962), the half-life of catalase derived from the rate of recovery from aminotriazole inhibition was about 1.5 days, as was the apparent half-life of the heme prosthetic groups measured with ^{14}C-δ-aminolevulinic acid. The discrepancy between the apparent turnovers of the polypeptide and heme components of catalase was subsequently shown by Poole (1971) to be a consequence of extensive reutilization of the ^{3}H-leucine label.

Several possible models for peroxisome biogenesis have been considered. The morphological observations of Novikoff and Shin (1964) indicating that rat liver microbodies may be attached to the smooth endoplasmic reticulum suggested that this organelle might be formed by budding from the endoplasmic reticulum. This theory received some support from the biochemical observations of Higashi and Peters (1963b) which implied that catalase was synthesized by rough microsomes and subsequently transferred to the peroxisomes. de Duve and Baudhuin (1966) pointed out in their review that were peroxisomes to have a fixed lifetime (of 4.4 days) during which they grew linearly (by budding, for example), and if at the end of this lifetime they were rapidly destroyed, the resulting kinetics would be compatible with the results of Price

et al. (1962) on changes in catalase activity after aminotriazole or allylisopropylacetamide treatment. This model was tested by Poole et al. (1969) and ruled out by their observation that the peroxisomes are destroyed randomly.

A second model, that of prolonged, independent growth of peroxisomes but with random destruction, was then considered. This theory predicts that the specific activity of catalase should be higher in smaller than in larger peroxisomes a short time after pulse-labeling with ^{3}H-leucine, and that at longer times the ratio should become reversed. Poole et al. (1970) discovered that the specific activity of catalase is independent of peroxisome size from 3 hours to 1 week after administration of ^{3}H-leucine. This rules out the second hypothesis. Therefore either the peroxisomes are formed rapidly within liver cells (with a synthesis time less than 3 hours), or else peroxisomes are capable of exchanging their contents.

In view of this information, we decided to investigate events in the formation of peroxisomes on a much shorter time scale. In particular we planned to study the biosynthesis of catalase, and its transport to peroxisomes. We used isotopic labeling techniques to tag the newly-synthesized catalase. We employed sucrose gradient centrifugation and biochemical analysis to fractionate the livers and identify the cell components. Finally, we used immunochemical methods to separate the catalase (which represents a minor part of the cell's biosynthetic efforts) from the other cell proteins, in order to determine the location of the labeled catalase in the cell as a function of time.

II. MATERIALS AND METHODS

A. Enzyme Assays

1. Automated assays

Protein, catalase, glucose-6-phosphatase, acid phosphatase, D-amino acid oxidase and L-α-hydroxy acid oxidase were measured by the automated methods described by Leighton et al. (1968), with the help of Technicon Auto-Analyzer units (Technicon Corp., Ardsley, N.Y.). The method of assay of the latter two enzymes was modified to decrease the noise level (Fig. 1). The amount of protein is expressed in mg based on a bovine serum albumin standard. The unit of activity for the phosphatases and oxidases is μmoles per minute.

Catalase was also measured by a new automated method with a 15-fold greater sensitivity (Fig. 2). The range of this assay was about 0.002 to 0.030 units per ml. Its linearity with respect to enzyme concentration is shown in Figure 3.

2. Hand assays

Catalase was sometimes measured by hand, as described by Baudhuin et al. (1964). Hand determinations served to calibrate the two automated methods. Catalase activity is proportional to the H_2O_2 concentration from 10^{-6} to 10^{-1} M (Bonnichsen, Chance, and Theorell, 1947). One unit of activity (defined by Baudhuin et al., 1964) will decrease the H_2O_2 concentration 10-fold per minute in a reaction volume of 50 ml at 0°. This unit equals the "Reaktionskonstante K" in the numerator of the classical expression of von Euler and Josephson (1923, 1927) for the specific activity of catalase, Kat.f. (Katalasefähigkeit, or catalatic capacity):

$$\text{Kat.f.} = \frac{\text{Reaktionskonstante K}}{\text{g Enzympräparat}}$$

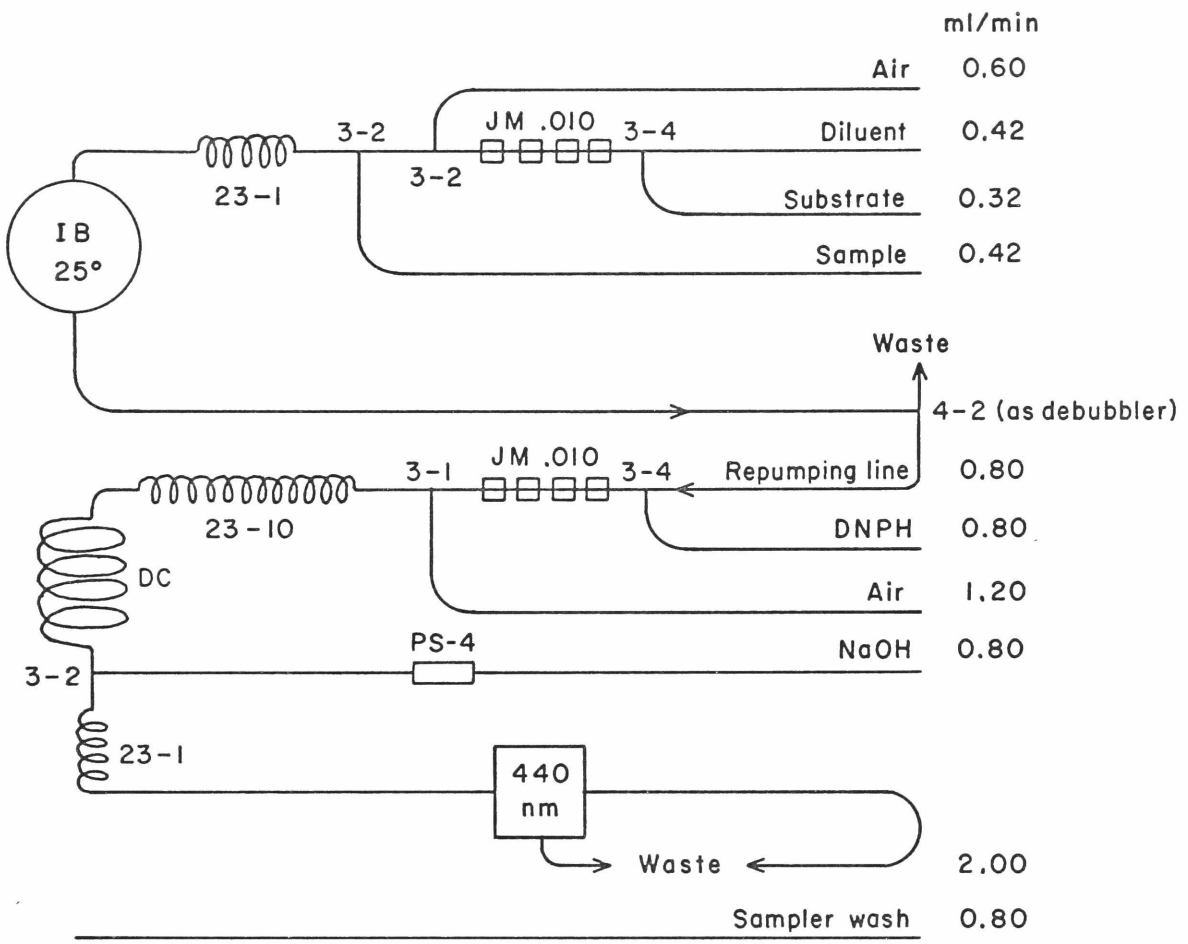


Figure 1. Automated assay of D-amino acid oxidase and of L-α-hydroxy acid oxidase.

IB incubation bath, 25°, 80-ft coil, 1.6 mm I.D.; JM .010, jet mixer, alternate segments of tygon tubing of 0.010 and 0.0625 in. I.D.; DC, delay coil, 8 ft, 2 mm I.D.; other symbols refer to Gradko catalogue. Diluent: Freshly prepared mixture of one volume of 2% Triton X-100 with five volumes of 0.15 M sodium pyrophosphate-HCl buffer pH 8.6 containing 80 mg/l of FAD (D-amino acid oxidase), or with five volumes of 0.10 M sodium pyrophosphate-HCl buffer pH 8.0 (L-α-hydroxy acid oxidase). Substrate: (D-amino acid oxidase): 0.2 M D-alanine, brought to pH 8.6 with NaOH. Substrate: (L-α-hydroxy acid oxidase): 0.025 M sodium glycolate. DNPH solution of 0.01% 2,4-dinitrophenylhydrazine in 2 N HCl containing 0.2% of Triton X-100. NaOH 3.0 N Washing fluid: Water. Standard: 0.2 mM redistilled pyruvic acid (D-amino acid oxidase) or glyoxylic acid (L-α-hydroxy acid oxidase). Sampling rate: 30 samples per hour. Colorimetry at 440 mμ with tubular flow cell of 15 mm light path. Recording: transmission on log paper.

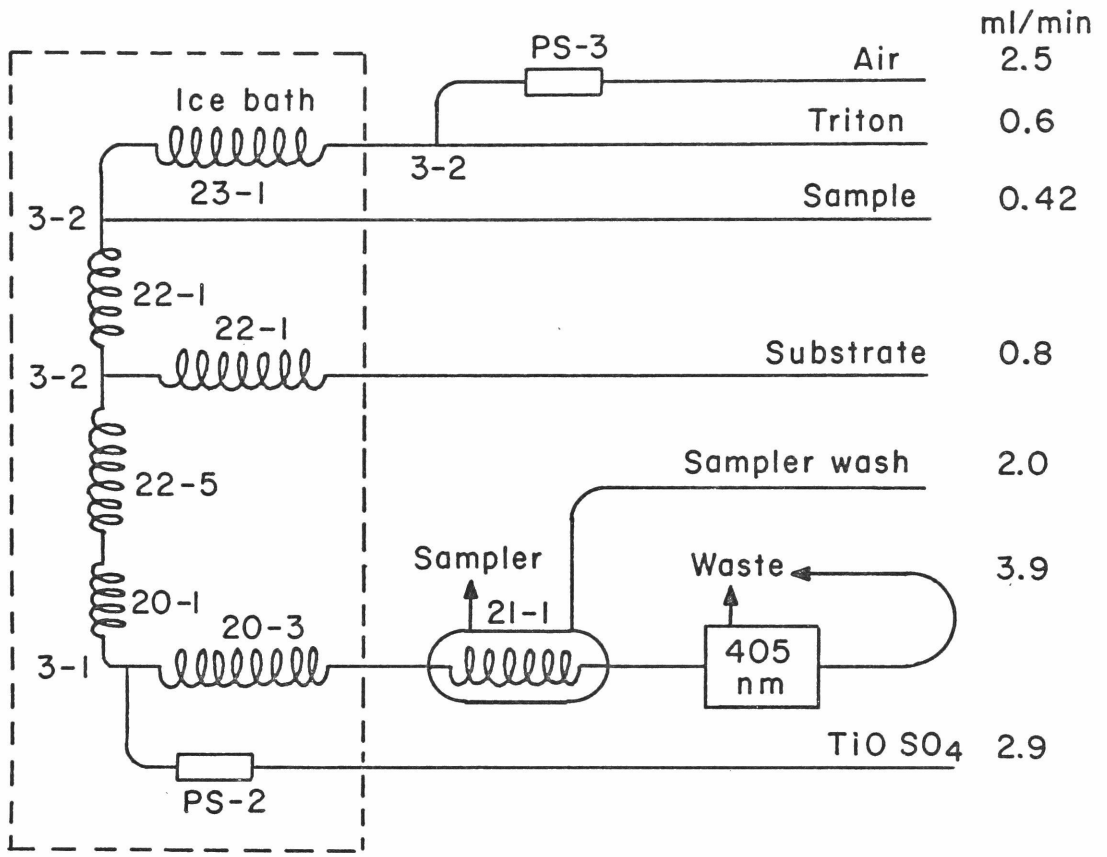


Figure 2. Automated sensitive catalase assay.

For symbols, refer to the Gradko catalogue. Triton, solution of 0.67% Brij-35 and of 0.33% bovine serum albumin in 2% NaCl. Substrate: Solution containing 0.032% of H_2O_2 in 0.045 M imidazole-HCl buffer pH 7.0. $TiOSO_4$: Prepared by dissolving 27 g of titanium oxysulfate in 4 liters of boiling 2 N H_2SO_4, filtering on Whatman #42 paper after cooling, and diluting the filtrate with 2 liters of 2 N H_2SO_4. Washing fluid: 0.05% of Brij-35 (Technicon) in water. Sampling rate: 20 samples per hour. Colorimetry at 405 mμ in Beckman DB spectrophotometer with rectangular flow cell of 6 mm light path.

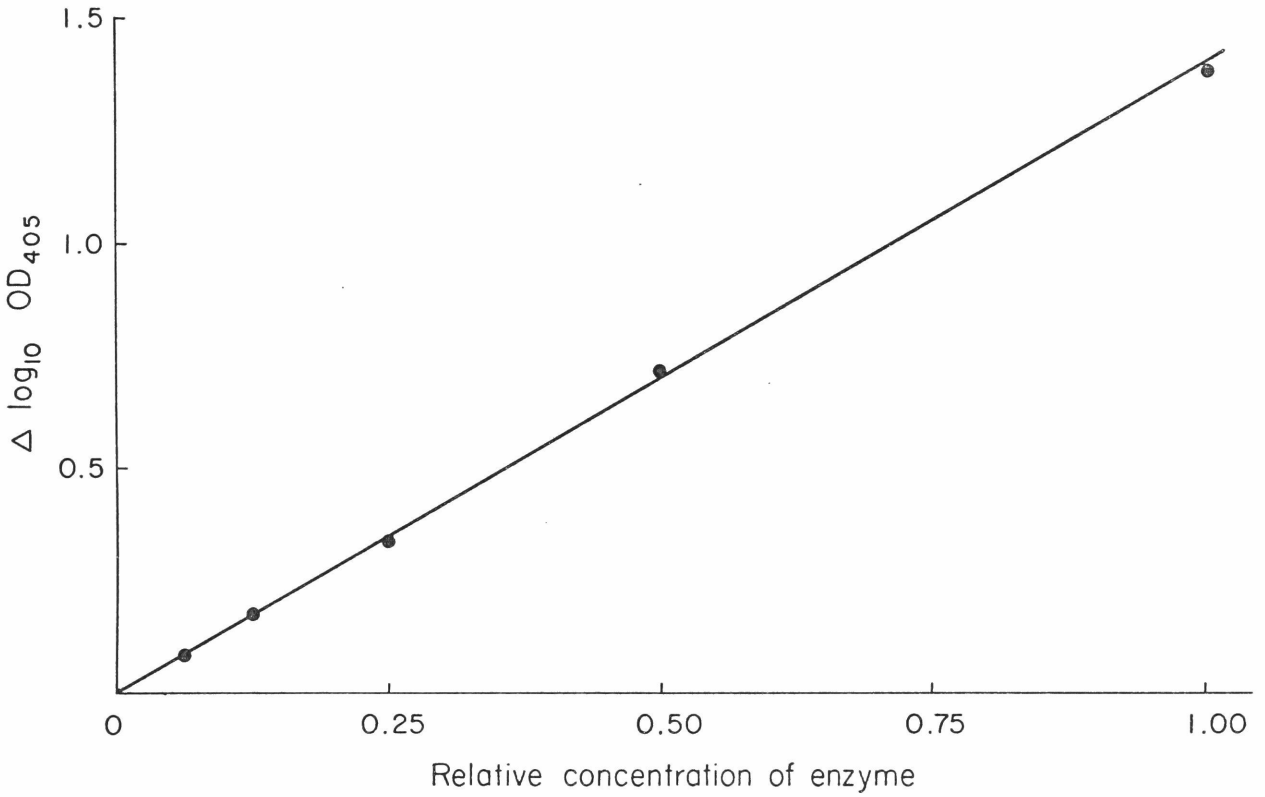


Figure 3. Linearity of automated sensitive catalase assay with respect to enzyme concentration.

The enzyme preparation was a rat liver extract. A relative concentration of 1.0 is 0.045 units of catalase/ml.

Cytochrome oxidase was determined by the method of Cooperstein and Lazarow (1951) as modified by Leighton et al. (1968). The activity of this enzyme is also proportional to its substrate concentration; the unit is Δ log O.D. per minute at 25° (for a reaction volume of 100 ml).

RNA was determined as described by Blobel and Potter (1968), employing the method of Schmidt and Thannhauser (1945) as modified by Fleck and Munro (1962). The amount of RNA was calculated using a mass extinction coefficient of 21.3 $cm^{-1}mg^{-1}$ ml (Fleck and Munro, 1962).

Phosphoglucomutase was assayed by coupling it to glucose-6-phosphate dehydrogenase as described by Slein (1955) for phosphohexoisomerases. The unit of activity is μmoles per minute.

In the assay of esterase, 0.1 ml of suitably diluted enzyme was mixed with 1 ml of 0.40 mM α-naphtyl acetate in 50 mM phosphate buffer, pH 6.8, and incubated for 6 minutes at 25° (Shibko and Tappel, 1964; M. Baggiolini, personal communication). The liberated α-naphtol was determinted by adding 1 ml of ice cold 1.0 M citrate buffer, pH 4.0, containing 5 μl of diazotized aniline yellow dye (Weissman, 1969). The product is water soluble and its optical density at 487 nm is measured 10 minutes after dye addition. The linearity of this assay with respect to enzyme concentration is shown in Figure 4. The unit of activity is μmoles per minute.

The linearity of all of the above assays with respect to enzyme concentration has been verified. Appropriate standards were included every time an assay procedure was used.

B. Radioactivity Determinations

Samples were dissolved in 0.5 ml of 0.1 M NaOH and counted with 10 ml of scintillation fluid containing Triton X-100. The exact composition of the scintillation fluid depends on the batch of Triton X-100 used, but most of the work described below employed a fluid consisting of 200 ml Triton X-100, 800 ml toluene, 5 g PPO, 0.2 g dimethyl POPOP, and 1 ml glacial acetic acid (recipe of B. Poole). The samples were

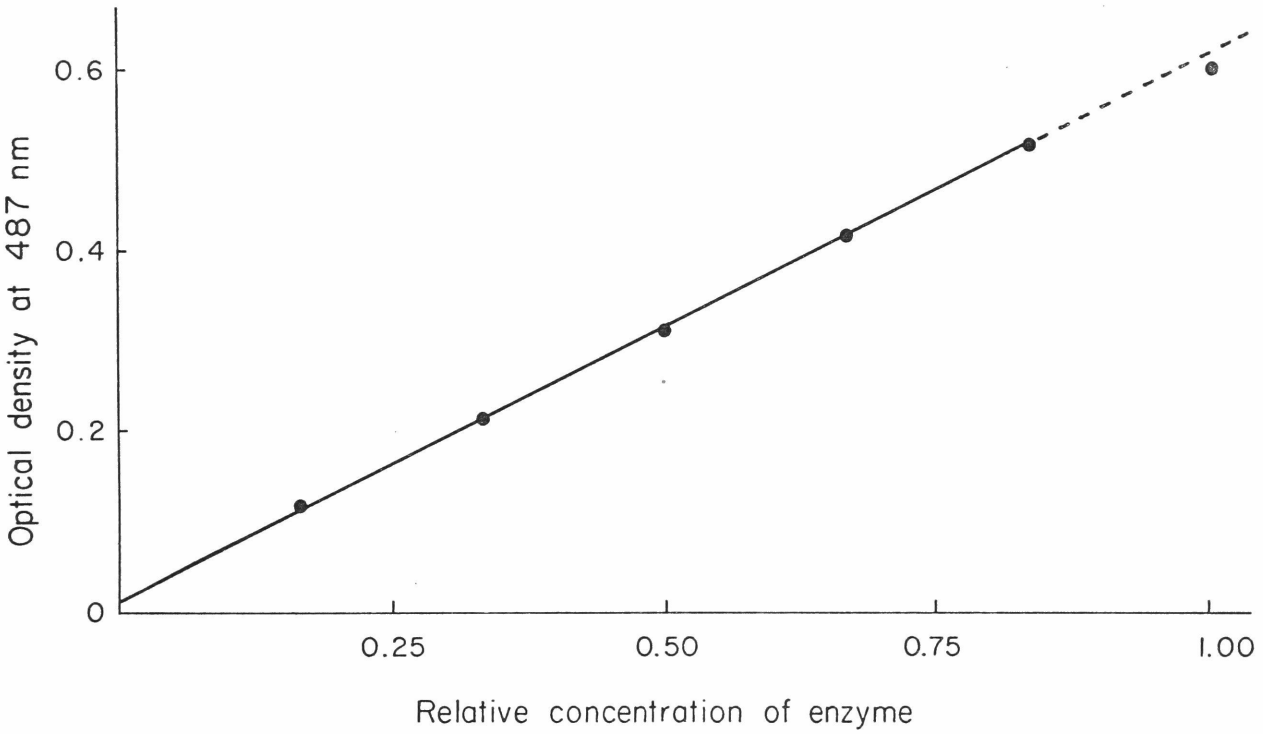


Figure 4. Linearity of esterase assay with respect to enzyme concentration.

The enzyme preparation was a rat liver extract. A relative concentration of 1.0 is 0.5 mg of liver/ml.

counted in a Packard #3375 refrigerated liquid scintillation spectrometer, equipped with automatic external standardization and Packard #7400056 bialkali phototubes. When optimized for double label counting, the efficiencies for tritium and carbon 14 were 37 and 60 percent, respectively, in their own counting channels. Their efficiencies in the cross channels were 0.3 and 14 percent, respectively. Observed counting rates were converted to dpm of each isotope by computer.

When total proteins of cell fractions were to be counted, they were precipitated with cold 10% trichloroacetic acid (TCA) containing 1 mM leucine and 0.5 mM δ-aminolevulinic acid (ALA). The precipitates were collected by centrifugation and washed twice with cold 5% TCA, containing leucine and ALA in the same concentrations.

Triton X-100 was obtained from Ruger, PPO and dimethyl POPOP were from Packard, and Mallinckrodt supplied the toluene.

Isotopes were obtained from Schwarz/Mann in 0.01 N HCl. They were neutralized and adjusted to isotonicity by the addition of 1/10 volume of 0.55 M $NaHCO_3$ in 1.14 M NaCl. Sometimes they were lyophilized and dissolved in saline in order to reduce the volume.

C. SDS Polyacrylamide Gel Electrophoresis

SDS gel electrophoresis was performed as described by Maizel (1971).

Samples were prepared in 0.1 M sodium phosphate buffer, pH 7.1, containing 1% sodium dodecylsulfate (SDS), 0.1% β-mercaptoethanol, and 4% sucrose. They were heated at 100° for five minutes or at 37° for two hours. Immunoprecipitates dissolve nicely in this solvent.

Four percent polyacrylamide gels were polymerized in 6 mm (i.d.) x 110 mm glass tubes from a solution containing 4% acrylamide, 0.2% N,N'-methylenebisacrylamide (BIS), 0.03% ammonium persulfate, and 0.03% N,N,N',N'-tetramethylethylenediamine (TEMED). Occasionally 7% acrylamide gels were prepared (using 0.35% BIS). The electrophoresis chamber reservoirs contained 0.1% SDS in 0.1 M sodium phosphate buffer,

pH 7.1. Electrophoresis was at 4 volts per cm for 3.5 hours with a heat dissipation of about 0.3 watt per gel.

The gels were fixed in 15% TCA, stained with 0.2% Coomassie Brilliant Blue in 50% methanol plus 8% acetic acid, and destained in 7.5% acetic acid containing 30% methanol, as described by Lizardi (1971). The migrations of the proteins were measured and expressed relative to an internal standard of cytochrome c to facilitate comparison of different experiments. The absolute migration rate of a protein in several gels prepared and run at the same time was very reproducible.

For radioactivity determinations the gels were sliced, and each slice was solubilized by an overnight incubation at 60° with 0.5 ml of 6% H_2O_2. Ten ml of dioxane containing 0.8% Butyl PBD and 10% naphthalene were added (Lizardi, 1971). The slices were then counted in a Packard #2002 room temperature liquid scintillation spectrometer with a 45% efficiency for tritium.

Acrylamide, BIS and TEMED were obtained from Eastman. Ammonium persulfate and Coomassie Brilliant Blue were obtained from Baker and Mann, respectively. SDS, recrystallized naphthalene and scintillation quality dioxane were obtained from Matheson, Coleman and Bell. Butyl PBD [2-(4'-t-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxdiazole] is a primary fluor manufactured by Beckman. Its fluorescence is detected by the RCA phototubes in the Packard #2002 spectrometer without the necessity of spectral shifting by a secondary fluor.

D. Immunochemical Procedures

The preparation of antigen, injection of animals, and collection and testing of sera were performed as described by Chase (1967). Immunodiffusion and immunoelectrophoresis were carried out according to the methods of Munoz (1971), Williams (1971), and Williams (personal communications). Details are given in the text and in figure legends.

Chapter V describes the development of methods for the specific precipitation of small amounts of catalase out of crude cell fractions.

E. Rats

Female Sprague-Dawley rats were used exclusively. They were generally obtained from Charles Rivers Breeding Laboratories (North Wilmington, Mass.) and weighed 220-260 grams. Purina Laboratory Chow and water were provided *ad libitum* and the animal room was maintained on a schedule of alternating 12 hour periods of light and dark.

F. Calculations

Data reduction, theoretical simulations, and plotting of complex functions were performed on a PDP-15 computer with a Calcomp 165 on line. Curve fitting was performed on Dr. Hartline's Control Data 160A computer with an on-line oscilloscope display.

III. PURIFICATION OF CATALASE

Catalase was purified from rat liver by a modification of the methods described by Greenfield and Price (1956), Price et al. (1962), and Higashi and Peters (1963a). All operations were carried out at a temperature of 0-4°. A large granule fraction, corresponding to the sum of the M and L fractions of de Duve et al. (1955) was isolated from rat liver by differential centrifugation, and resuspended in 2 ml of eluting solution per gram of liver (eluting solution consists of 220 ml of ethanol mixed with 44 ml of 1 M sodium acetate buffer, pH 4.1, and brought to 1 liter with water). After 30 minutes, the suspension was mixed vigorously with one-tenth its volume of chloroform, and left to stand for 10 minutes, following which it was centrifuged for 15 minutes at 5,000 rpm. To the supernatant was added 0.5 M Na_2SO_4 to a final concentration of 0.01 M, and the precipitate formed was separated by a 10 minute centrifugation at 5,000 rpm. The pellet was taken up in a small volume of 0.01 M potassium phosphate buffer, pH 7.0, and dialyzed overnight against 1 liter of this buffer. The undissolved proteins in the dialysis bag were removed by a 10 minute centrifugation at 5,000 rpm, and the supernatant was purified further by gel filtration through a column of Sephadex G-200 with a solution containing 0.5 M NaCl and 0.1 M potassium phosphate buffer, pH 6.0, as solvent.

In Table I are shown the results of one purification experiment. The spectrum of the purified catalase is shown in Figure 5 and some extinction coefficients are listed in the first line of Table II for comparison with literature values. The shape of the spectrum in the UV region is very similar to that published by Price et al. (1962) for their rat liver catalase and, as may be seen from Table II, the mass extinction coefficients and ratios thereof are quite comparable. On this basis we may infer that our catalase is reasonably pure.

So far as I know, the visible spectrum of rat liver catalase has not been published. Our visible spectrum (Fig. 5) is similar in shape

Table I

Purification of Rat Liver Catalase

Fraction	Activity	Protein	Specific activity	Recovery
	units	mg	units/mg*	%
Homogenate	562	2285	0.25	100
M + L fraction	332	375	0.89	59
After chloroform	220	28	7.9	39
After Na_2SO_4 and dialysis	163	6.2	26.3	29
After Sephadex G-200 chromatography	135	2.6	52.0	24

* Equal to 10^{-3} Kat.f.

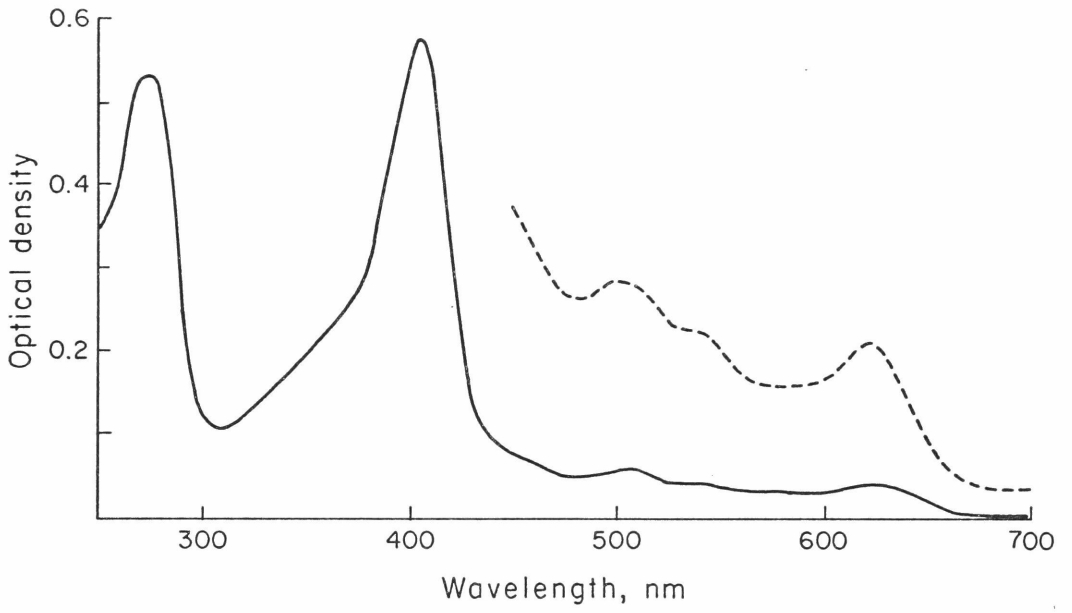


Figure 5. UV and visible spectrum of rat liver catalase.

The sample contained 18.8 units of catalase activity per ml and 0.360 mg of protein per ml (specific activity = 52) in a solvent of 0.5 M NaCl, 0.1 M potassium phosphate buffer, pH 6.0. The spectrum was determined on a Cary model 14 spectrophotometer, using both the 0-2.0 and the 0-0.2 optical density slidewires. The cuvette in the reference compartment contained solvent only. The spectrum in the visible region is also shown amplified 5 times (dotted line). The extinction coefficients are shown in line 1 of Table II.

Table II

Comparison of Properties of Purified Catalase with Literature Values

Catalase preparation	Specific activity†	Activity extinction coefficient*	Mass extinction coefficients††				
	units/mg	407 nm	407 nm	312 nm	276 nm	252 nm	$\frac{\varepsilon_{407}}{\varepsilon_{276}}$
Purified from M + L							
Experiment 1	52	0.031	1.58	0.30	1.47	0.96	1.08
Experiment 2	51	0.032	1.64	0.32	1.46	0.90	1.12
Purified from peroxisomes							
Experiment 1	58	0.032	1.88	0.34	1.67	1.10	1.13
Experiment 2	60	0.031	1.86	0.36	1.83	§	1.02
Calculated from the data of Price et al. (1962)	73	0.023	1.68	0.29	1.55	1.04	1.085

†Equal to 10^{-3} Kat.f.

*Absorbance per cm of a solution containing 1 unit of catalase activity per ml.

††Absorbance per cm of a solution containing 1 mg protein per ml (ε).

§ε_{252} not measured, but $\varepsilon_{260} = 1.57$, compared to 1.27 for peroxisome experiment 1. This may be due to a small amount of light scattering; then the ε_{276} is slightly overestimated, and $\varepsilon_{407}/\varepsilon_{276}$ correspondingly underestimated. This correction decreases toward the longer wavelengths, since scattering goes as the fourth power of the frequency of the light.

to that obtained by Keilin and Hartree (1951) for horse liver catalase, but our mass extinction coefficients are 68-79% of theirs. In addition, the rat liver enzyme has a definite peak of absorbance at 500 nm, whereas the horse liver enzyme shows only a shoulder at this wavelength.

The purity of our catalase was tested further by electrophoresis in PAMU-polyacrylamide gels (kindly performed by D. Wood). PAMU stands for phenol, acetic acid, mercaptoethanol and urea; in these gels protein migration depends mainly on molecular weight, but charge also plays some role (Wood, 1970). Most of the protein in our catalase preparation migrated as a single band, but several minor bands were also detected.

In order to obtain purer catalase preparations, we decided to exploit further the cell biology of our system, and take advantage of the fact that Nature has conveniently localized catalase in peroxisomes, along with only 2.5% of the total liver proteins. We purified peroxisomes according to the method of Leighton et al. (1968) and thereby achieved a 35-fold purification of the catalase, although only a 15% recovery. The peroxisomes then served as starting material for the chemical purification described above. The results of the last step in the purification, namely molecular sieving on Sephadex G-200, are shown in Figure 6, along with similar results obtained previously with catalase purified from an M+L fraction. In contrast to those earlier results, the protein and catalase profiles are almost identical in the catalase peak; the specific activity is nearly constant all the way across the peak. Therefore, the proteins that are observed eluting just after catalase when an M+L fraction serves as starting material are removed in the preparation of peroxisomes.

When catalase prepared in this way from peroxisomes is electrophoresed on an SDS-polyacrylamide gel, it appears to be homogeneous, as shown in Figure 7. The catalase migrates with an apparent molecular weight of 60,000, as determined by comparison with standards of known molecular weight (Figs. 7 and 8 and Table III). The migration of this rat liver catalase is identical to that of beef liver catalase, whose

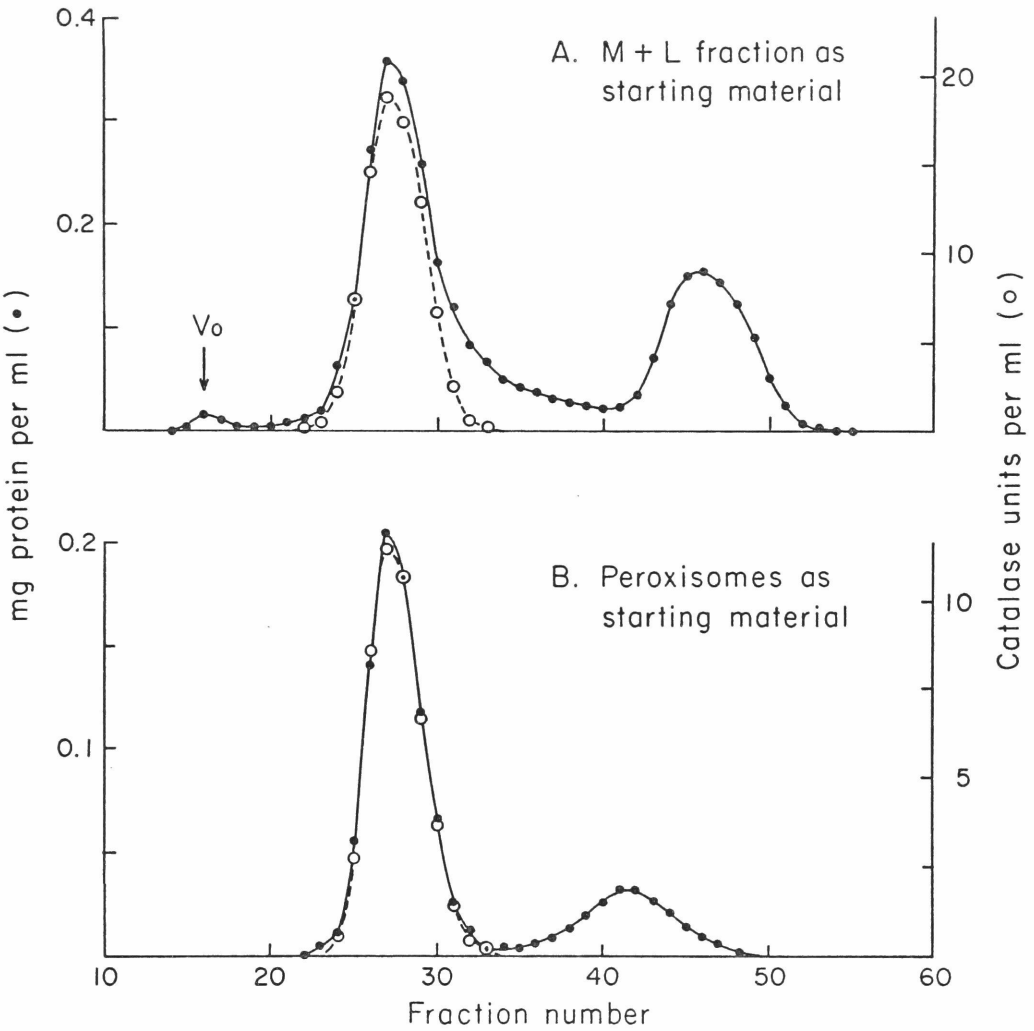


Figure 6. Final purification of catalase by gel filtration on Sephadex G-200.

The column was 2.5 cm in diameter and 91 cm long. The solvent was 0.5 M NaCl, 0.1 M potassium phosphate buffer, pH 6.0, and the flow was upward at 20 ml per hour. A 5 ml sample was applied and 10 ml fractions were collected. The ordinates are drawn so that the protein (solid circles) and the catalase (open circles) coincide at a specific activity of 58 units per mg. A) The protein in the catalase peak was also measured by the ninhydrin method after alkaline hydrolysis and these values agreed with those determined in the usual way by the automated Lowry method. Bovine serum albumin was used as standard with both methods.

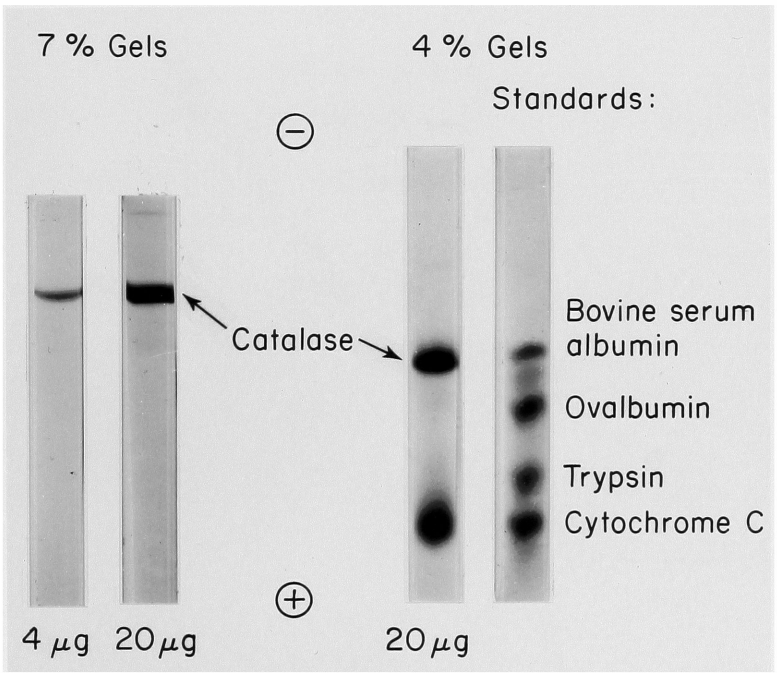


Figure 7. SDS gel electrophoresis of purified rat liver catalase prepared with peroxisomes as starting material.

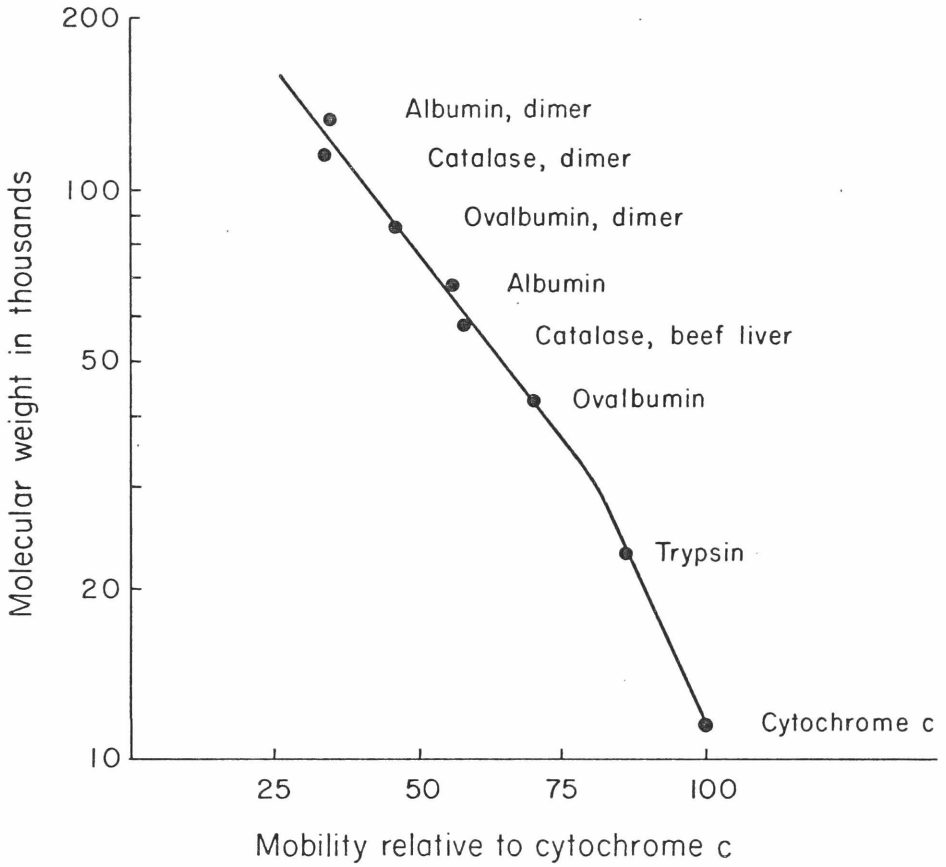


Figure 8. Calibration of 4 percent SDS gels.

The proteins used are described in Table III and were electrophoresed separately as well as together. The results (including the appearance of ovalbumin and BSA dimers) are similar to those of Shapiro, Viñuela and Maizel (1967). The break in the curve is reported by Dunker and Rueckert (1969) to be a reproducible feature of these gels: the breakpoint shifts to higher molecular weights with decreasing acrylamide concentrations.

Table III

Proteins Used to Calibrate SDS Gels

Protein	Species	Tissue	Molecular weight*
Albumin	beef	serum	68,000
Catalase	beef	liver	58,000
Ovalbumin	chicken	egg white	43,000
Trypsin	beef	pancreas	23,300
Cytochrome c	horse	heart	11,700

*According to Weber and Osborn (1969) except for that of catalase which is from Schroeder et al. (1969).

All proteins were obtained from Sigma except for serum albumin which was obtained from Armour.

molecular weight was reported by Schroeder et al. (1969) to be 58,000 after nearly complete analysis of its primary amino acid sequence. A slowly migrating, minor band may be seen in the second gel from the left in Figure 7. A similar band is sometimes seen in 4% gels; its migration is identical to that of beef liver catalase dimers. Note that whereas during analysis by SDS gel electrophoresis catalase migrates mainly as a monomer, in the last preparative step (G-200 chromatography) it migrates as a tetramer. Therefore SDS gel electrophoresis is a good test of purity.

In addition to meeting these physical criteria of homogeneity, our rat liver catalase prepared from peroxisomes appears to be pure by the very different criterion of immunochemical analysis. As described below, when this catalase is injected into a goat, the resulting antibody does not react with any rat liver component other than catalase (Fig. 13). Therefore, we may be confident of the purity of this rat liver catalase preparation.

The specific activity and spectral properties of catalase prepared from peroxisomes is shown in lines 3 and 4 of Table II. Our mass extinction coefficients agree with those of Price et al. (1962), or if anything are a littler higher than theirs (indicating greater purity). However, it is disturbing that whereas our catalase has a specific activity of 58-60 units per mg, a value of 73 units per mg can be calculated from their data. It must be noted that a similar difference exists for the total catalase activity of the liver. The number of catalase units per gram liver reported by Price et al (1962) is 50-100% higher than the values found in our laboratory (Leighton et al., 1968), as well as in the Louvain laboratory (Beaufay et al., 1964).

The most likely explanation of these discrepancies would appear to be a difference in assay procedure. To test this possibility, we assayed some purified catalase both by our method and by the spectrophotometric method of Beers and Sizer (1952), employed by Price et al. (1962). The observed activity is the same whether examined spectrophotometrically, or after reaction with $TiOSO_4$. It is also the same

with 1.5 mM H_2O_2 (our conditions) or 10 mM H_2O_2 as used by Price et al. (1962). Finally, we can exclude the possibility that the difference in assay temperature (we work at 0°, they at 22°) could account for the discrepancy, since catalase has a very low temperature coefficient. We find a Q_{10} of 1.13, in substantial agreement with the value of 1.10 assumed by Price et al. (1962).

Barring a loss of activity in the course of purification--an explanation we cannot rule out, but consider unlikely since the difference between our data and those of Price et al. (1962) also affects the total liver activity--we are left with the possibility of a genuine difference in the specific activity of catalase in the strains of rats used in our work and in that of Price et al. (1962). Such a difference in specific activity has been detected by Ganschow and Schimke (1969) in two strains of inbred mice. The difference might be due in part to the presence of some degraded heme in catalase (Bonnichsen, Chance and Theorell, 1947; O'Carra and Colleran, 1969; Colleran and O'Carra, 1970). Conceivably there might also be a sex difference in the specific activity of catalase; we use exclusively female rats whereas Price uses both sexes.

In summary, then, the G-200 chromatography, spectral analysis, SDS gel electrophoresis, and immunochemical observations all indicate that our catalase purified from rat liver peroxisomes is pure. Its specific activity indicates that it is somewhat less active than the preparation isolated by Price et al. (1962).

IV. PREPARATION OF ANTICATALASE

During the course of this work two anticatalase preparations were used. The first was made in rabbits and found to be impure; it was purified by affinity chromatography. Later a monospecific goat anticatalase antiserum was prepared.

A. Preparation of Rabbit Anticatalase

Eight mg of purified catalase were emulsified in Freund's adjuvant and injected into each of 2 rabbits. Each rabbit was injected at 4 sites: 2 intramuscular in the thighs and 2 subcutaneous near the shoulder. They were bled, beginning 2 months later, taking 30 ml from each once a week for 5 weeks. Fluid balance was maintained by intraperitoneal saline injections. Antibody titers were estimated by semi-quantitative immunodiffusion (see below, Fig. 10), and the first 3 bleedings from each rabbit were pooled. The titers were redetermined by quantitative immunoprecipitation and 4 ml of serum were found to be equivalent to 1 mg of catalase.

The catalase used for immunization was prepared from an M+L fraction and had an $O.D._{407}/O.D._{280}$ ratio of only 0.9, and the resulting antiserum was found to be impure. Ouchterlony double diffusion showed the presence of two antigenic systems, including one involving a mitochondrial component (see below, Fig. 12a). Therefore it was necessary to purify the anticatalase.

B. Purification of Rabbit Anticatalase by Affinity Chromatography

Anticatalase was purified by a modification of the solid phase immunoadsorbent method of Avrameas and Ternynck (1969). This technique uses glutaraldehyde to polymerize a protein into a gel. The gel is then used to adsorb the antibody specific for this protein out of a multivalent antiserum. The gel is washed, and finally the antibodies are eluted from the gel with a solution of low pH or high salt concentration.

When only a small amount of a protein is available, Avrameas and Ternynck (1969) report mixing it with a carrier protein, such as bovine serum albumin (BSA), before polymerization. In the resulting gel, much of the protein of interest is buried internally and inaccessible to the antibody. In applying this technique to the catalase-anticatalase system, we first polymerized some BSA, and then used additional glutaraldehyde to attach the catalase to the preformed gel, in the hope of forming a catalase-coated gel, with most of the catalase accessible.

1. Formation of BSA gel

5% BSA, in 0.2 M acetate buffer, pH 5.0, was mixed with 2/5 its volume of 2.5% glutaraldehyde. A gel formed in about 10 minutes. After sitting half an hour, it was dispersed into small pieces in a loose fitting Potter homogenizer and washed 6 times with acetate buffer.

2. Attachment of catalase

Preliminary experiments with commercial beef liver catalase showed that as much as 3 mg of catalase could be attached in 2 hours to 30 mg of gel protein in 4 ml of acetate buffer, pH 5.0, using 0.2% glutaraldehyde at room temperature. The method of following the attachment was to observe the loss of catalase activity, of optical density at 407 and 280 nm, and of protein from the gel supernatant. The last 2 measurements were corrected for the presence of glutaraldehyde which both absorbs at 280 nm and reacts with the Lowry reagent. In practice, visual control turned out to be sufficient: as the reaction proceeded, the gel turned green and the solution became colorless. The beef liver catalase attached to the gel was not removed by washing with acetate buffer, nor with 0.1 M glycine buffer, pH 2.8, to be used later in eluting the antibodies. However, the catalase had lost 99% of its enzymatic activity.

Experiments with purified rat liver catalase gave similar results, and showed that pH 7 was inferior to pH 5 for the attachment reaction. In an experiment using 1 mg of catalase and 20 mg of gel

protein, all of the catalase was attached to the gel in 50 minutes when the reaction was carried out in pH 5.0 acetate buffer, but only 40% attachment occurred at pH 7.0 in 0.05 M phosphate buffer (criterion was loss of $O.D._{407}$ from the supernatant). Furthermore, the catalase insolubilized at pH 7 had the same poor retention of its enzymatic activity (1%) as did that insolubilized at pH 5, so there is no reason to work at the higher pH.

3. Blockage of unreacted glutaraldehyde groups

The gels described above, despite their lack of enzymatic activity, successfully adsorbed all of the anticatalase out of a rabbit antiserum, while removing less than 10% of the total protein. However, none of the anticatalase could be subsequently eluted from the gel, possibly because it had become trapped by glutaraldehyde groups attached at only one end to the gel. Indeed, the defect was corrected by washing the gel with several rinses of 100 mM ethanolamine in acetate buffer, pH 5.0, before adsorbing the antibody. The first ethanolamine wash sometimes eluted a small amount of green material from such gels. This material showed an abnormal catalase spectrum, with maximum Soret absorbance at 430 instead of 407 nm.

4. Purification of antibody

Purification of antibody was carried out as described by Avrameas and Ternynck (1969), except that antibody was adsorbed onto the gel at 0° instead of at room temperature. Elution was done with 0.1 M glycine-HCl buffer, pH 2.8 at room temperature.

5. Rapid assay of antibody

We developed a semiquantitative method for assay of anticatalase which employs a micro-immunodiffusion technique. Two µl of antiserum are placed in the center of a square pattern, containing known amounts of catalase in the holes around the outside, as shown in Figure 9. Four such assays can be run on one microscope slide; immunodiffusion

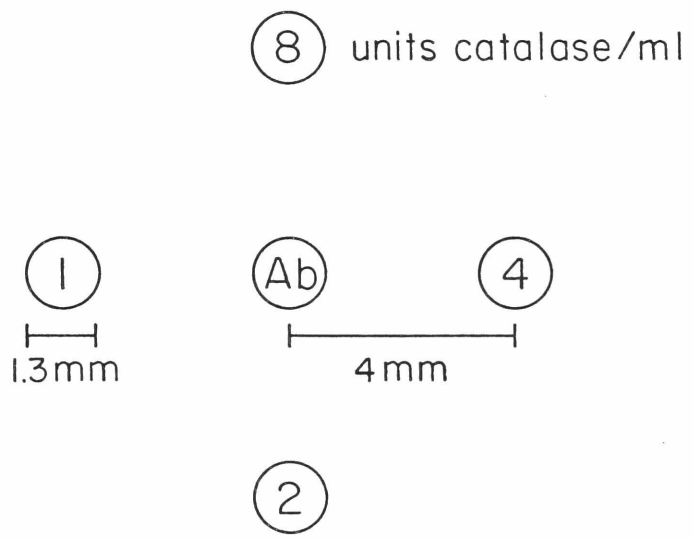


Figure 9. Pattern used for semiquantitative estimation of anticatalase by immunodiffusion.

Four such patterns are cut in a 1.5 mm layer of 2% agar (in 0.9% saline) on a microscope slide. The volume of each well is 2 µl.

takes place overnight at 37°. The resulting pattern of precipitate is compared with those obtained using known amounts of antiserum (Fig. 10). The size of the square, the presence of the fourth side and the sharpness of each line depend on the amount of anticatalase present. If necessary, the center well can be refilled 2 or 4 times (doubling or quadrupling the sensitivity). This technique is rapid, sensitive, and suffices to estimate the antibody concentration to the nearest factor of 2, over a total concentration range from about 20 to 1000 µg of anticatalase per ml.

6. Preparative purification of anticatalase

Pure rat catalase (prepared from purified peroxisomes and of much higher quality than that used to immunize the rabbits) was employed with the methods described above to prepare several batches of purified anticatalase. The details of one preparation are given in Table IV. As shown in Table V, about 50% of the antibody was adsorbed onto the gel. This amounts to about 7 mg of antibody bound to 5 mg of catalase for a weight ratio of 1.4. This ratio compares favorably with those reported by Avrameas and Ternynck (1969) of 0.2-1.0. Therefore, use of a catalase-coated BSA gel is an improvement over co-polymerized mixed gels. The elution of antibody off the gel was roughly 75%. The gel was reused twice with similar results, although there seemed to be some decrease in antibody binding capacity. Altogether, purified anticatalase equivalent to about 14 ml of the original serum was obtained.

C. Analysis of Potency and Specificity of Rabbit Anticatalase

The exact potency of the purified anticatalase was determined by quantitative immunoprecipitation as shown in Figure 11. The 0.3 ml of antibody used is seen to be equivalent to about 1.0 unit of catalase. In the equivalence zone, and in the region of slight antigen excess, the precipitate contains about 58 µg of protein, of which approximately

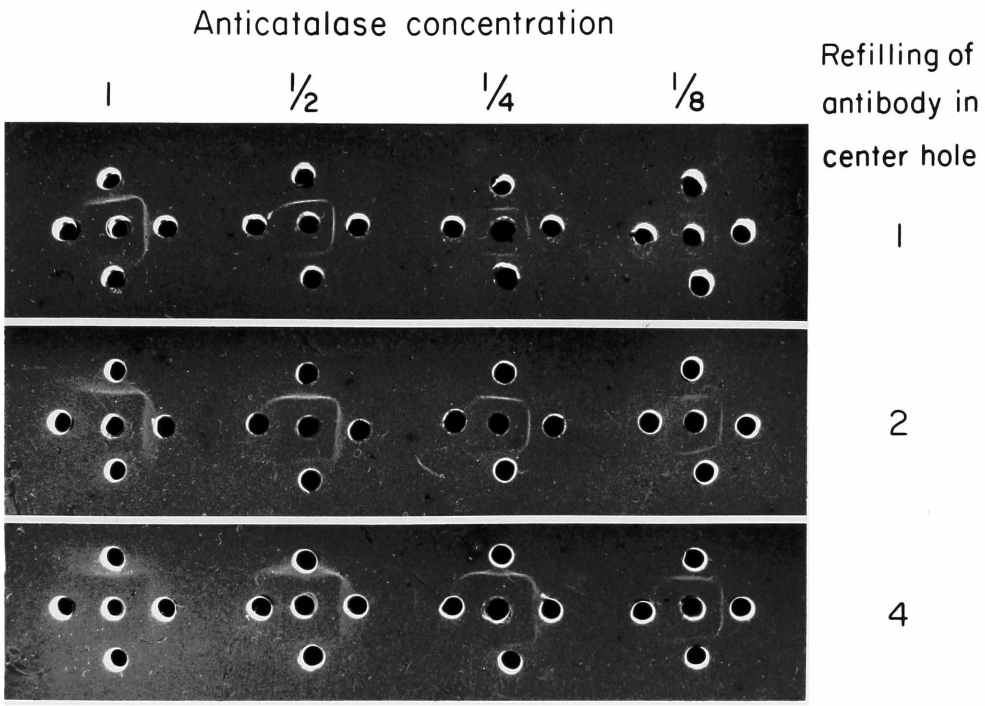


Figure 10. Calibration of semiquantitative immunodiffusion.

The center holes were filled once, twice or four times with rabbit anticatalase (whole serum) or dilutions thereof. One ml of undiluted serum is equivalent to 15 units of catalase. The outer holes were filled with purified rat liver catalase, in the concentrations indicated in Figure 9. Immunodiffusion took place overnight at 37°.

Table IV

Protocol for Anticatalase Purification

1. 7.5 ml of 5% BSA and 3 ml of 2.5% glutaraldehyde, both in acetate buffer pH 5.0 were mixed; the resulting gel was homogenized and washed 3 times with acetate buffer.
2. 4.8 mg of pure rat liver catalase were added to 300 mg of gel protein in 25 ml of acetate buffer containing 0.2% glutaraldehyde. After stirring for 50 minutes at room temperature, the $O.D._{407}$ of the supernatant was 0, indicating complete attachment of the catalase.
3. The gel was rinsed once in acetate buffer and then 3 times in 20 ml of 100 mM ethanolamine in acetate buffer, stirring at room temperature for 10 minutes the first times and 3 hours the last two times. The gel was left overnight in ethanolamine in acetate buffer in the cold room.
4. The gel was washed twice with 50 mM phosphate buffer, pH 7.0 and then twice more with 0.15 M NaCl containing 10 mM phosphate, pH 7 (PBS).
5. 20 ml of rabbit antiserum #1 were added to the gel. This contained approximately 15 mg of anticatalase protein and was equivalent to the catalase on the gel according to quantitative immunoprecipitation. It was stirred in the cold room for 13 minutes.
6. The gel was washed in the cold twice with 6 ml and 3 times with 20 ml of PBS; the $O.D._{280}$ of the last rinse was only 0.06. Approximately 95% of the serum protein appeared in the washes.
7. The gel was eluted at room temperature 4 times with 6 ml of glycine buffer, pH 2.8, stirring for 15 minutes each time. The eluates were filtered through a 0.22 μ Millipore filter and dialyzed overnight against several changes of PBS.
8. The original serum, adsorbed serum, PBS washes, and glycine eluates were all assayed for anticatalase with the semiquantitative immunodiffusion method.
9. The gel was washed once more with 20 ml of glycine buffer and then several times with PBS, and stored in the cold room for reuse.

Table V

Recovery of Antibody in Purification

	Relative anticatalase concentration	Volume ml	Amount of anticatalase*
Whole antiserum	1	20	20
Antiserum after adsorption by gel	$\frac{1}{2}$	20	20
PBS wash #1	$\frac{1}{16} - \frac{1}{8}$	6	0.5
#2	0	6	0
Glycine eluate #1	$\frac{1}{4}$	6	1.5
#2	$\frac{1}{2}$	6	3
#3	$\frac{1}{4} - \frac{1}{2}$	6	2
#4	$\frac{1}{16}$	6	0.5

Total recovery = 17.5/20 = 88%
Anticatalase adsorbed to gel = 20 - 10 - 0.5 = 9.5
Recovery from gel = 7/9.5 = 74%

* 1 unit = that amount of anticatalase present in 1 ml of whole antiserum.

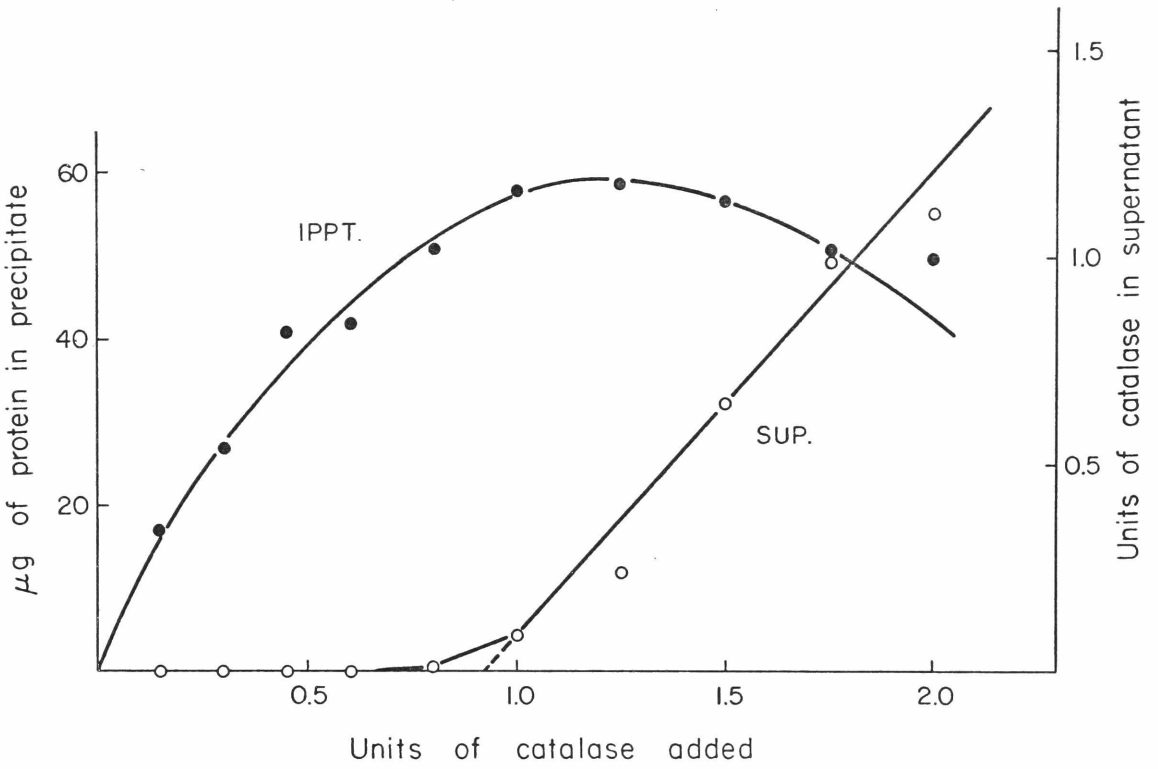


Figure 11. Quantitative immunoprecipitation with purified rabbit anticatalase.

300 μl of anticatalase were incubated with various amounts of purified rat liver catalase for 1 hour at 37° and 36 hours at 0°. The immunoprecipitates were washed twice and dissolved in 0.1 M NaOH for protein determinations. Control tubes lacking anticatalase contained negligible precipitated protein. Control tubes with anticatalase alone had 4 μg of precipitated proteins; this was subtracted from the protein determined in the immunoprecipitates. The catalase remaining in the supernatant after immunoprecipitation was also measured, using both the sensitive and classical automated catalase assays.

15 μg belong to catalase. The molar ratio of γ-globulin to catalase is then $\frac{58 - 15}{15} \times \frac{230,000}{150,000} = 4.4$ (assuming a molecular weight of 150,000 for the goat γ-globulins). This is a reasonable value for a protein of the size of catalase, according to the results of Kabat (1961). When only 0.15 unit of catalase is added (so that the antibody is in large excess), the molar ratio is 8.7, suggesting that there are about 9 antigenic sites per catalase molecule. The next two points in Figure 11 imply molar ratios of 6.6 and 6.7, respectively. Since catalase is tetrameric, a value of 8 sites per molecule appears to be reasonable.

The 300 μl of anticatalase used in this quantitative immunoprecipitation experiment contained 118 μg of protein. Of this, a maximum of 43 μg precipitated; therefore this anticatalase is 37% pure. In contrast, the original serum contained about 0.7 mg of precipitating anticatalase per ml and roughly 70 mg/ml of total protein. Consequently it was about 1% pure. Therefore the affinity chromatography removed more than 97% of the total serum proteins, resulting in a 40-fold purification of the rabbit anticatalase.

The measurements of catalase activity remaining in the supernatant (Fig. 11, open circles) complement the data on the size of the precipitates (Fig. 11, closed circles). Precipitation of the catalase is 99% complete when up to 0.8 units are added. Furthermore, in the region of catalase excess, the catalase found remaining in the supernatants is 0.8 to 0.9 units less than the amount added; a line drawn through these activities extrapolates back to approximately 0.9 units.

The purity of these antibodies was determined by immunodiffusion in chambers with reservoirs of antigens and antibodies (manufactured by Cordis Laboratories, Miami, Florida 33137). As shown in Figure 12b the second line of antibody activity (against a mitochondrial protein) was removed by purification, and the resulting preparation is specific for catalase. This was also checked in additional immunodiffusion experiments against various concentrations of purified catalase and rat liver

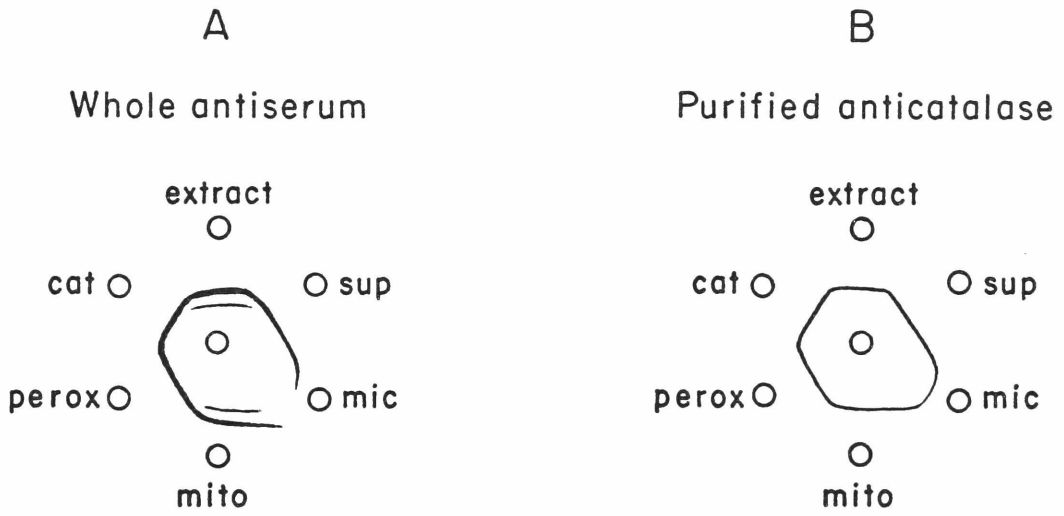


Figure 12. Specificity of rabbit anticatalase.
A. Whole serum.
B. Purified anticatalase.

The outer holes contained: purified rat liver catalase prepared from peroxisomes (cat), rat liver extract (postnuclear supernatant) with 0.2 grams of liver per ml (extract), and four fractions derived from the extract by the method described below in Chapter VII. These were supernatant (sup), microsomal (mic), mitochondrial (mito) and peroxisomal (perox) fractions. The immunodiffusion was performed in cells manufactured by the Cordis Company (Miami, Fla.). The main precipitin line evolves bubbles when flooded with H_2O_2 and stains with the DAB reaction of Graham and Karnovsky (1966) as modified by Novikoff and Goldfischer (1969). The minor line seen in A does neither.

cell extracts. No contaminating antibody system was ever detected. The serum was sterilized by filtration through a 0.22 μ Millipore membrane and stored in the cold room. Its potency was unchanged for at least 6 months.

D. Goat Anticatalase: Preparation and Analysis

Seven mg of pure catalase, prepared from rat liver peroxisomes (described in line 4 of Table II) were emulsified in complete Freund's Adjuvant, and injected into a female goat. Injection was at four sites: two deep intramuscular in the hind legs, and two subcutaneous near the scruff of the neck over the shoulders. The goat was bled two months later (and various times thereafter) from the external jugular vein. A globulin fraction was prepared from the serum by precipitation with half-saturated $(NH_4)_2SO_4$ (final concentration at 25°), washing by reprecipitation, and extensive dialysis against 0.15 M NaCl. The final volume of this globulin preparation was 1/2 that of the serum from which it was derived. The preparation was passed through a 0.22 μ Millipore filter, and frozen in many small lots of 1 to 2 ml each.

Control globulins were similarly prepared from serum obtained from the goat before immunization.

The anticatalase titer in the globulin preparation was determined by quantitative immunoprecipitation (method similar to that of Fig. 11): one ml of globulins is equivalent to 50 units of catalase. This is twice as much anticatalase per ml blood as we ever obtained in rabbits (in four animals), despite the fact that roughly the same absolute amount of catalase was injected into each animal, while the goat outweighed the rabbits approximately ten times.

The specificity of the goat antiserum was determined by immunodiffusion against purified catalase and rat liver extracts (Fig. 13). The relative concentrations of the reactants were varied more than 200-fold (ranging from great antibody excess to great antigen excess) without revealing any immunoprecipitate other than that formed by catalase

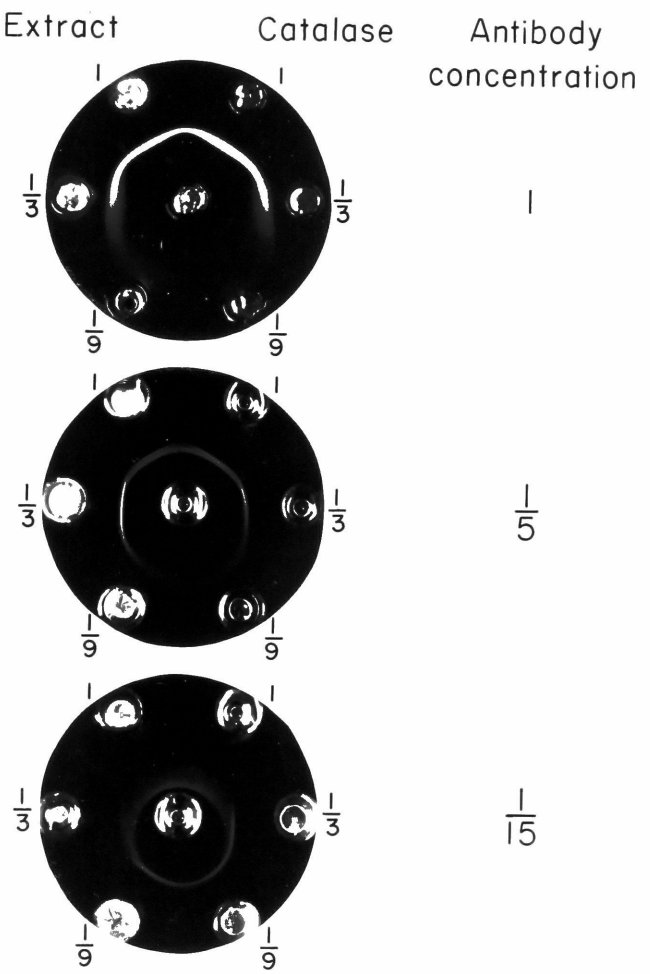


Figure 13. Immunodiffusion of goat anticatalase versus rat liver extract and purified catalase.

The undiluted extract contains 0.2 g liver/ml, the undiluted catalase contains 11 units/ml, and the undiluted anticatalase is the goat globulins preparation described in the text. Immunodiffusion was for 6 days at 0° in cells manufactured by the Cordis Co. (Miami, Fla.). Duplicate sets of cells were incubated for 3 days and for 9 days, without ever detecting a second immunoprecipitation line. A cell containing control goat globulins revealed no immunoprecipitation whatsoever.

and anticatalase. This precipitate causes the evolution of O_2 bubbles when flooded with H_2O_2, and stains with the DAB reaction of Graham and Karnovsky (1966), as modified by Novikoff and Goldfischer (1969). When the anticatalase is replaced with control goat globulins no immunoprecipitate whatsoever can be detected.

In principle, however, a minor contaminating immunoprecipitate might be missed, if it coincided exactly with that of catalase. To test this possibility we performed immunoelectrophoresis and detected no contaminating antibody-antigen system. Therefore we may be confident of the specificity of our goat anticatalase.

V. DEVELOPMENT OF METHODS FOR IMMUNOPRECIPITATION

We wished to use our antibody to precipitate catalase from homogenates, and various cell fractions derived from homogenates, in order to determine its radioactivity (after in vivo labeling). However, catalase constitutes only 0.5% of the liver cell proteins. Therefore it was necessary to modify the standard methods of immunoprecipitation in order to reduce nonspecific precipitation to very low levels while still permitting quantitative precipitation of small amounts of catalase solubilized from cell fractions.

A. Solubilization and Filtration of Cell Fractions

Supernatant, microsomal, mitochondrial, and peroxisomal fractions were brought to 1% concentration in Triton X-100, causing a prompt clearing of the turbidity. They were then filtered through a 0.22 μ Millipore filter. 95-100% of the catalase activity was recovered after filtration. A similar recovery of radioactive proteins was obtained, even when the rat was killed only 3 minutes after administration of the ^{3}H-leucine, such that about half of the radioactivity was in nascent polypeptides attached to ribosomes. Therefore 1% Triton X-100 suffices to solubilize cell fractions.

B. Immunoprecipitation Medium and Collection of Precipitates

Throughout this work the immunoprecipitation medium used is phosphate buffered saline containing 0.1 mM leucine and 0.01 mM δ-aminolevulinic acid (PBSLA). The NaCl concentration is 0.15 M, the phosphate (potassium salts) concentration is 10 mM and the pH is 7.0. In the few exceptional cases where a different medium was used, this fact is explicitly noted.

In all reported experiments, immunoprecipitates were collected by centrifugation for 1 hour at 2000 rpm in a refrigerated International centrifuge. Immunoprecipitates were washed by resuspending them in

fresh medium (same volume as used originally, generally 2.5 ml) and repeating the centrifugation.

C. Effect of Tube Shape and Rotor Angle

Erratic results sometimes occurred in preliminary experiments when immunoprecipitates were collected in round-bottom tubes spun in angle-head rotors. This was probably due to the loss during decantation of material which had been centrifuged onto the wall of the tube.

Conical glass tubes, when spun in swing-out rotors, deposit all of the precipitate in the conical tip. When the supernatants are decanted a small drop of fluid remains stationary in the tip, protecting the precipitate against accidental loss. Use of these tubes (in swing-out buckets) allowed uniform, quantitative collection of catalase immunoprecipitates.

D. Effect of Triton X-100 and Sucrose on Immunoprecipitation

Since both of these agents were to be present in our samples, but are not normally used in immunoprecipitations, their possible interference was checked. Neither 0.4% Triton X-100 nor 0.41 M sucrose interfere with the formation of a 60 µg catalase immunoprecipitate (Table VI). This conclusion is based on the observation that in no case did more than 1% of the catalase activity appear in the immunoprecipitate supernatant or wash; however, there appeared to be a slight, 6% decrease in the protein in the precipitate.

However, it should be noted that these agents are not equally harmless for all immunoprecipitations. We found that both 0.4% Triton X-100 and 0.41 M sucrose interfere with the formation of a similarly sized precipitate from ovalbumin and antiovalbumin (75% and 50% inhibition, respectively).

Table VI

Non-effect of Triton X-100 and Sucrose on the Formation of a Catalase Immunoprecipitate

Incubation was for 1 hour at 37° and then 1.5 days at 0°. The precipitates were washed once and dissolved in 0.1 M NaOH for protein determinations.

#	Components added						Results		
	PBSLA	2% Triton X-100	2.05 M Sucrose	Double Strength PBSLA	Purified Catalase	Purified anti-catalase	Protein in precipitate	Catalase in supernatant	Catalase in wash
	ml	ml	ml	ml	mUnits	µl	µg	mUnits	mUnits
1	2.5	--	--	--	560*	300†	62	1	6
2	2.5	--	--	--	560	300	63	3	1
3	0.5	0.5	0.5	1.0	560	300	58	1	2
4	0.5	0.5	0.5	1.0	560	300	59	2	3
5	2.5	--	--	--	560	--	2	420	1
6	2.5	--	--	--	--	300	12	0	0

* Equal to 9.5 µg protein

† Contains 118 µg protein of which about 43 µg are precipitating anticatalase.

E. Adsorption of Radioactive Material to Container

Various tubes received 0.1, 0.5 or 2.5 ml of a radioactive liver extract (prepared from a rat labeled for 10 minutes *in vivo* with ^{3}H-leucine; approximately 10 mg of protein and 70,000 cpm per ml; 1% Triton X-100) and phosphate buffered saline (PBS) to a total volume of 2.5 ml. The extract was poured out, the tubes were rinsed 3 times with PBS, 1 ml of 0.1 N NaOH was added to dissolve any adsorbed material, and the radioactivity in the NaOH solution was determined. Glass tubes adsorbed about 0.5% of the label. However, siliconized glass tubes, as well as polyallomer and polypropylene tubes retained less than 0.01% of the radioactivity put in them.

F. Number of Washes

Figure 14 shows that 2 washes of the immunoprecipitates are necessary and sufficient.

G. Use of a Preliminary Ovalbumin-antiovalbumin Immunoprecipitate

Peters (1962a, b) has reported the use of one or more chicken serum albumin-anti-chicken serum albumin immunoprecipitates to clear rat liver extracts of nonspecific radioactive proteins that would otherwise appear in specific immunoprecipitates. We tried using ovalbumin-antiovalbumin immunoprecipitates for this purpose (the antiovalbumin did not react with rat liver proteins, according to micro Ouchterlony immunodiffusion). However, in our hands a preliminary immunoprecipitation did not decrease the nonspecific precipitation in subsequent immunoprecipitations.

H. Controls to Determine Nonspecific Precipitation

If the cause of nonspecific radioactivity is proteins which denature and stick to the walls of the tube, then this can be determined in control tubes with no immunoprecipitate at all. If, on the other hand, the presence of an immunoprecipitate increases the amount

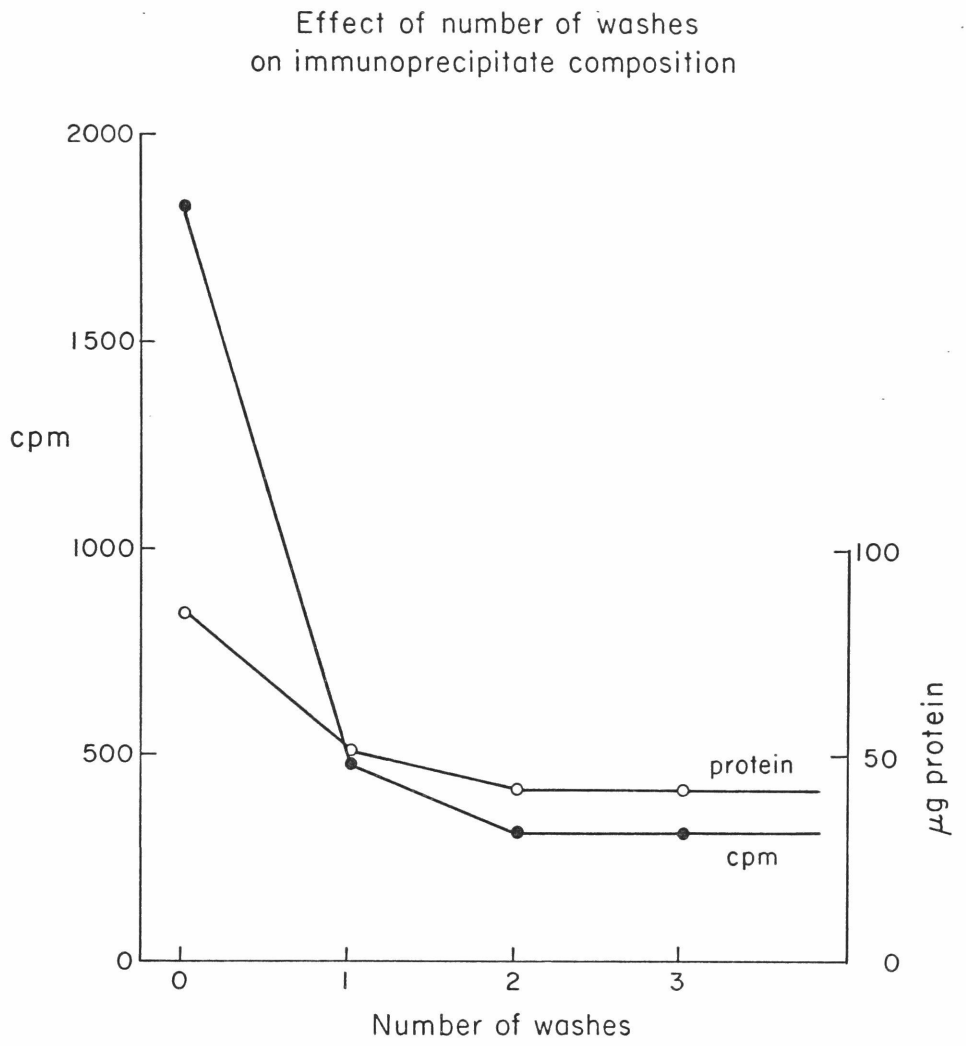


Figure 14. Effect of number of washes on immunoprecipitate composition.

300 μl of purified rabbit anticatalase and 2.5 ml PBSLA were mixed with 100 μl of a rat liver extract containing 0.53 unit of catalase, 1.1 mg of protein, 62,000 TCA-precipitable cpm and 1% Triton X-100. The rat had been labeled in vivo for 15 minutes with ^{3}H-leucine and the extract had been passed through a 0.22 μ Millipore filter. The tubes were incubated for 1 hour at 37° and 36 hours at 0°. The precipitates were washed 0 to 3 times and dissolved in 0.1 M NaOH for cpm and protein determinations. The supernatant of the third wash contained 15 cpm.

of nonspecific radioactivity, this should be sensitive to manipulation of the size of the precipitate.

We tested this by taking aliquots of a labeled rat liver extract and adding anticatalase. Purified non-radioactive catalase was added to some tubes to double the size of the immunoprecipitate. This did not affect the radioactivity precipitated.

To measure nonspecific radioactivity we routinely use control tubes lacking antibody, but having a composition as similar as possible to the experimental, anticatalase containing tubes. Thus in those experiments where purified rabbit anticatalase immunoglobulins were used, control tubes received saline. In other experiments employing goat anticatalase globulins, control tubes received globulins prepared from serum taken from the goat before it was immunized with catalase.

I. Use of Rabbit Serum Globulins to Reduce Nonspecific Precipitation

The methods described up to this point allowed the precipitation of catalase out of cell extracts or homogenates in a reasonably clean and reproducible fashion: control precipitates (in tubes receiving control goat globulins) generally contained 5 to 30% of the label found in the specific immunoprecipitates obtained from homogenates labeled with ^{3}H-lecuine or ^{3}H-ALA. Unfortunately, when employed with subcellular fractions isolated by sucrose gradient centrifugation, the method gave very poor results. Mitochondrial fractions, in particular, deposited large precipitates in control tubes, containing roughly 50 times as much radioactivity as might be expected in the catalase in these fractions. This effect did not occur with homogenates containing the same amount of mitochondrial protein, suggesting that we were dealing with a denaturation artifact that was somehow prevented by the soluble proteins of the homogenate. Indeed, as can be seen from Figure 15, rabbit serum globulins (prepared by ammonium sulfate precipitation), added at a concentration of 4 mg/ml, successfully reduced the nonspecific precipitation from 25% to 2% of the added radioactivity. Bovine

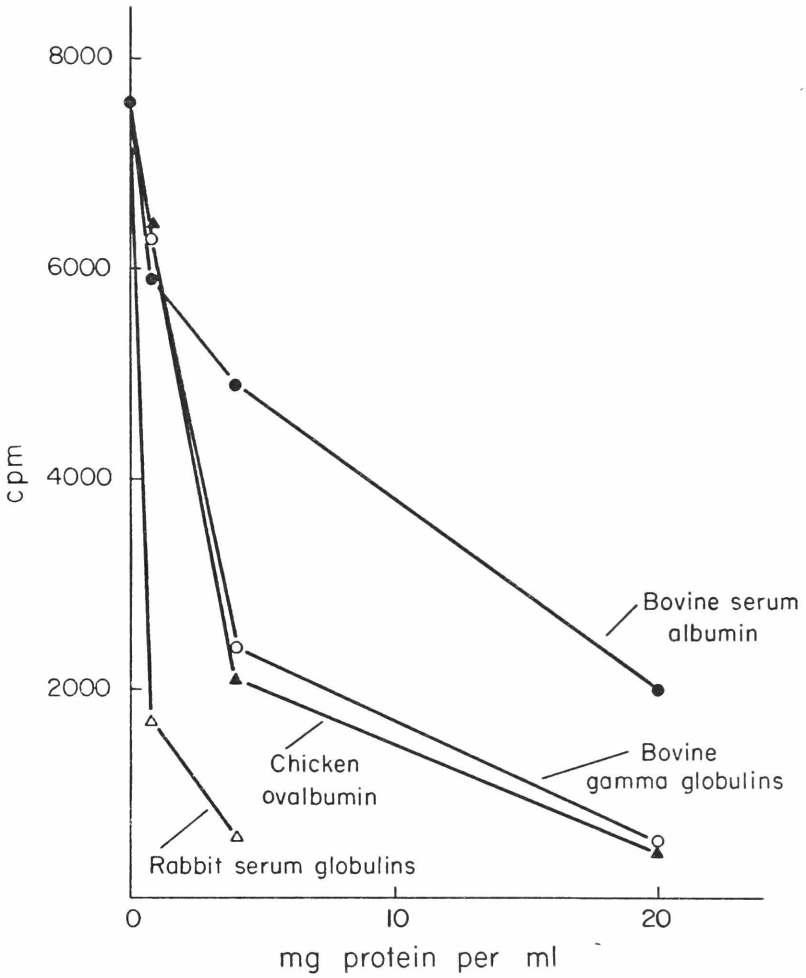


Figure 15. Effect of various proteins on the nonspecific precipitation of mitochondrial proteins.

A rat was injected with ^{3}H-leucine, killed 15 minutes later, and an M+L fraction was prepared from the liver. This was mixed with 1/10 its volume of 11% Triton X-100 and 9/10 its volume of double strength PBSLA and filtered through a 0.8 μ Millipore filter. 0.5 ml (containing 3.3 mg protein and 30,000 cpm) was added to tubes containing various amounts of different proteins dissolved in PBSLA; the final volume was 2.0 ml in all cases. The tubes were incubated at 37° for 1 hour and then at 0° for 36 hours. The precipitates were collected, washed twice, dissolved in 0.1 M NaOH, and their radioactivity determined.

γ-globulins and ovalbumin were as effective when used in higher concentrations. The decrease in radioactivity was accompanied by a dramatic decrease in the amount of precipitate seen in the tubes.

We also checked the effect of two of these proteins on the formation of catalase immunoprecipitates. As shown in Table VII, neither rabbit serum globulins nor bovine γ-globulins increase the amount of catalase activity remaining in the supernatant after collection of the immunoprecipitate. The rabbit globulins have the added beneficial effect of protecting the catalase activity in control tubes. The bovine γ-globulins have the disadvantage of giving rise to a small precipitate, both in the control tube, and in the antibody-containing tube. Therefore, the rabbit globulins seemed the protein of choice, and these were included in the immunoprecipitation milieu of fractions obtained by sucrose gradient centrifugation. Using this technique, we were able to determine the distribution of radioactive catalase in gradient fractions with sufficient accuracy to show a rapid appearance of labeled catalase in peroxisomes, for example (see results, below). Unfortunately, the control tubes for some fractions still contained 50% or more of the label obtained in the specific immunoprecipitates. We did not obtain really clean data until we began omitting the 37° incubation from our immunoprecipitation protocol.

J. Immunoprecipitation at 0°

Higashi and Kudo (1971) reported performing immunoprecipitation at 0°, when attempting to precipitate nascent catalase polypeptides still attached to ribosomes. Table VIII and Figure 16 show that omitting the 37° incubation step solves the nonspecific precipitation problem, without impairing the efficiency of precipitation, which is 98% complete after 6 hours. Addition of rabbit globulins is not necessary when the 37° incubation is omitted.

Table VII

Effect of Serum Globulins on Completeness of Immunoprecipitation

0.56 unit of purified catalase was added to each tube and the tubes were incubated for 1 hour at 37° and 2 days at 0°. After collection of the precipitates, the catalase remaining in the supernatants was assayed.

Added protein	Per cent of catalase remaining in supernatant	
	+ Purified anticatalase	+ Saline
None	1	45
Rabbit globulins (2 mg/ml)	1	80
Bovine γ-globulins (20 mg/ml)	1	46

Table VIII

Effect of Omitting the 37° Incubation on the Nonspecific Precipitation of Mitochondria

Some of the labeled M+L fraction described in the legend to Figure 15 was diluted with PBSLA and filtered through a 0.22 μ Millipore filter. 2.5 ml aliquots containing the same amount of protein as used in the experiment shown in Figure 15 were taken for incubation. The precipitates were washed twice in PBSLA.

#	One hour 37° Incubation	Overnight 0° Incubation	Turbidity after Incubation*	CPM in Precipitate
1	+	+	++++	2920
2	+	+	++++	3000
3	-	+	0	40
4	-	+	0	30

* The turbidity in tubes 1 and 2 was apparent after the first hour.

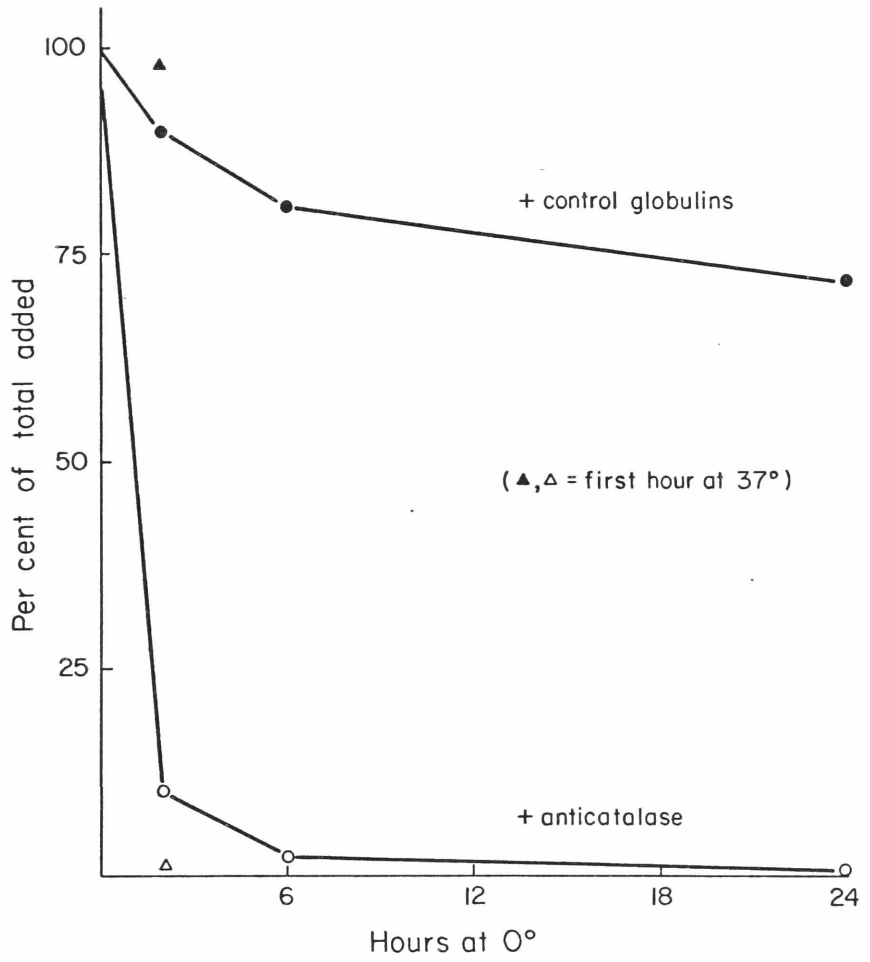


Figure 16. Immunoprecipitation kinetics at 0°: amount of catalase remaining in the supernatant.

The homogenate from 20 mg of liver (treated with 1% Triton X-100 and passed through a 0.22 μ Millipore filter) was incubated with goat anti-catalase or control goat globulins for various lengths of time. The precipitates were removed by centrifugation and the amount of catalase remaining in the supernatant was determined. 100% equals 0.82 unit of catalase.

K. Final Immunoprecipitation Method

The routine procedure which was adopted is summarized in Table IX. It was used for most of the work presented below; occasional exceptions are noted in the figure legends.

Table IX

Final Immunoprecipitation Method

1. Tared, siliconized, conical 3 ml glass centrifuge tubes are used.
2. The medium is phosphate buffered saline containing leucine and ALA (PBSLA); no rabbit globulins are added.
3. Sample is added to duplicate tubes; 50 µl of goat anticatalase are added to one and 50 µl of control goat globulins to the other. The final volume is 2.5 ml and the final sucrose and Triton X-100 concentrations do not exceed 0.41 M and 0.4%, respectively.
4. The tubes are incubated overnight at 0°; there is no 37° incubation.
5. The tubes are spun for 1 hour at 2000 rpm in swing-out rotors in a refrigerated International centrifuge to collect the precipitates.
6. The precipitates are washed twice by resuspending them in 2.5 ml PBSLA and respinning as above.
7. The precipitates are dissolved by adding 0.5 ml of 0.1 M NaOH to the tubes (in whose conical tips 50-100 µl of PBSLA remain). The total volume is determined by weighing the tubes and subtracting the tare weight.
8. 500 µl are taken for determination of the radioactivities. The cpm in the aliquots are converted to dpm in the total precipitates.
9. The original supernatants (from step #5) are assayed for catalase activity to check for quantitative catalase immunoprecipitation, or complete recovery of catalase, in the supernatant in the antibody-containing and control tubes, respectively.

VI. KINETICS OF INCORPORATION OF LEUCINE AND ALA INTO CATALASE

0.5 to 1.0 mC ^{3}H-4,5-L-leucine (6 to 15 C/mmole) or 0.25 mC ^{3}H-2,3-δ-aminolevulinic acid (ALA, 22 C/mmole), in 0.5 to 1.0 ml saline, was injected into the portal vein of 220 to 260 g, female, Sprague-Dawley rats under Nembutal anesthesia (40 mg/kg). Rats were killed after various lengths of time and the livers homogenized in 0.25 M sucrose containing 0.1% ethanol (to protect the catalase, Chance, 1950). Incorporation of leucine and ALA into TCA insoluble material was determined.

Immunoprecipitation was carried out both on the whole liver homogenates, dispersed with 1% Triton X-100 and filtered through a 0.22 μ Millipore filter, and on partially purified catalase from the same homogenates prepared by a modification of the procedure described above (Fig. 17, Table XI). In the former case, both catalase activity and radioactivity were recovered quantitatively in the filtered homogenate, and less than 1% of the catalase activity remained in the supernatant after immunoprecipitation. The precipitate obtained in this manner, to be designated "whole liver immunoprecipitate," contains all the material present in the homogenate that is recognized antigenically by the anticatalase antibodies. With the second procedure, 50 to 70% of the catalase and 3 to 4% of the protein are recovered after the chemical purification, resulting in a 14- to 17-fold increase in specific activity (7 to 9% pure). This preparation retained 5 to 15% of the total TCA precipitable radioactivity in the case of leucine labeling. This partially purified catalase was treated with antibody to prepare the "purified catalase immunoprecipitate," which contained antigenically recognizable catalase that survived the purification procedure. Its radioactivity was corrected for the loss of catalase enzymatic activity during purification, which was measured in each case. That is, if 70% of the catalase activity was recovered after purification, the measured radioactivity was divided by 0.70.

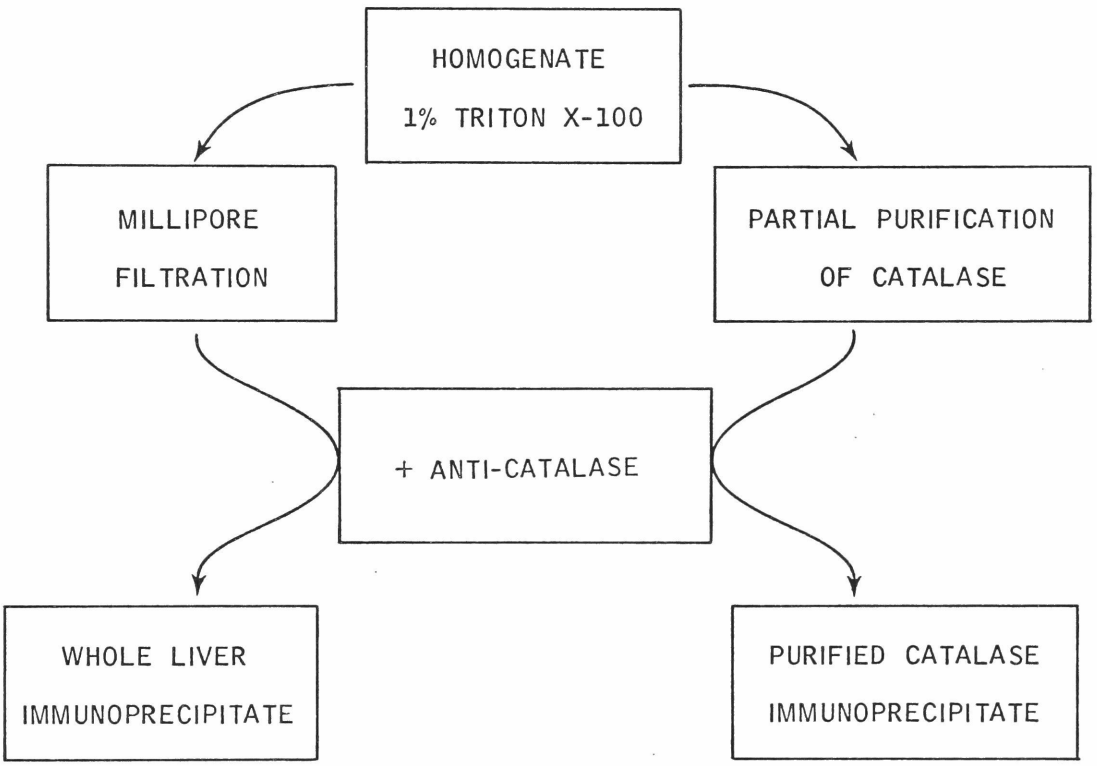


Figure 17. Preparation of the two types of immunoprecipitates. (See also: Table XI in Chapter VIII).

In order to approximate pulse-chase conditions, we administered the leucine or ALA by intraportal injection. As shown in Figure 18, incorporation of both labeled precursors into total TCA-insoluble material was practically completed within a few minutes. Therefore our expectations were reasonably fulfilled. In the case of leucine labeling, we may estimate the half-life of the free leucine pool as 2 minutes. The plateau value is 1.3% of the dose per gram liver; since the livers weigh about 8 g, fully 10% of the injected leucine has been incorporated into liver protein in 8 minutes.

With leucine labeling (Fig. 19), the whole liver immunoprecipitate acquired label with much the same kinetics as did total liver proteins (Fig. 18a). Again the time to reach one-half the plateau labeling is about 2 minutes. The plateau value is 0.0059% of the dose per gram liver. This is 0.45% of the incorporation into TCA precipitable material; catalase constitutes about 0.5% of the total liver proteins. By contrast, the time course of incorporation of labeled leucine into the purified catalase immunoprecipitate is very different. There is a suggestion of an initial lag of 2 to 3 minutes; then the radioactivity increases gradually, approaching the value of the whole liver immunoprecipitate after 2 hours. As shown by the data of Table X, obtained on unanesthetized animals receiving the label by intraperitoneal injection, the radioactivities of the two immunoprecipitates eventually reach the same value.

These results indicate that the newly synthesized catalase protein differs from authentic catalase in an important way. It is antigenically recognizable, but it does not accompany catalase through purification. In 2 to 3 hours the difference in radioactivity between the two immunoprecipitates largely disappears. Since there is no further incorporation of ^{3}H-leucine into protein during this time, we are witnessing the conversion of the antigenic biosynthetic intermediate into purifiable catalase.

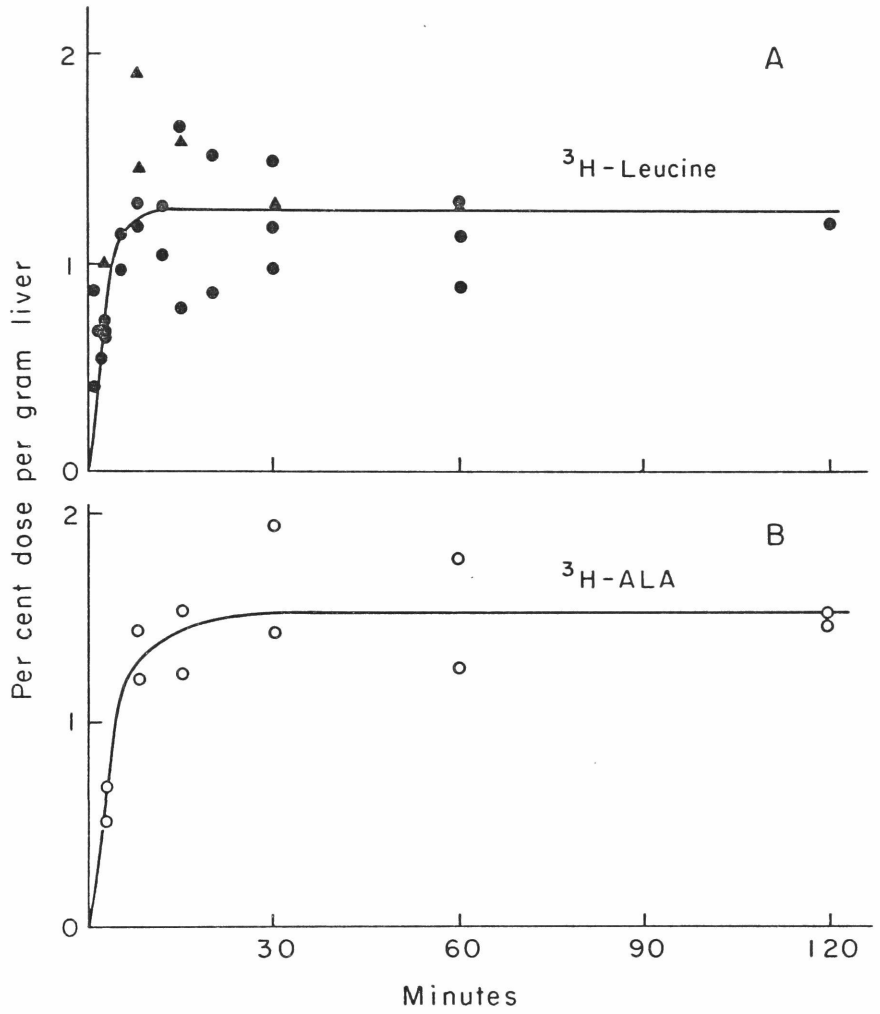


Figure 18. Kinetics of incorporation of ^{3}H-leucine (A) and ^{3}H-δ-amino-levulinic acid (B) into total material precipitated from liver with TCA.

A. The rats received 0.5 to 1.0 mC (circles) or 2 to 5 mC (triangles).
B. The rats received 0.25 mC.

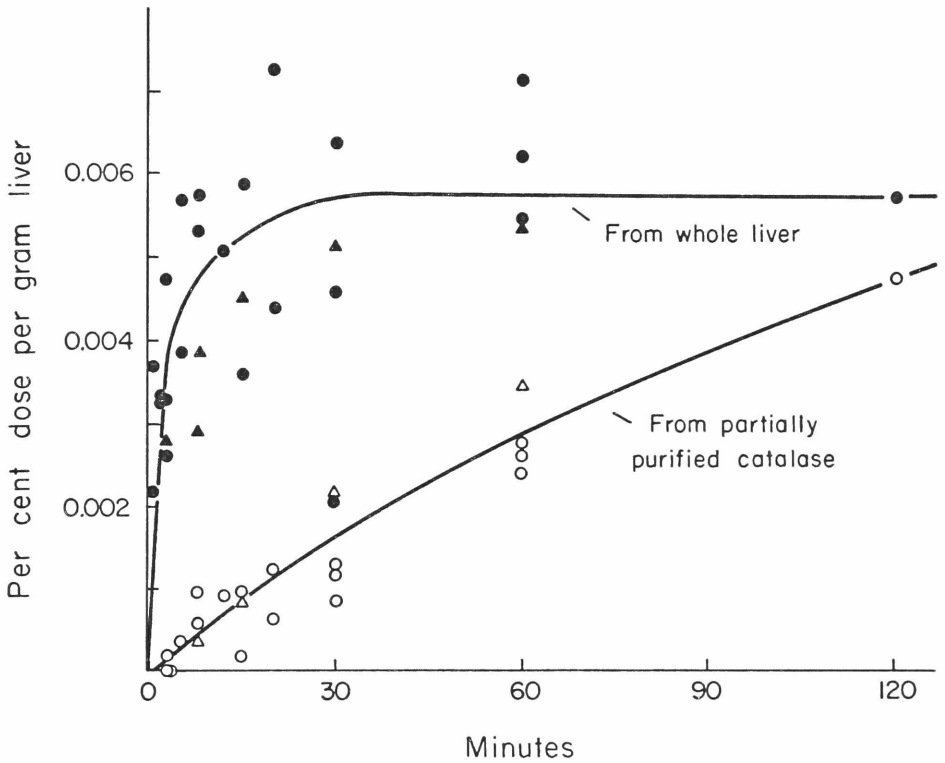


Figure 19. Kinetics of incorporation of ^{3}H-leucine into material precipitated by anticatalase.

The rats received 0.5 to 1.0 mC (circles) or 2 to 5 mC (triangles). The radioactivity in the controls (which has been subtracted) was generally 10 to 35% of the radioactivity in the immunoprecipitate-containing tubes.

Table X

Long-term Incorporation of Isotopes*

Precursor	Dose	Time	TCA PPT	Whole Liver IPPT	Purified Catalase IPPT	Percent purifiable
	mC	hours	% dose/gram liver			
Leucine	0.5	3	0.55	0.0039	0.0029	74
		18	0.38	0.0034	0.0033	97
ALA	0.25	3	1.42	0.059	0.042	71
		3	1.09	0.043	0.034	79
		18	0.59	0.072	0.071	98
		18	0.61	0.074	0.075	101

* After intraperitoneal injection.

It should perhaps be emphasized that the kinetics shown in Figure 19 argue strongly that the radioactivity found in the whole liver immunoprecipitate belongs to some form of catalase. On the one hand, the whole liver immunoprecipitate values, which are the most open to criticism since they were obtained by precipitating catalase out of crude homogenates, are exactly what we expect from the kinetics of labeling of the total liver proteins. On the other hand, the purified catalase immunoprecipitate values, which are intrinsically more reliable since 90% of the radioactivity was eliminated before performing the immunochemistry, continue to increase long after the free amino acid pool is depleted of radioactivity. The simplest interpretation of our results is the one given above, namely that two steps are involved in the biosynthesis of catalase: first amino acid assembly with the formation of catalase-specific antigenic determinants, and then some additional step rendering a biosynthetic intermediate purifiable.

We have also confirmed the above interpretation in another way. The labeled material that is precipitated by anticatalase, but that does not survive purification, migrates on SDS polyacrylamide gels exactly as does catalase. This is illustrated in Figure 57, Chapter IX, for one fraction obtained by sucrose gradient centrifugation from the liver of a rat labeled for 8 minutes with ^{3}H-leucine. In this example, only 4 percent of the labeled material could survive purification. Therefore, we may be confident that the anticatalase is precipitating an intermediate in the biosynthesis of catalase.

The data of Figure 19 may be used to make a quantitative estimation of the size of the metabolic pool of the biosynthetic intermediate. The data are replotted in a slightly different way in Figure 20 and an exponential function is fitted to them by the method of least squares as shown in Figure 21. We find the label in the intermediate disappearing (into authentic catalase) with a half-life of 53 minutes (46 to 64 minutes give the 95% confidence limits of the least squares fit). The half-life of liver catalse is 36 hours

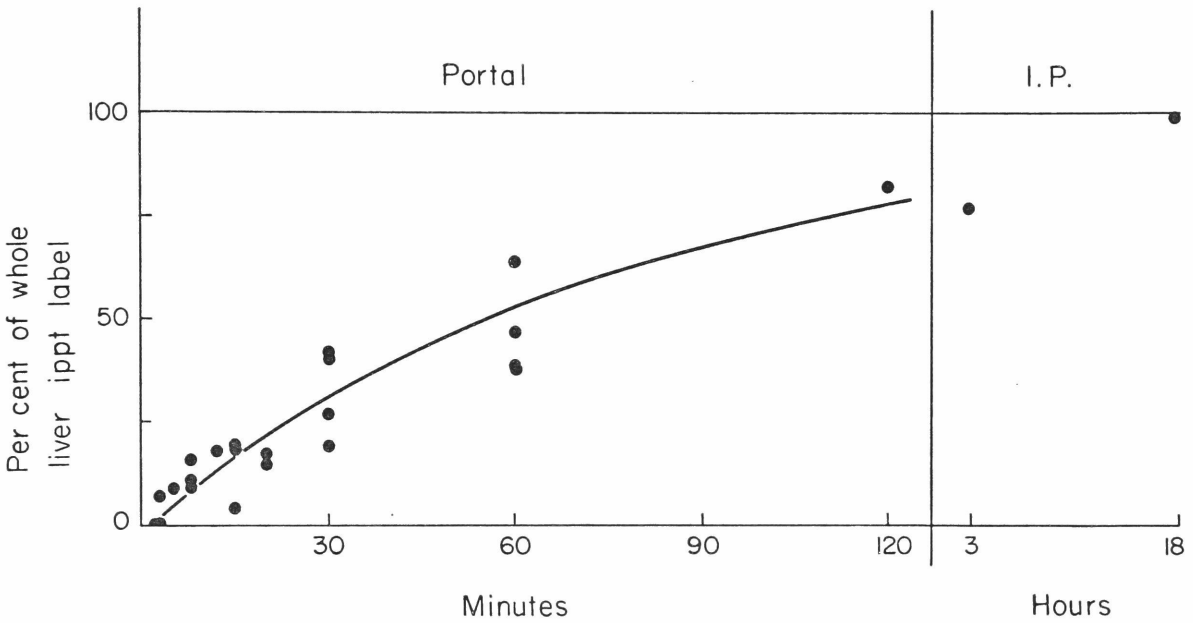


Figure 20. ^{3}H-leucine in purifiable catalase, expressed as a percentage of the label in total catalase, including the nonpurifiable biosynthetic intermediate.

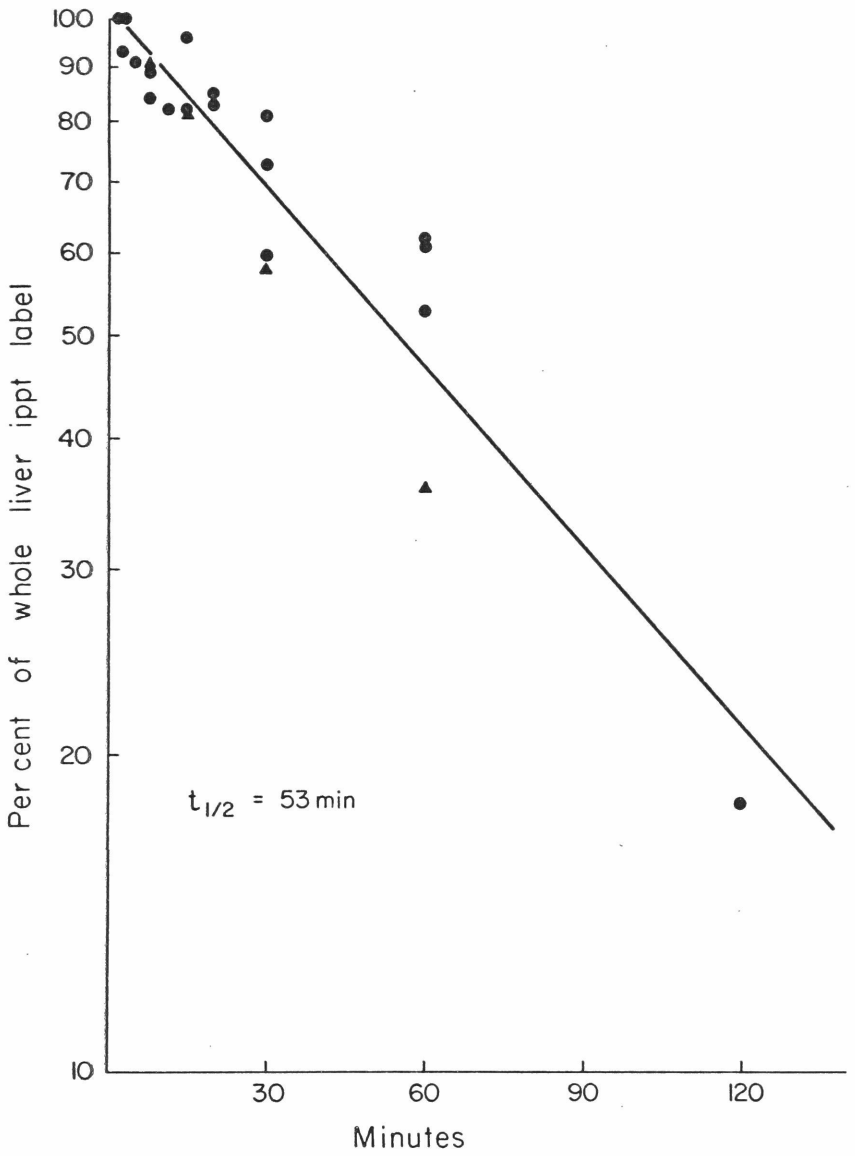


Figure 21. Semilogarithmic plot of ^{3}H-leucine in the catalase biosynthetic intermediate, expressed as a percentage of the label in total catalase.

The regression line was fitted by computer by minimization of the sum of the squared deviations.

(Poole et al., 1969; Poole, 1971). Therefore, this intermediate constitutes $\frac{\frac{53}{60}\text{ hour}}{36\text{ hours}} \times 100 = 2.5\%$ of the liver catalase.

What is the nature of this biosynthetic intermediate? In order to determine whether or not it contains heme, we measured the incorporation of δ-aminolevulinic acid into the immunoprecipitates (Fig. 22). Unlike ^{3}H-leucine, label from ALA continues entering the whole liver immunoprecipitate for much longer than it does the TCA precipitate (Fig. 18b), indicating that ^{3}H-heme remains available for catalase synthesis long after the ALA pool itself has become depleted of radioactivity. We may consider three explanations, which are schematically depicted in Figure 23. First, since heme is formed from ALA by way of porphyrin intermediates, these porphyrins would first trap and then slowly release label to the heme pool if their combined pool sizes were large with respect to the steady state flux through them. However, this would also result in a lag in the appearance of label in catalase, which is not observed. Therefore we may eliminate this alternative. Secondly, the heme pool itself may be large with respect to the flux through it. This is the simplest hypothesis consistent with our data. Thirdly, and more speculatively, there might be a small free heme pool exchanging with a large pool of heme in hemoproteins (Garner and McLean, 1971). Whatever the reason may be, the kinetics of Figure 18b indicate that the intermediates must be TCA insoluble. In any event, we do not have "pulse-chase" labeling of catalase using ALA as precursor.

The kinetics of appearance of label derived from ALA in the purified catalase immunoprecipitate differs from that for the whole liver immunoprecipitate (Fig. 22). The former shows a lag during the first 8 minutes not seen in the latter. Furthermore there is a gap between the two curves which persists beyond 2 hours but disappears after a longer time period, as it does after leucine (Table X). This indicates that there is a heme-containing biosynthetic intermediate that does not accompany catalase through purification.

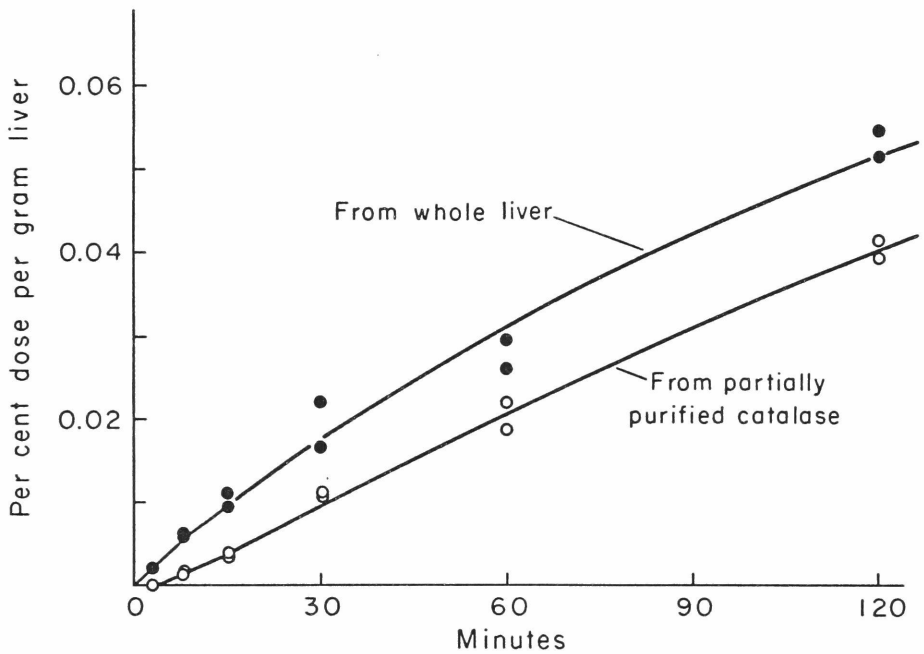


Figure 22. Kinetics of incorporation of ^{3}H-δ-aminolevulinic acid into material precipitated by anti-catalase.

The radioactivity in the controls (which has been subtracted) ranged from 0.0011 at 3 minutes to 0.0021 at 120 minutes for the whole homogenate determinations; for the partially purified catalase it was less that 0.0001% dose/gram liver.

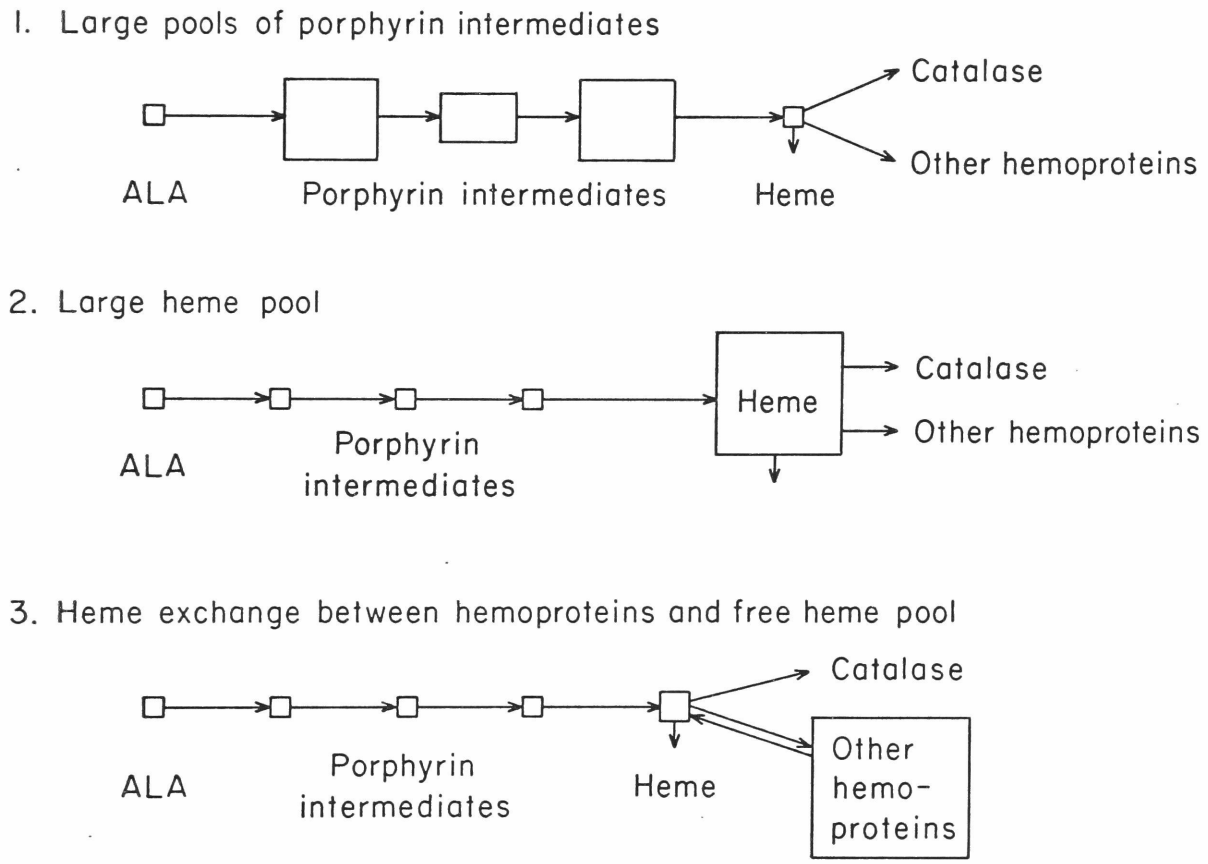


Figure 23. Hypothetical models for the prolonged labeling of catalase after ^{3}H-ALA injection.

The data of Figure 22 are replotted in Figure 24. A simple exponential may be fitted to the first 10 points as shown in Figure 25. This ALA-labeled intermediate has an apparent half-life of 32 minutes (27 to 39 minutes give the 95% confidence limits of the least squares fit). However, the effect of the prolonged labeling on this apparent time constant must be evaluated.

We consider first the simplest possible model, which assumes the incorporation of a precursor such as leucine or heme into a single biosynthetic intermediate, and the conversion of the latter to catalase. This model is shown in Figure 26. The differential equations for the transport of radioactivity through this system are solved, assuming simple exponential decay of the label in the precursor.

The solutions to the equations are illustrated in Figure 27 for one value of τ_i; other values have also been examined. It is obvious that the value of τ_p has a profound influence on the kinetics of appearance of label in the finished protein. Clearly it is advantageous to try to arrange pulse-chase conditions if one wishes to demonstrate an intermediate/product relationship. This conclusion applies equally to biosynthetic intermediates and to intermediate localizations in transport through the cell.

The ratio of I to T (Figs. 26 and 28) corresponds to our experimental parameter, percent of radioactivity in catalase that is not purifiable (Figs. 21 and 25). When τ_p is small, that is, when one has "pulse-chase" kinetics, the expression for I/T simplifies to e^{-t/τ_i} (Fig. 26). Therefore, in our experiments with leucine (τ_p = 3, half-life = 2 minutes) we were justified in equating the slope of the best line through the data of Figure 21 with the lifetime of the biosynthetic intermediate (τ_i = 76 minutes; half-life = 53 minutes).

However, when τ_p is large, as in the case of labeling with ALA, the full expression for I/T (Fig. 26) applies. The behavior of this complex function is illustrated in Figure 28 for one value of τ_i. It is seen to be pseudo-exponential, that is, $I/T \simeq Ae^{-t/\tau_{apparent}}$.

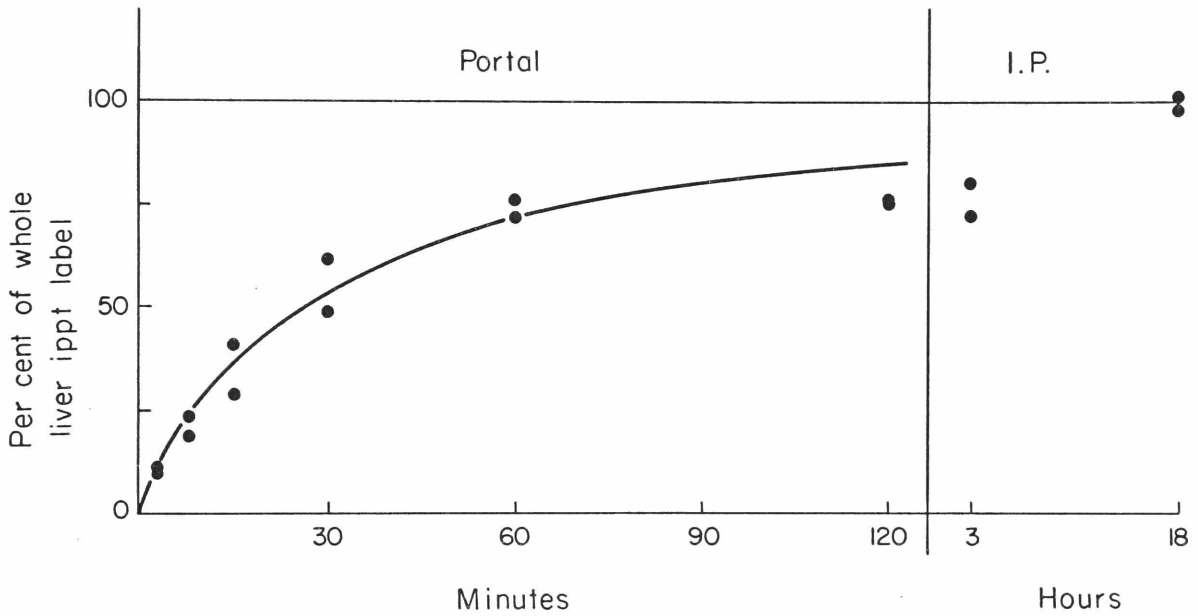


Figure 24. ^{3}H from ALA in purifiable catalase, expressed as a percentage of the label in total catalase, including the non-purifiable biosynthetic intermediate.

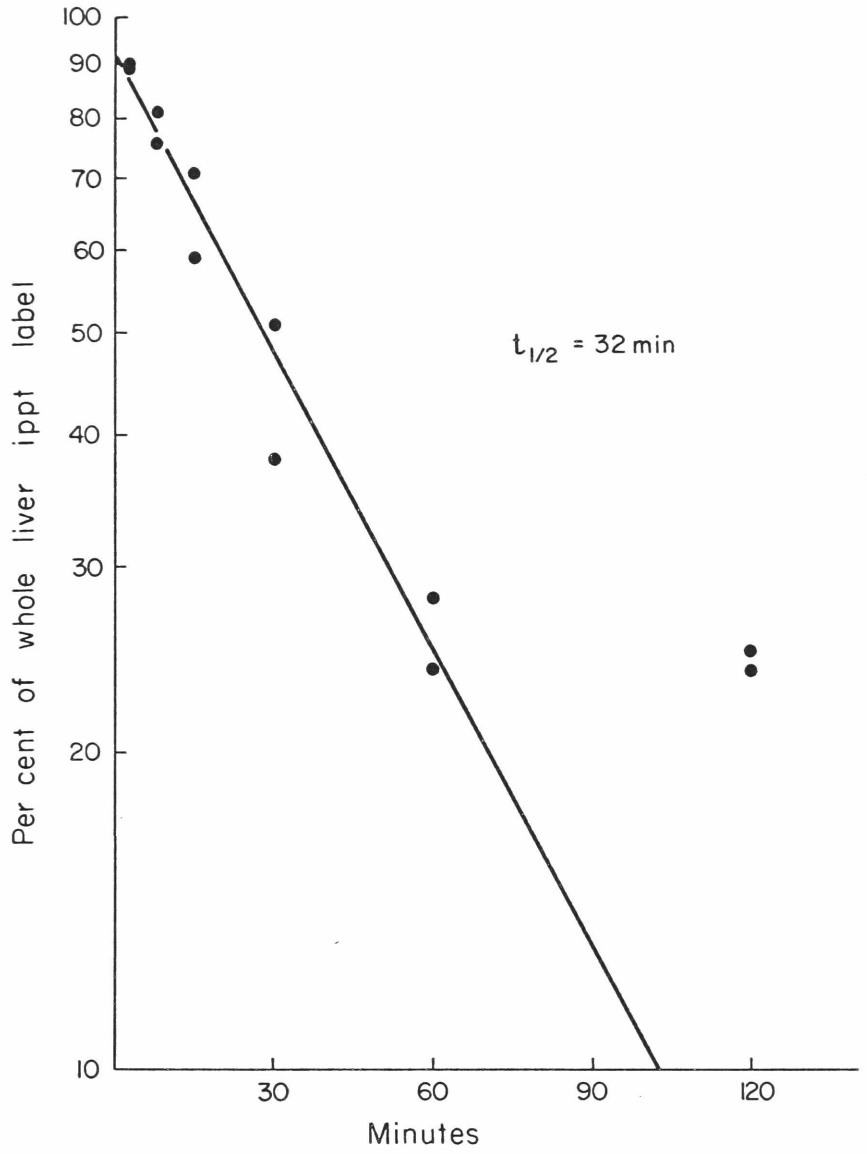
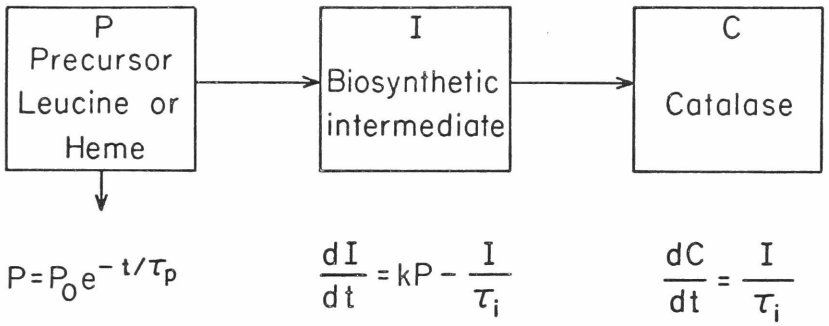


Figure 25. Semilogarithmic plot of ^{3}H from ALA in the catalase biosynthetic intermediate, expressed as a percentage of the label in total catalase.

The regression line was fitted by computer by minimization of the sum of the squared deviations. The two points at 120 minutes were omitted from the fit.



P, I, and C are the radioactivities in the Precursor, Intermediate, and Catalase compartments. P is assumed to decay with simple exponential kinetics; the loss of radioactivity from the system due to destruction of catalase is negligible during the time scale of these experiments. Then:

$$I = \frac{P_I \cdot \tau_p \cdot \tau_i}{\tau_p - \tau_i} \left(e^{-t/\tau_p} - e^{-t/\tau_i} \right)$$

$$C = \frac{P_I \cdot \tau_p}{\tau_i - \tau_p} \left(\tau_p \cdot e^{-t/\tau_p} - \tau_i \cdot e^{-t/\tau_i} \right) + P_I \cdot \tau_p$$

$$T = I + C = P_I \cdot \tau_p \left(1 - e^{-t/\tau_p} \right)$$

$$\frac{I}{T} = \frac{\tau_i}{\tau_p - \tau_i} \left(\frac{e^{-t/\tau_p} - e^{-t/\tau_i}}{1 - e^{-t/\tau_p}} \right)$$

$$\lim_{\tau_p \to 0} \frac{I}{T} = e^{-t/\tau_i} \qquad \text{where } P_I = k \cdot P_0$$

Figure 26. One intermediate labeling kinetics - theory.

Note that the time constant, τ, is related to the corresponding half-life, $t_{1/2}$, by $t_{1/2} = \tau \times \log_e 2 = 0.693\tau$.

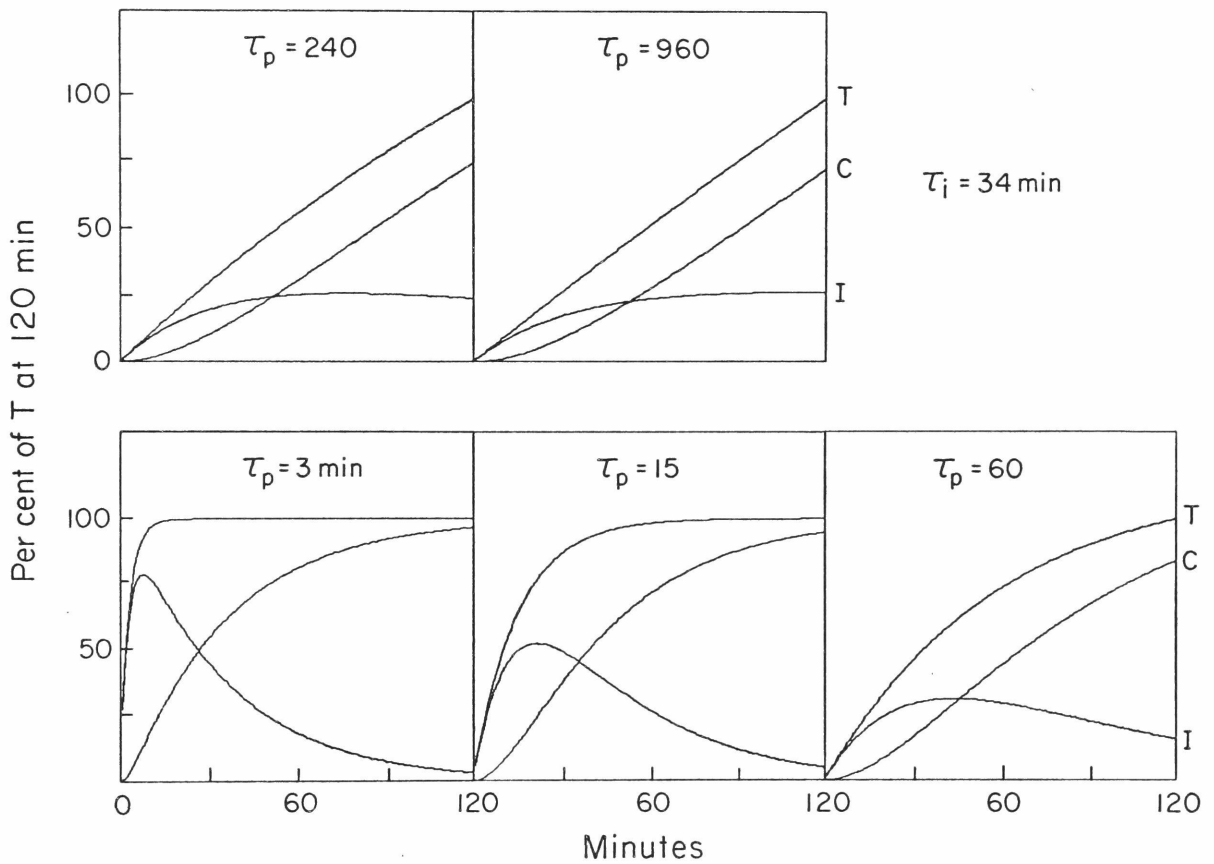


Figure 27. One intermediate labeling kinetics.

Plots of I, C and T for τ_i = 34 minutes.

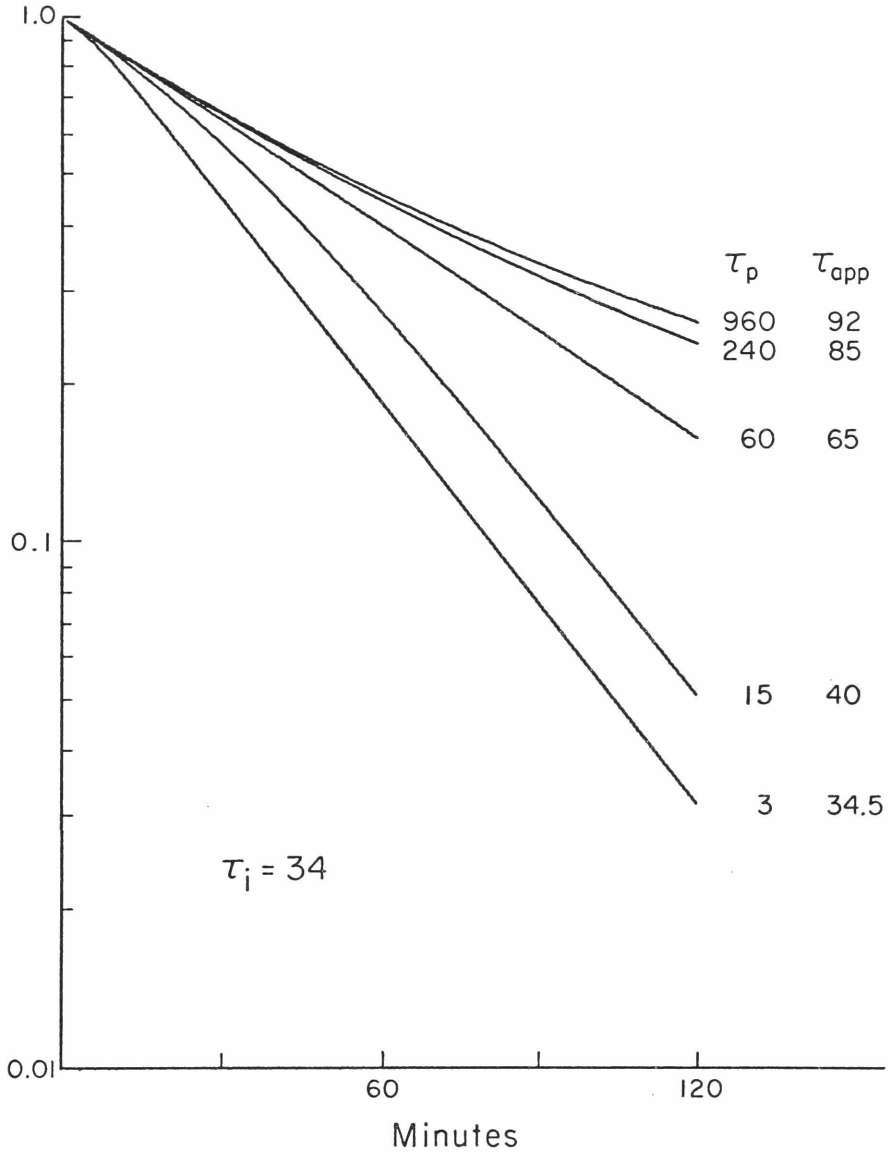


Figure 28. One intermediate labeling kinetics.

Plots of I/T for τ_i = 34 minutes. $\tau_{app} = \tau_{apparent}$ in $I/T \simeq Ae^{-t/\tau_{apparent}}$. It was approximated by measuring the slope of the straight line segment connecting the values of I/T at 3 and 120 minutes.

$\tau_{apparent}$ increases with increasing values of τ_p. Therefore the $\tau_{apparent}$ we measured in Figure 25 of 46 minutes (half-life of 32 minutes) is not the true τ_i, but is larger. In order to determine the true τ_i we fitted the complete expression for I/T to the data of Figure 25, using the estimated value for τ_p of 140 minutes (half-life of 100 minutes). A value of 18 minutes for τ_i gives the best fit, again neglecting the two experimental points at 120 minutes. This τ_i corresponds to a half-life of 12 minutes from which we may calculate that the ALA-labeled biosynthetic intermediate constitutes

$$\frac{\frac{12}{60}\text{ hour}}{36\text{ hours}} \times 100 = 0.55 \text{ percent of the total liver cell catalase.}$$

What can we infer from the above data about the biosynthesis of catalase? First of all, there is a leucine-containing biosynthetic intermediate that does not survive purification; it has a half-life of 53 minutes and constitutes 2.5% of the total catalase. Secondly, there is also a heme-containing intermediate that does not survive purification. Therefore, heme addition does not complete catalase biosynthesis. The heme-labeled intermediate has a half-life of 12 minutes and constitutes only about 0.5% of the cell catalase. Therefore the intermediates identified with leucine and with ALA labeling are not identical. There are two leucine-containing intermediates. The first one lacks heme and constitutes about 2 percent of the liver catalase. The second contains both leucine and heme and represents 0.5 percent of the liver catalase. The differential equations for the corresponding two-intermediate model have been written and solved and have been shown to fit the experimental data.

We performed one experiment to determine whether the biosynthetic process can be completed *in vitro*.

Negishi and Omura (1970) have shown that after *in vivo* administration of ^{3}H-leucine to the rat, chemically isolated cytochrome b_5 is more highly labeled if heme is added to the homogenate before purification. Their interpretation is that the heme combines *in vitro* with

labeled apocytochrome b_5, rendering it purifiable. We added 5 µl of a 100 µM hematin solution (in 0.01 N NaOH) to 0.5 ml of a homogenate (0.2 grams of liver per ml, 1% Triton X-100). Thus the final concentration was 1 µM hematin, compared with a concentration of 0.003 µM catalase hematin. After a 5 minute incubation at room temperature, catalase was purified as usual, except that the eluting solution also contained 1 µM hematin. In one experiment the hematin was reduced with dithionite before addition. As shown in Figure 29, neither hematin nor heme had any effect on the amount of ^{3}H-leucine recovered in the purified catalase. The simplest interpretation is that heme addition failed to occur *in vitro*. However, in view of the successful addition described above for b_5 (under similar conditions), this experiment may indicate that the step subsequent to heme addition fails to occur *in vitro*.

What do these results tell us about the nature of the catalase intermediates? In view of the known structure of catalase, we may consider three simple models (Fig. 30). The first model, that of simultaneous leucine and heme incorporation, has already been excluded on the basis of the incorporation kinetics, which showed that heme is added after leucine.

That catalase should be synthesized in the form of apocatalase is not particularly surprising, since Kadenbach (1970) has described an apocytochrome c, and Hara and Minakami (1970) and Negishi and Omura (1970) have presented evidence for an apocytochrome b_5. The finding of a hemoprotein precursor of catalase was unexpected. Two alternatives for its nature are shown in models 2 and 3 (Fig. 30). In model 2 the aggregation of four heme-containing monomers constitutes the final step in catalase biosynthesis. In model 3 the addition of four hemes to an apotetramer finishes the protein; tetramers with 1, 2 or 3 hemes constitute the heme-containing intermediate.

There are, however, several additional facts which must be kept in mind. First, while the iron in catalase has a valency of +3, we do

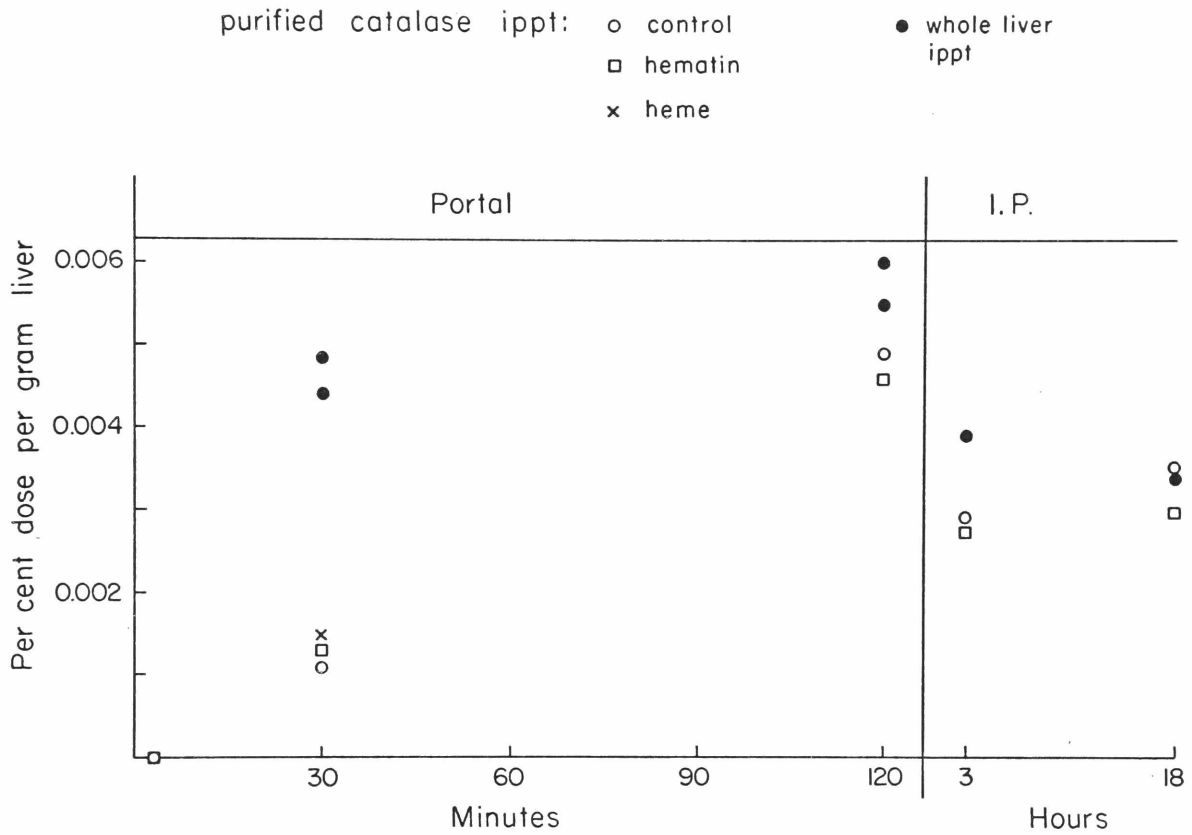


Figure 29. Non-effect of added hematin on ^{3}H-leucine in purified catalase immunoprecipitates.

The two solid circles at 30 and at 120 minutes represent duplicate determinations on the same animal.

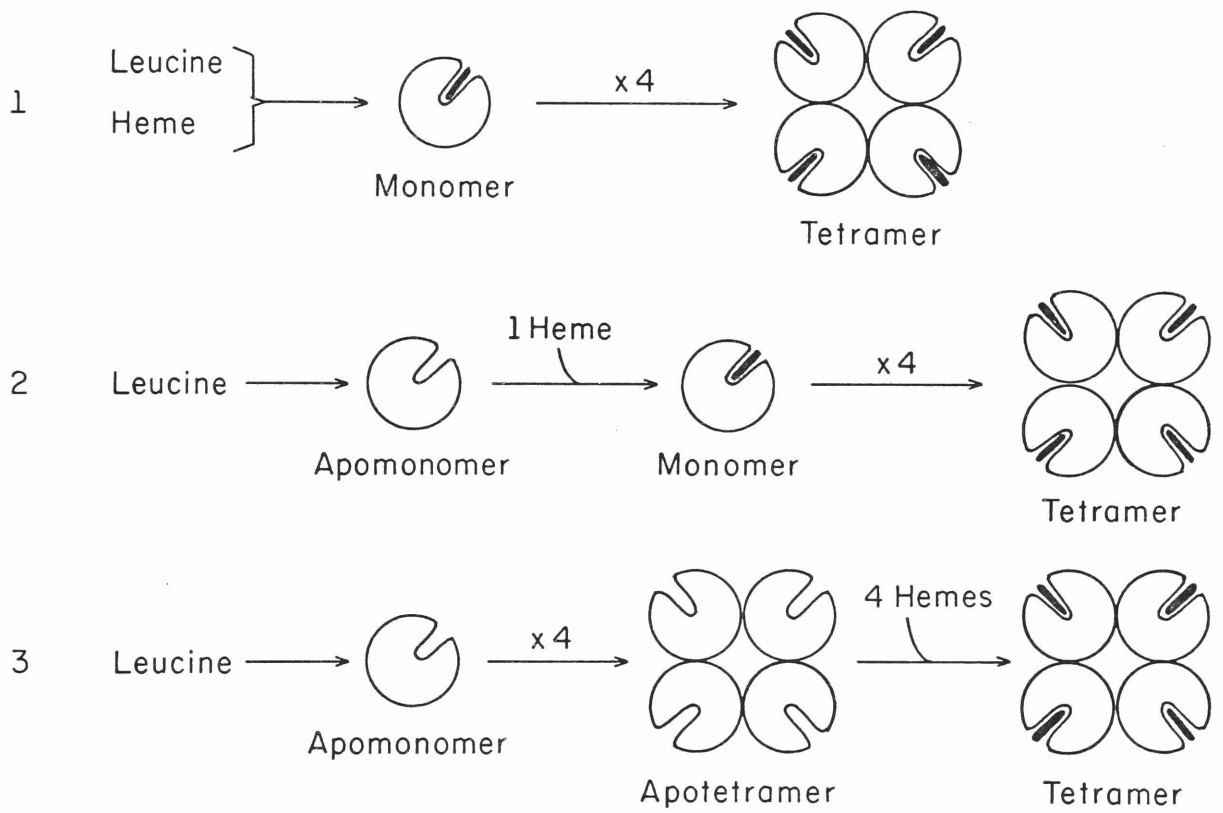


Figure 30. Hypothetical catalase intermediates--3 models.

not know the valency of the iron in the heme (hematin) which is inserted into the apoprotein. Conceivably the iron might require oxidation after insertion. Secondly, Schroeder et al. (1964) have reported that beef liver catalase contains 1 or 2 acetyl groups per monomer and that the N-terminal amino acid is blocked. If this is true of rat liver catalase also, then acetylation might be one step in the completion of catalase biosynthesis. Further work will be required to evaluate these possibilities.

VII. DESIGN OF ONE-STEP GRADIENT FRACTIONATION

We designed a rapid one-step procedure for fractionating rat liver extracts (post-nuclear supernatants) on continuous sucrose gradients, exploiting both differences in size and differences in density of the cell organelles. The general principle is to layer an extract over a linear sucrose gradient in the automatic rotor of Beaufay (1966), and spin just long enough to bring most of the peroxisomes to their equilibrium density of 1.23 at the bottom of the gradient. Under these conditions the microsomes sediment out of the layer, but do not reach their broad isopycnic distribution (as measured by Amar-Costesec et al., 1969, and Wibo et al., 1971). The result is a reasonable separation of the major cell components.

Beaufay's rotor contains a chamber in the shape of a cylindrical annulus 1.2 cm high with a maximum sedimentation path length of 1 cm. It can be loaded and unloaded while spinning and with the centrifuge chamber refrigerated and under vacuum; for further details see Beaufay's book (1966) and Leighton et al. (1968).

The design of the fractionation experiment is as follows: eight ml of a liver extract (0.2 g liver/ml) are injected into the rotor and underlayed with 26 ml of a linear sucrose gradient (1.10-1.27 gm/cm^3 density limits) followed by 6 ml of a dense sucrose cushion (1.32 gm/cm^3). The gradient solutions are prepared by dissolving sucrose in water containing 5% (w/w) Dextran-10 (thought to protect the peroxisomes, Leighton et al., 1968) and 0.1% ethanol (to protect the catalase, Chance, 1950); they are buffered at neutrality with 1 mM imidazole. The position of the layers at the start of the experiment is shown in Figure 31, taken from the paper of Leighton et al. (1968).

The sedimentation of the peroxisomes under the influence of a centrifugal field was simulated with a digital computer. The computation was based on the Svedberg equation which may be written (de Duve et al., 1959):

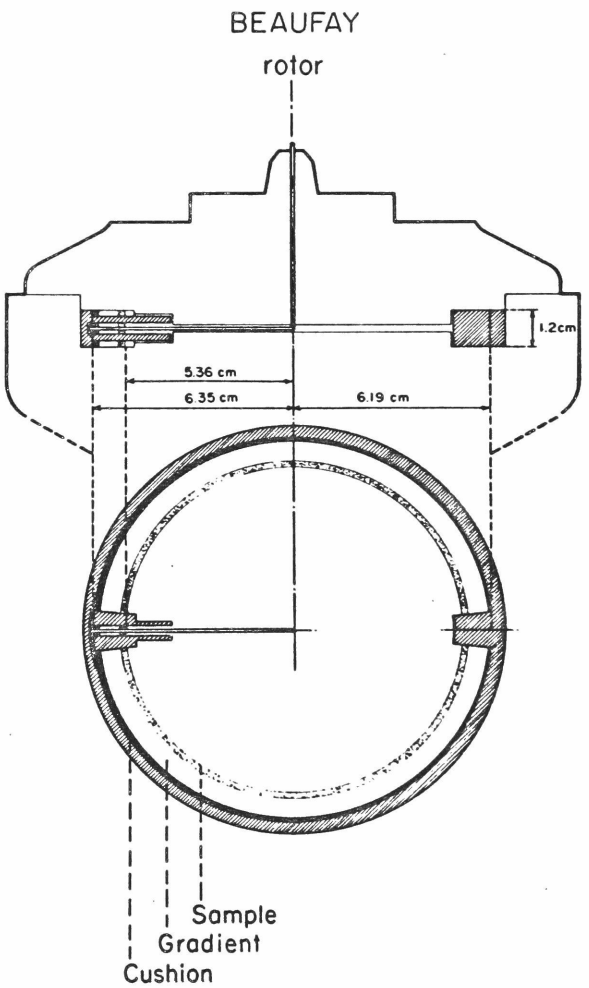


Figure 31. Shape of Beaufay's density gradient cell and initial disposition of layers.

A, axial cross-section. B, transverse cross-section. For further details, see Beaufay (1966).

$$\frac{dx}{dt} = \frac{\phi(\rho_p - \rho_m)}{f} \omega^2 x \qquad (1)$$

where x, ϕ, ρ_p and f are respectively the distance from the axis of rotation, volume, density and frictional coefficient of a sedimenting particle. The density of the medium is ρ_m, ω is the angular velocity of the rotor, and t is time. According to Stokes' law, the frictional coefficient of a small spherical particle is: $f = 6\pi\eta r$ (2)
where η is the viscosity of the medium and r the radius of the particle. Substituting this and $\phi = 4/3\ \pi r^3$ (3)
into equation (1) we obtain:

$$\frac{dx}{dt} = \frac{2r^2(\rho_p - \rho_m)}{9\eta} \omega^2 x \qquad (4)$$

This may be rearranged, separating the variables depending on x from those depending on t, as follows:

$$\frac{9\eta dx}{2r^2(\rho_p - \rho_m)x} = \omega^2 dt \qquad (5)$$

Since peroxisomes are osmotically inactive (Beaufay et al., 1964), r does not depend on x and we may integrate as follows:

$$\frac{9}{2r^2} \int_{x_0}^{x} \frac{\eta dx}{(\rho_m - \rho_m)x} = \int_{t_0}^{t} \omega^2 dt = W \qquad (6)$$

The right hand integral, which we call W, measures the total effective centrifugation, taking into account the variation in rotor speed during acceleration and deceleration. Experimentally it may be measured by means of an "Integrator Accessory" manufactured by Beckman Instruments.

The left hand integral summarizes the properties of the gradient as they affect the sedimentation of the particle; we will call this integral G (for gradient). Each of its parameters depends on the radial distance, x. We assume that the density of the gradient, ρ_m, is linear with respect to x. Hence:

$$\rho_m(x) = 1.10 + \frac{x - 5.57}{6.19 - 5.57}(1.27 - 1.10) \qquad (7)$$

where 5.57 and 6.19 are the radial positions of the ends of the gradient. The viscosity of a sucrose solution depends both on the sucrose concentration (or density) and on the temperature. We may express the logarithm of the viscosity (in centipoise) at one temperature, 5°, as a power series in the density:

$$\log_e \eta(x,5°) = \sum_{i=1}^{9} C_i(\rho_m)^{i-1} \qquad (8)$$

The constants, C_1 to C_9 are: -7.985, 14.47, -6.711, -4.827, 1.825, 10.06, -4.980, -5.196, 3.728. The constants were determined by the method of least squares; the viscosities calculated by means of this equation agree with those interpolated from the data of Landolt-Bornstein (1923) and of Bates (1942) by de Duve et al. (1959) with a mean deviation of 0.15% (0.64% deviation at most). The third parameter in the integral, the peroxisome density, ρ_p, has been shown by Beaufay et al. (1964) to be expressed by

$$\rho_p = \frac{1.23 + 2.55\rho_m}{3.55} \qquad (9)$$

For the purposes of this calculation we assume that all peroxisomes have an equilibrium density of 1.23. Using equations 7 to 9 we make a numerical integration of

$$G(x) = \int_{x_0}^{x} \frac{\eta dx}{(\rho_p - \rho_m)x} \qquad (10)$$

We may then rewrite equation 6 as follows:

$$\frac{9}{2r^2} G(x) = W \qquad (11)$$

which may be rearranged to yield

$$r = \sqrt{\frac{9G(x)}{2W}} \qquad (12)$$

Equation 12 allows us to calculate the radius, r, of the peroxisomes which will be found at any position in the rotor, x, after an amount of centrifugation, W. This radius distribution may be converted to a catalase distribution by utilizing the peroxisome size distribution measurements of Poole et al. (1970). We replot the data of their Figure 3 as cumulative activity versus radius, slightly truncated at either end (Fig. 32). These data cannot be adequately fitted by a single polynomial, even of degree 9; we use two degree 4 polynomials, applied in different regions of the domain, $c = f(r)$ (13)

If $r<0.174\mu$ $\quad c = 0.0$

If $0.174<r<0.3136$ $\quad c = -0.5998 + 17.04r - 161.9r^2 + 602.6r^3 - 693.5r^4$

If $0.3136<r<0.434$ $\quad c = -11.12 + 87.72r - 225.4r^2 + 231.4r^3 - 67.96r^4$

If $r>0.434$ $\quad c = 1.0$

This complex function adequately fits the data, as shown by the solid line in Figure 32. We used functions 12 and 13 to determine the distribution of catalase activity with respect to radial distance in the rotor at various values of W. The results are shown in Figure 33. We see that the computer simulation predicts that most of the peroxisomes will reach their equilibrium density when W equals 1.2 to 1.8 x 10^{10} sec^{-1} (12 to 18 $nsec^{-1}$).

This prediction was tested by performing the simulated experiment using a rat liver extract and W = 1.4 x 10^{10} sec^{-1} (14 $nsec^{-1}$). The catalase distribution, as measured in the fractions removed from the rotor, is shown in the upper right square of Figure 34. The catalase distribution is broader than predicted by the computer simulation. However, when this distribution is compared with that reported by Leighton et al. (1968) for peroxisomes at equilibrium, we find that the main reason for our observed peak width is not incomplete equilibration, but rather true heterogeneity of peroxisomes with respect to density. Therefore, the prediction of the computer simulation is basically borne out: most of the peroxisomes are at or near their equilibrium densities, but there is a fair dispersion in these densities

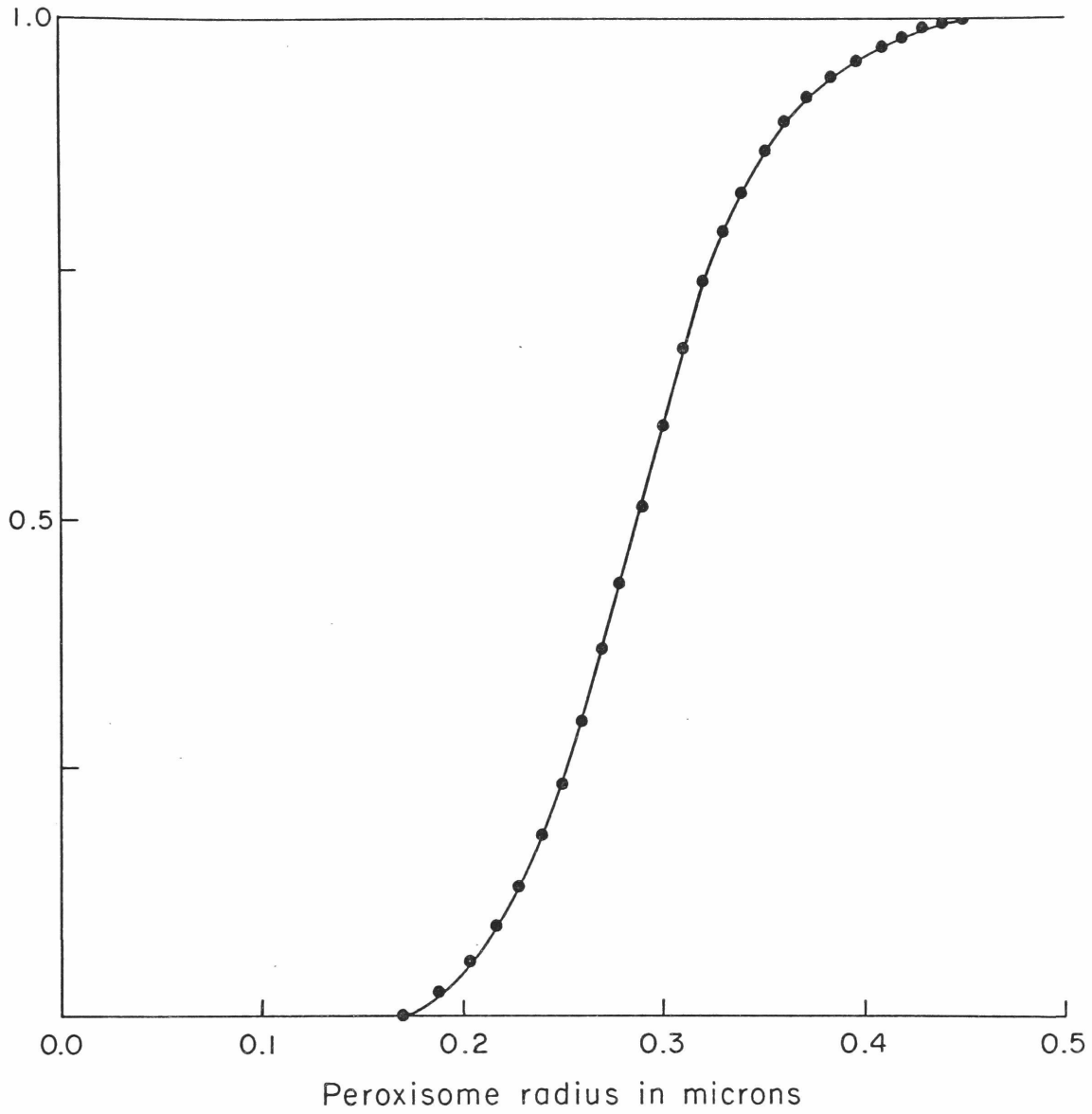


Figure 32. Cumulative size distribution of catalase.

The points were computed from the data of Poole et al. (1970). The solid line represents the complex function fitted to the points.

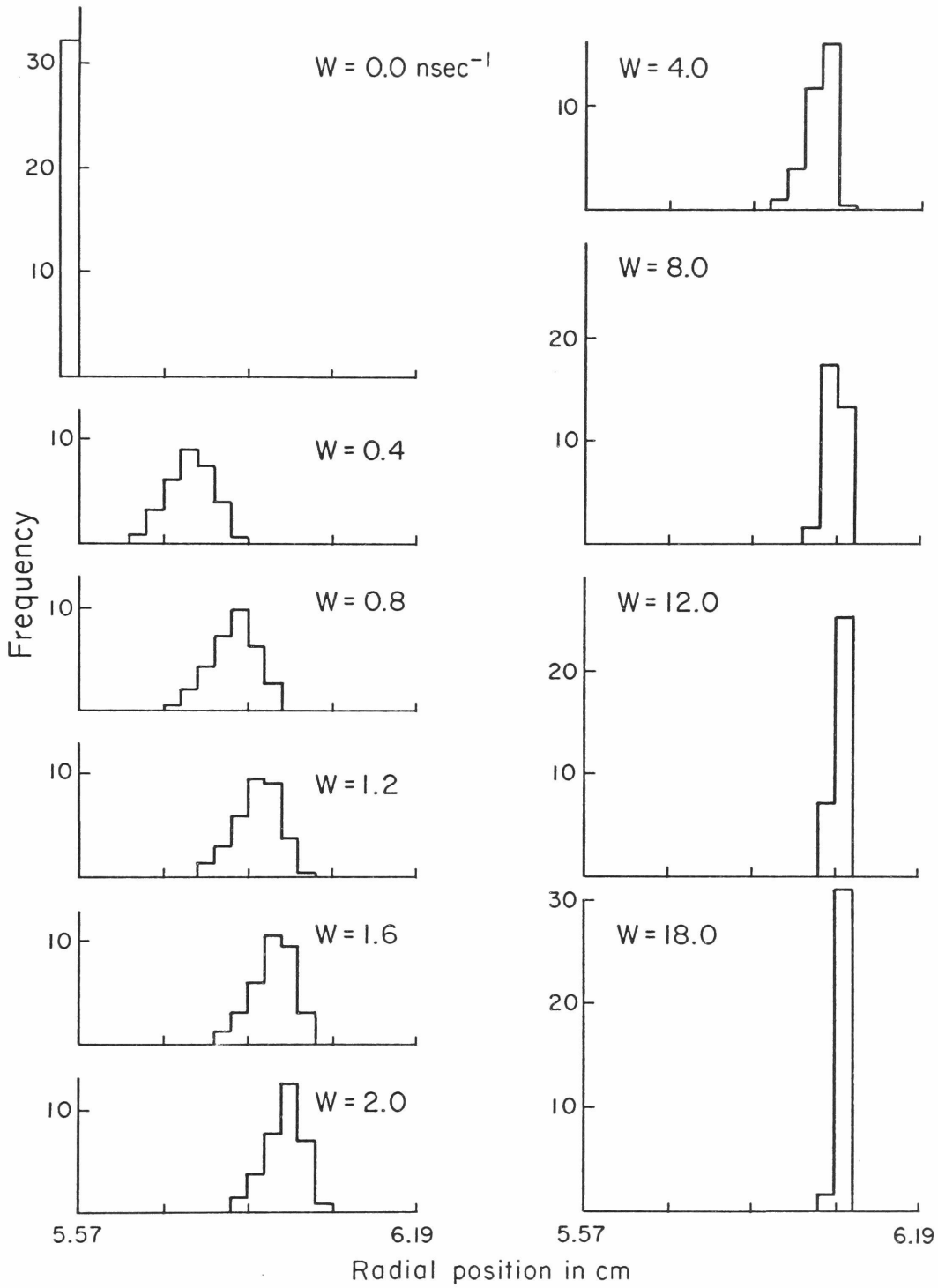


Figure 33. Computer simulation of peroxisome sedimentation. W is the time integral of the square of the angular velocity.

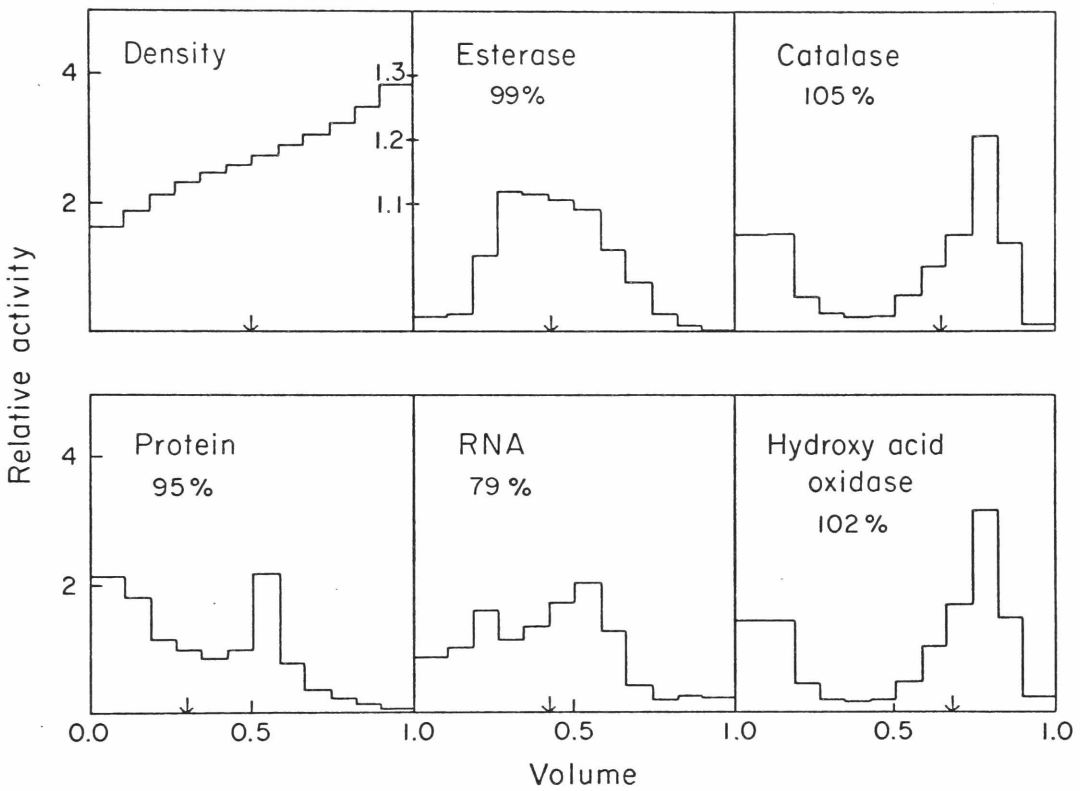


Figure 34. Distribution of marker enzymes in gradient fractions.

which was neglected in the simulation. In a second experiment (Fig. 35) the peroxisomes are seen to be somewhat less well equilibrated at their equilibrium densities. The most likely reason is a gradient temperature lower than the 5° assumed in the calculation, resulting in increased viscosities.

The locations of other cellular organelles in these gradients were determined by measuring the concentration of various marker enzymes in each fraction. The weights and densities of the fractions were also measured (Beaufay et al., 1964). The results were calculated and plotted as histograms by the methods described by Beaufay et al. (1964) and Leighton et al. (1968). Briefly, the total volume of the fractions is calculated and set equal to unity, and similarly the total enzyme activity found in the fractions is set equal to unity. The abcissa is cumulative, normalized volume. The ordinate is the observed enzyme concentration divided by the enzyme concentration which would have been found had the enzyme been uniformly distributed throughout all the fractions. The ordinate values are calculated by dividing the normalized enzyme activity of each fraction by its normalized volume. Consequently, the area of each histogram bar is equal to the proportion of the total enzyme activity found in the corresponding gradient fraction. The form of these distributions is the same as would be obtained simply by plotting absolute enzyme concentrations versus absolute cumulative volumes. In each histogram the top of the initial layer is at the left and the bottom of the cushion at the right. The recovery, defined as

$$\text{Recovery} = \frac{\text{total enzyme activity in fractions}}{\text{enzyme activity applied in layer}} \times 100$$

is shown for each enzyme. The median volume of the distribution is indicated with an arrow.

Figure 35 shows the results obtained in this way for a number of enzymes. As already discussed, the peroxisomes (catalase) are near their equilibrium density of 1.23. The mitochondria (cytochrome oxidase) are banded at their equilibrium density of 1.18 and coincide

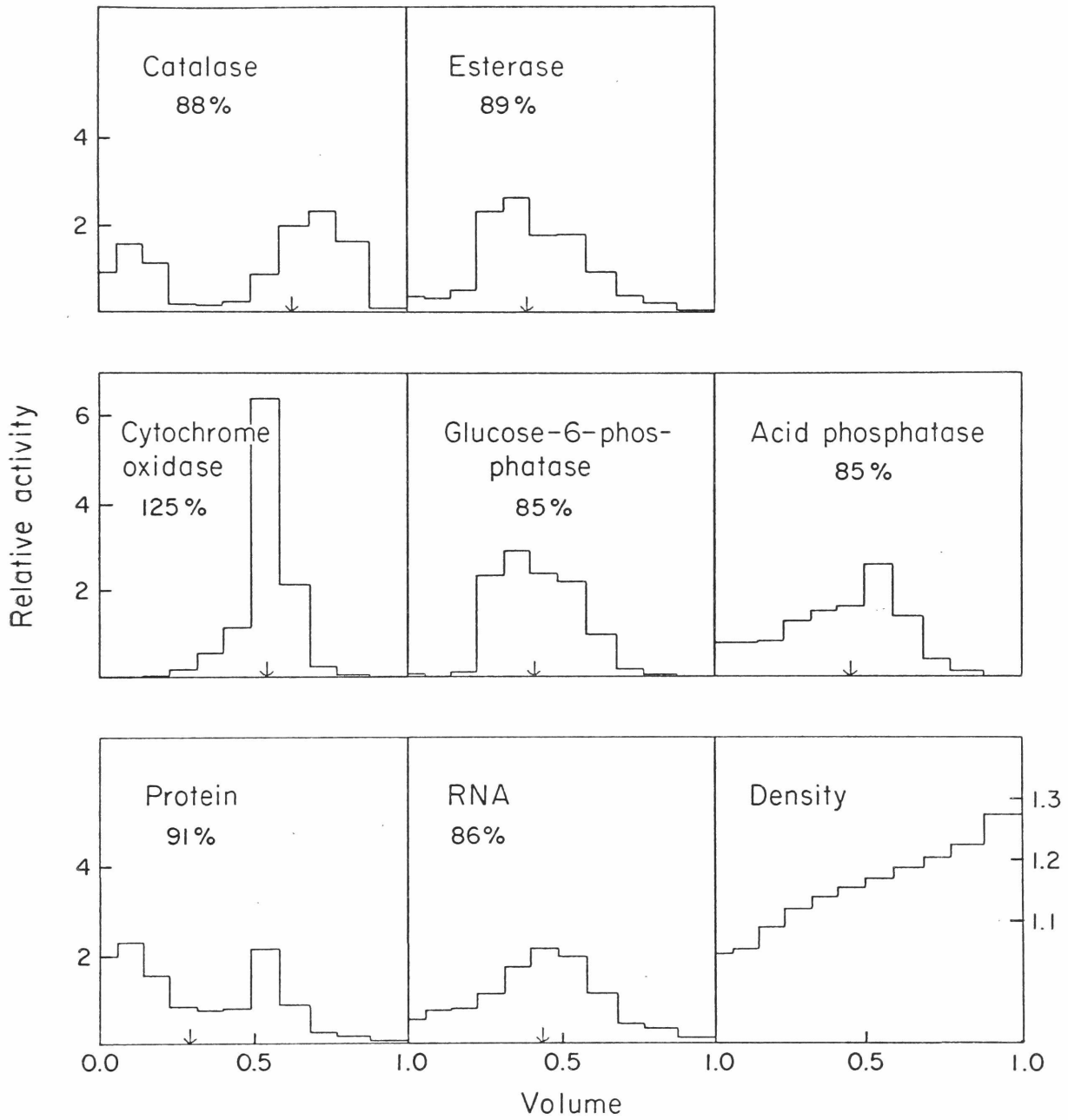


Figure 35. Distribution of marker enzymes in gradient fractions.

with, and in fact are responsible for, one of the two peaks of protein. The other protein peak is due to soluble proteins remaining in the layer. The microsomes (esterase, glucose-6-phosphatase, RNA) are seen to have moved out of the layer and into the top half of the gradient. They overlap somewhat with the mitochondria but only very slightly with the peroxisomes. The microsomes have by no means reached their equilibrium density distribution, as reported by Amar-Costesec et al. (1969) and by Wibo et al. (1971). We note that esterase and glucose-6-phosphatase have essentially the same distributions, in agreement with the results of Amar-Costesec et al. (1969). The ratio of RNA to either enzyme is seen to increase somewhat with increasing cumulative volume, indicating some separation of rougher from smoother microsomes. The lysosome distribution (acid phosphatase) overlaps both those of the mitochondria and microsomes but has a significantly different shape. It would be possible to manipulate the lysosome distribution by pretreatment of the rats with Triton WR-1339 (Wattiaux et al., 1963; Wattiaux, 1966; Leighton et al., 1968) but this is irrelevant to our purposes.

The enzyme distributions shown in Figure 34 are essentially the same as those of Figure 35. In addition to catalase, a second peroxisomal enzyme, L-α-hydroxy acid oxidase, was also measured. The two distributions are extremely similar, in agreement with all previous studies (Leighton et al., 1968; Poole et al., 1970). About 30% of each enzyme is found at the top of the gradient. Previous papers from this laboratory have provided good evidence that this soluble activity is due to ruptured peroxisomes (Beaufay et al., 1964; Leighton et al., 1968). This will be discussed further, below.

These gradients are extremely reproducible. The results of 12 experiments were averaged by the method of Leighton et al. (1968). The mean distributions, together with their standard deviations, are shown in Figure 36.

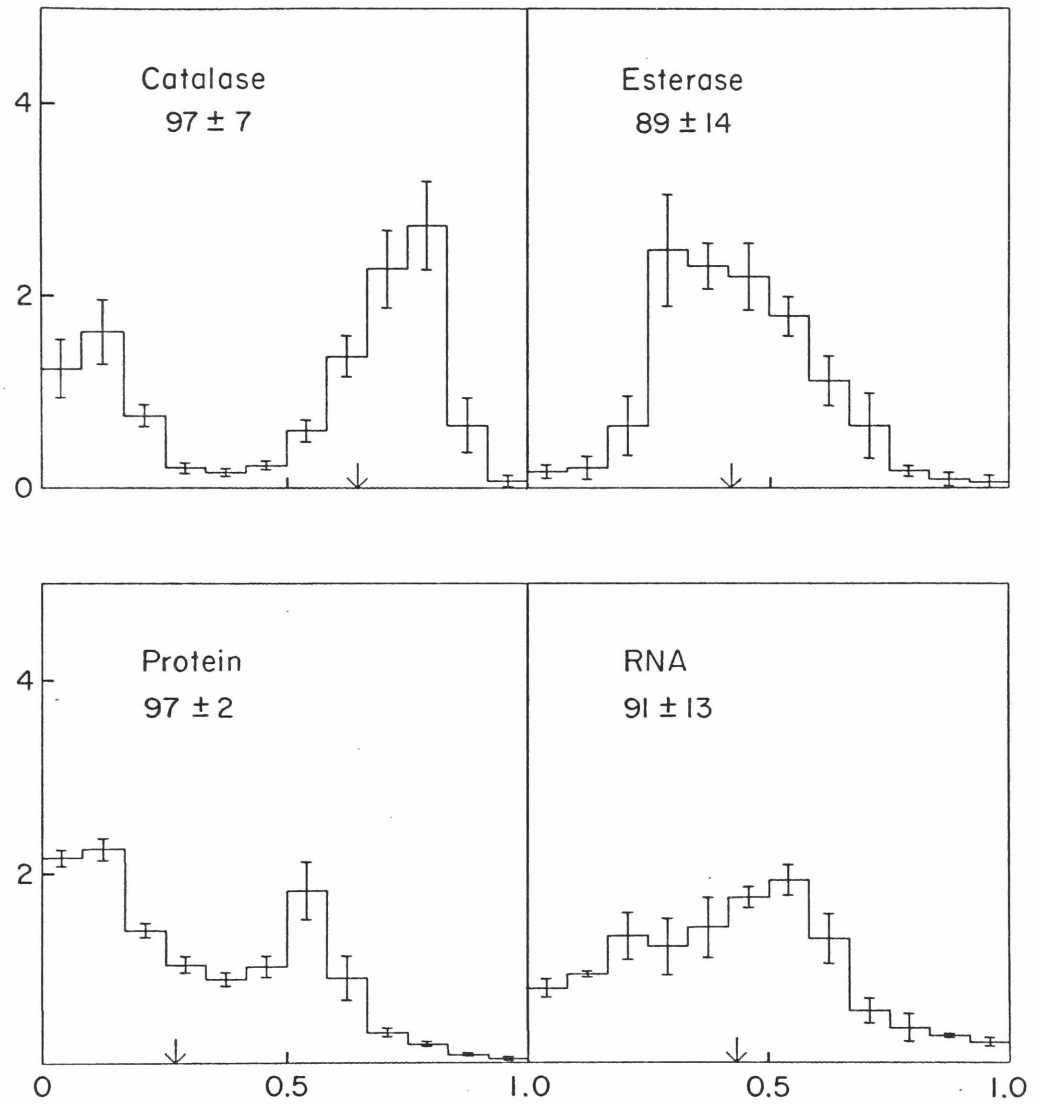


Figure 36. Mean distributions of marker enzymes in fractions from 12 gradients.

RNA was measured on only 3 gradients. The vertical bars indicate standard deviations. The mean and standard deviation of the recoveries is also indicated.

These results indicate that this method of fractionation largely separates the cell sap, microsomes, and peroxisomes from each other, and also produces distinctly different distributions of mitochondria and lysosomes. The entire fractionation requires only two hours, from decapitation of the rat to collection of the fractions. The actual spin in Beaufay's rotor takes about 15 minutes at 35,000 rpm top speed. This combined rate/isopycnic fractionation system is therefore a rapid and convenient means of studying subcellular localizations in rat liver. It has been applied both to localizing enzymes within liver cells, and for the transport studies described below. Furthermore, this fractionation system may be adapted for use in swinging-bucket rotors. Satisfactory results are obtained if 0.5 ml of a liver extract is layered over a 4.6 ml linear gradient (1.10 to 1.27 density limits) and 0.3 ml of a 1.32 density cushion and spun for $W = 1.5 \times 10^{11}$ sec^{-1} in a Spinco SW39 swinging-bucket rotor. (A homogenate can also be fractionated in this way, but the peroxisome region is contaminated by what are probably nuclei with attached pieces of rough ER.) Therefore this one-step fractionation of rat liver extracts may be accomplished using widely available, commercially produced, swinging-bucket rotors.

VIII. DISTRIBUTION OF LABELED CATALASE IN GRADIENT FRACTIONS AS A FUNCTION OF TIME

A. Procedure

Rats were anesthetized with Nembutal and received by intraportal injection a mixture of 2 to 5 mC of ^{3}H-4,5-leucine (6 C/mmole) and 0.1 to 0.5 mC of ^{14}C-ALA (46.5 mC/mmole), in a total volume of 1 ml saline. The livers were removed after 8, 15, 30, or 60 minutes, and homogenized in 0.25 M sucrose containing 1.0 mM imidazole, pH 7.0, and 0.1% ethanol. An extract (postnuclear supernatant) was prepared according to de Duve et al. (1955), and fractionated in the Beaufay rotor as described above. In each gradient fraction, as well as in the extract, we determined the concentrations of marker enzymes, total TCA-precipitable radioactivity from both leucine and ALA, as well as the radioactivity in unpurified and purified catalase immunoprecipitates, from both labels (Table XI).

B. Results

The overall biochemical results pertaining to the animals and liver extracts are summarized in Tables XII and XIII. Figures 37 to 48 show the observed distributions as a function of volume for all the components measured in the experiments. There are 3 figures for each animal; the first gives the normalized distributions of the marker enzymes and of the total TCA precipitable radioactivity. The remaining two give the absolute distributions of label in the whole liver immunoprecipitates and in the purified catalase immunoprecipitates, respectively. For each label, two graphs are given. One shows, superimposed upon each other, the values found in the immunoprecipitates, and the corresponding blank values obtained with the control goat serum, so as to permit an objective assessment of the dependability of the results. The second graph shows the difference between the two distributions depicted in the former, and therefore represents the distribution of the labeled materials specifically precipitated by anticatalase.

Table XI

Preparation of Immunoprecipitates[1]

Fraction from gradient (frozen)
Thaw. Add Triton X-100 to 1% final concentration (1/10 volume 11% Triton, or 1 volume 2% Triton to very dense fractions).

FOR WHOLE LIVER IPPTS:	FOR PURIFIED CATALASE IPPTS:
1.5 ml fraction	1.5 ml fraction in Spinco #50 polyallomer tube
+ 1.5 ml 2X-PBSLA + 4.5 ml PBSLA	+ 1.5 ml 2X-eluting solution[2] + 5.5 ml eluting solution[2] Shake several times in 30 minutes.
Filter through 0.22 μ Millipore filter.	+ 0.5 ml chloroform; shake Wait 10 minutes; shake. Spin 1 minute x 1500 rpm. Discard chloroform phase.
Take duplicate 2.5 ml aliquots.	+ 0.1 ml 0.5 M Na_2SO_4. Shake; wait 30 minutes. Spin 15 minutes x 40000 rpm. Discard supernatant and drain tube.
	Homogenize pellet in 1 ml 10 mM phosphate buffer, pH 7.0, with teflon pestle machined to fit the #50 tube. Transfer to a 2 ml tube which, with an adapter, fits in the #40 rotor. Rinse the #50 tube and pestle with a second 1 ml of buffer and combine with the first. Let sit overnight.
	Spin 30 minutes x 40000 rpm. Save supernatant.
	Take duplicate 0.7 ml aliquots and mix each with 0.7 ml 2X-PBSLA and 1.1 ml PBSLA.

Quantitative immunoprecipitation of catalase, as described in Chapter V, Table XVII

NOTES: 1) all steps at 0-4°. 2) "Eluting solution" = 44 ml 1 M acetate buffer, pH 4.1 + 220 ml ethanol diluted to 1 liter; 2X means double strength. 3) Microsomal fractions were supplemented with catalase as follows: 0.56 U/aliquot for Whole Liver Ippts. and 0.2 ml rat liver extract (0.1 g liver/ml), added before purification, for Purified Catalase Ippts. 4) When assaying homogenates or extracts 0.5 or 1.0 ml was taken, instead of 1.5 ml.

Table XII

Marker Enzyme Activities of Double-labeled Rats

Time	Body Weight	Liver			
		Weight	Protein	Catalase	Esterase
min	g	g	mg/g	U/g	U/g
8	220	9.0	162	52	147
15	250	8.1	180	57	173
30	235	7.8	185	53	163
60	240	8.8	195	56	153

Table XIII

Isotope Doses and Incorporation into TCA Precipitable Material

Time	Dose		Incorporation	
	^{3}H-leucine	^{14}C-ALA	^{3}H-leucine	^{14}C-ALA
min	dpm in billions		% dose/gram liver	
8	10.7	1.37	1.45	0.15
15	9.8	1.13	1.59	0.25
30	6.9	0.56	1.29	0.90
60	3.2	0.26	1.28	1.47

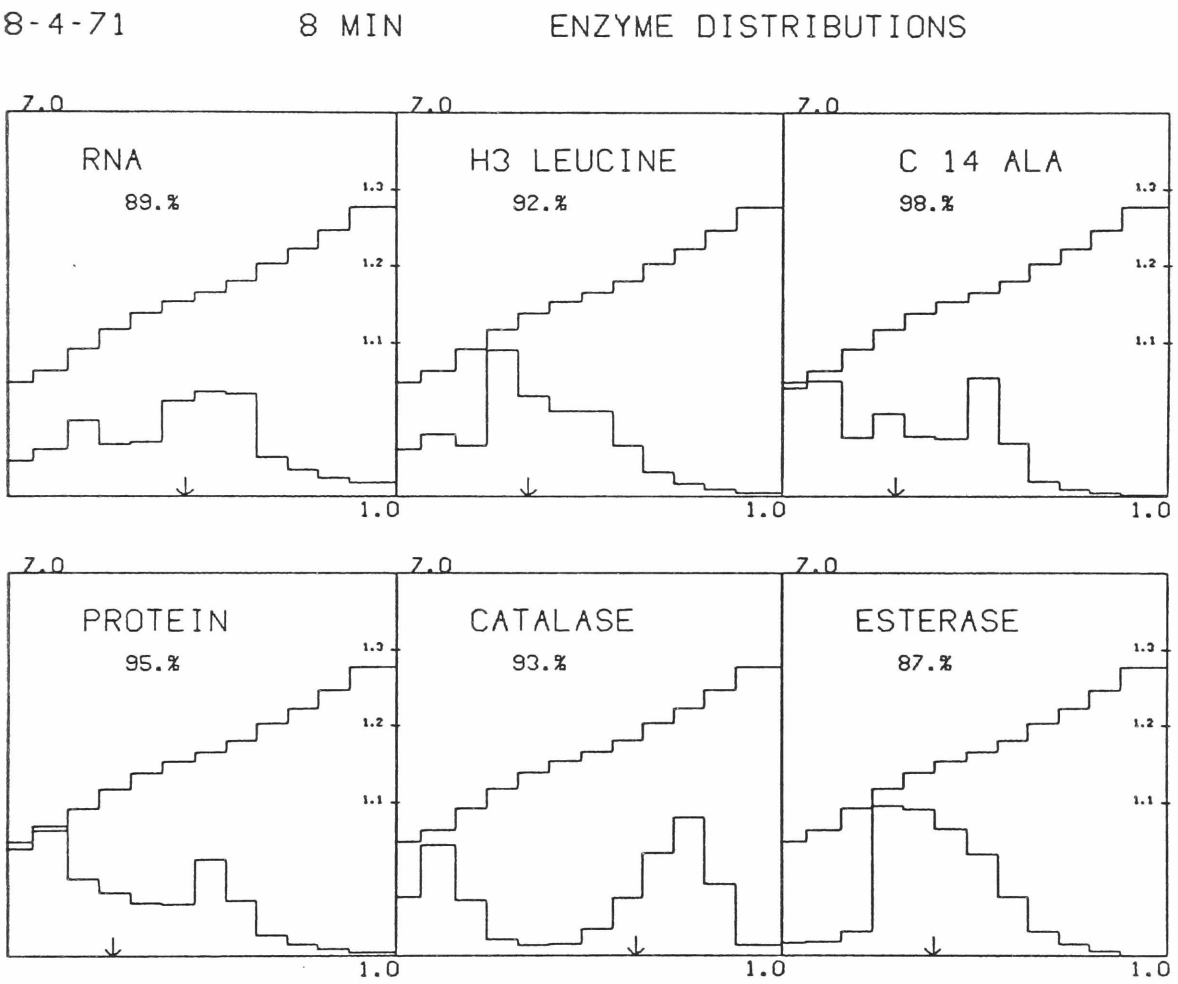


Figure 37. Distribution of marker enzymes and TCA precipitable radioactivity in the gradient - 8 minute rat.

This and the following 11 illustrations were drawn by the computer.

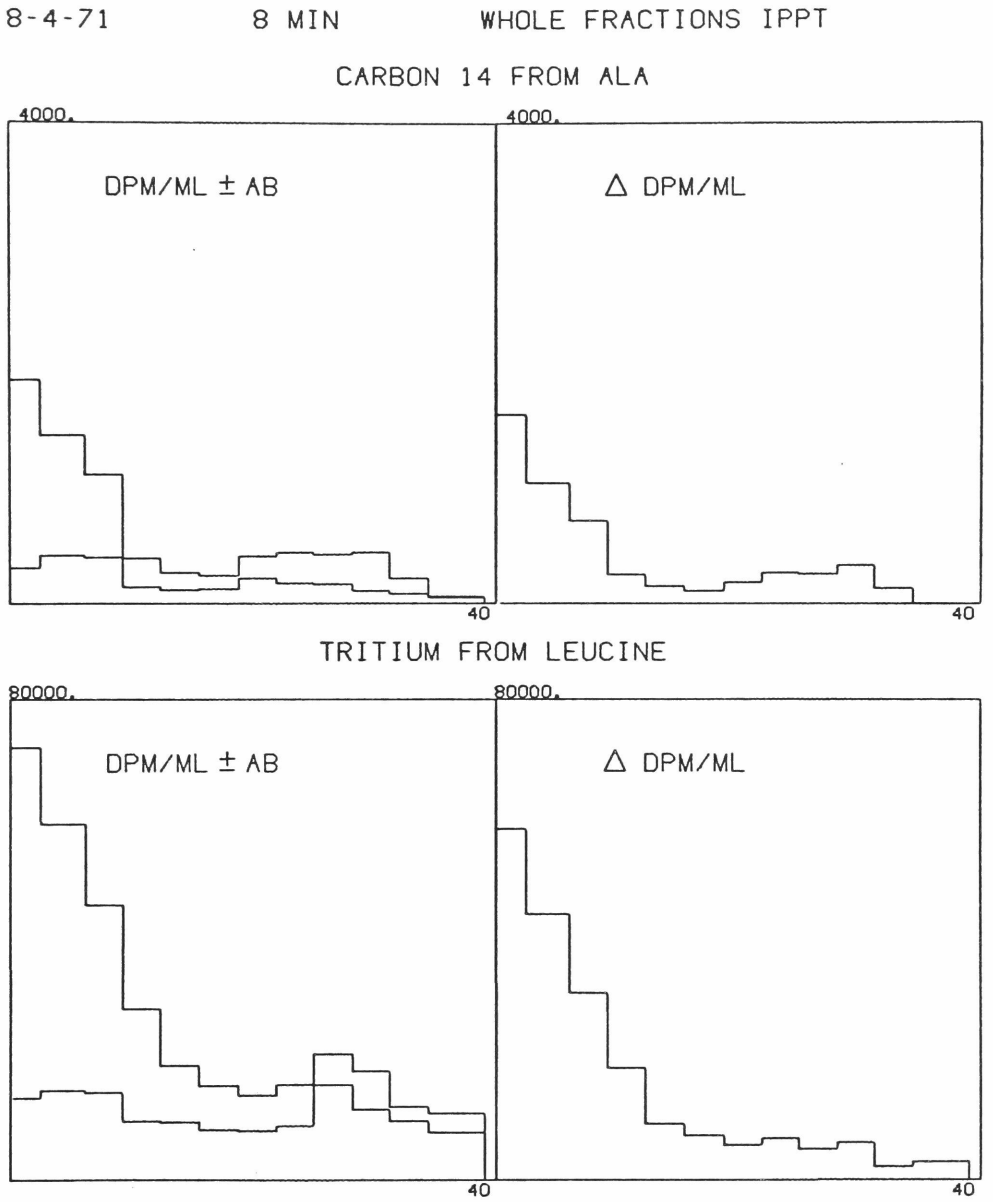


Figure 38. Distribution of label in the whole fraction immunoprecipitates - 8 minutes.

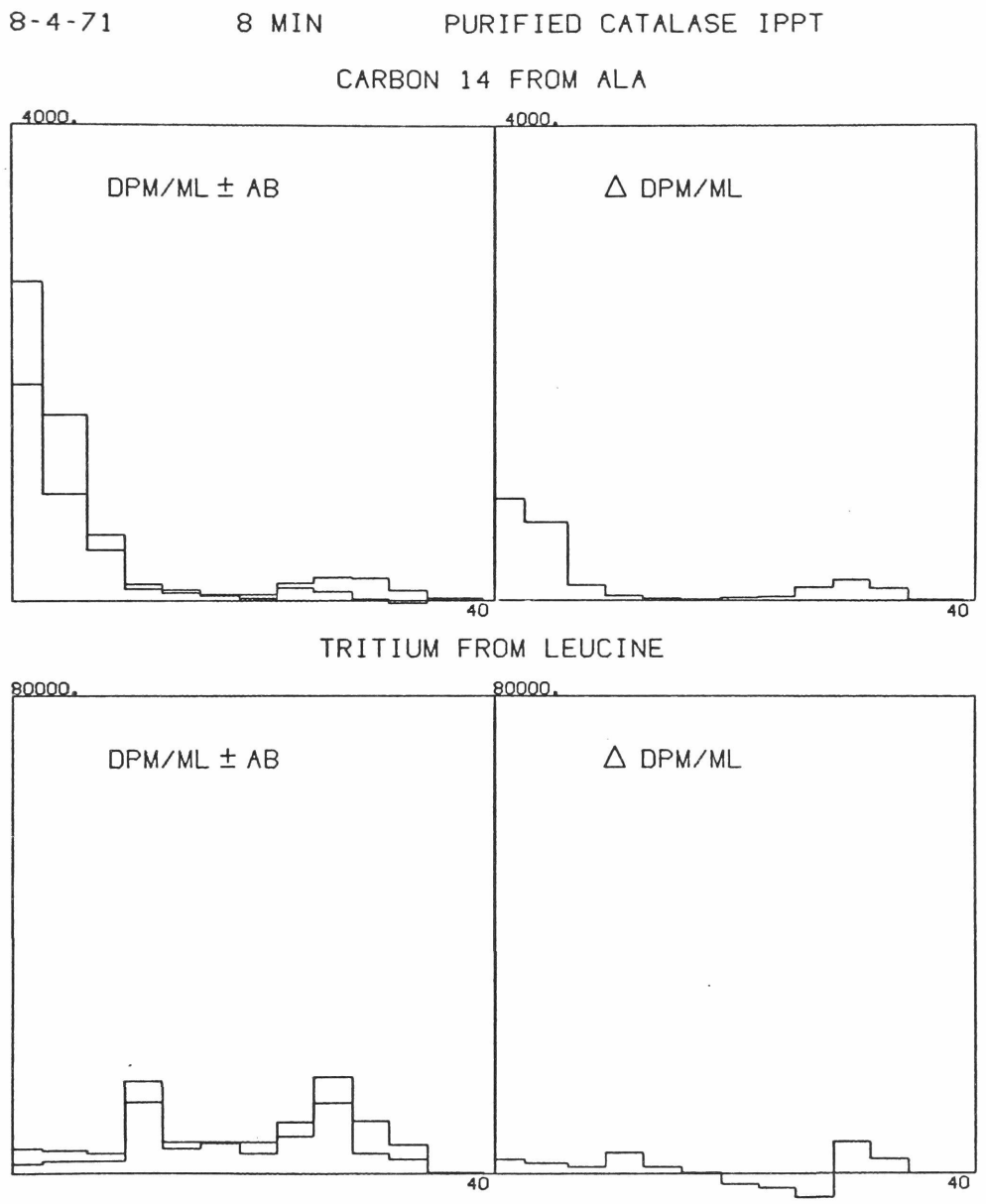


Figure 39. Distribution of label in the purified catalase immunoprecipitates - 8 minute rat.

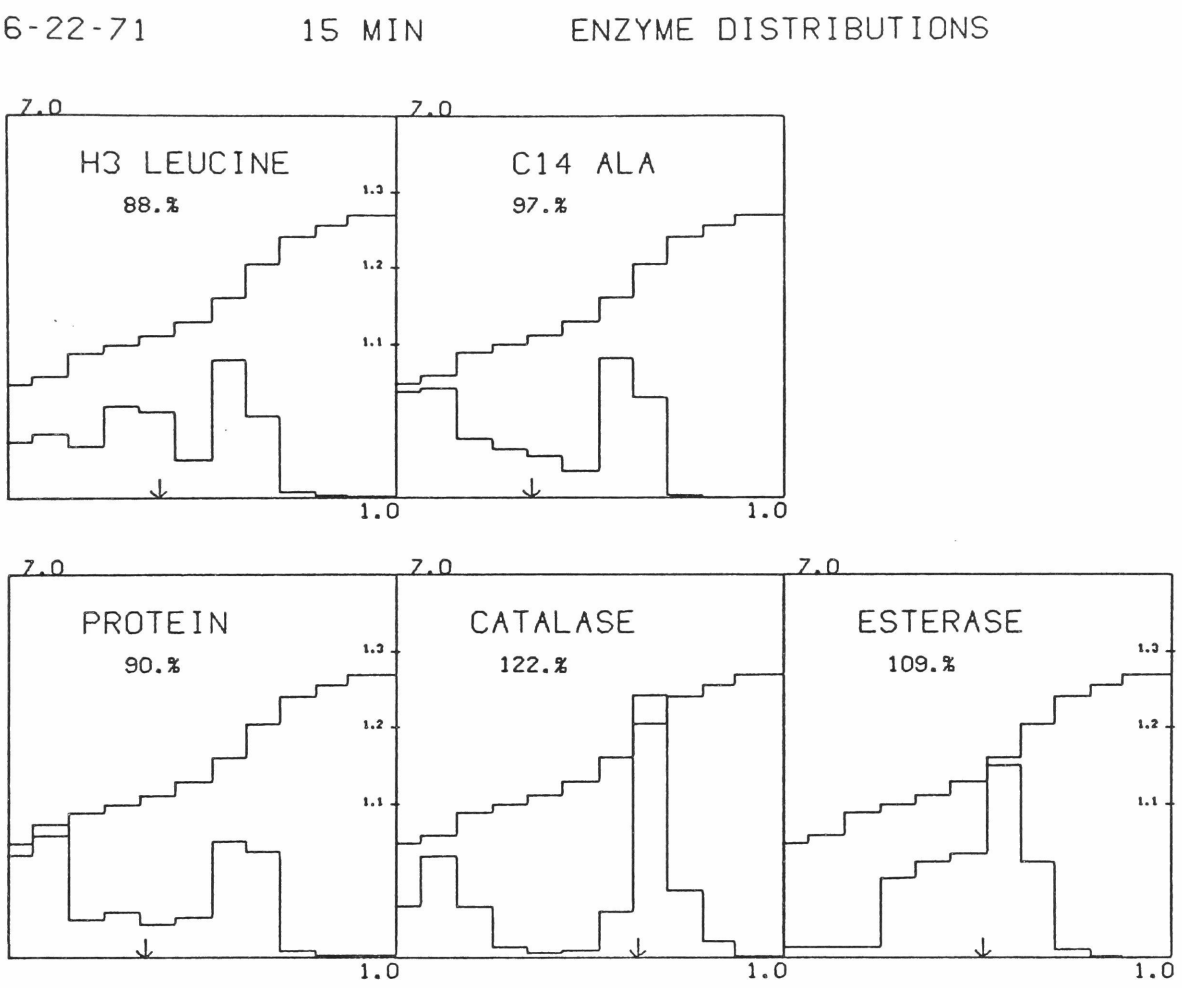


Figure 40. Distribution of marker enzymes and TCA precipitable radioactivity in the gradient - 15 minute rat.

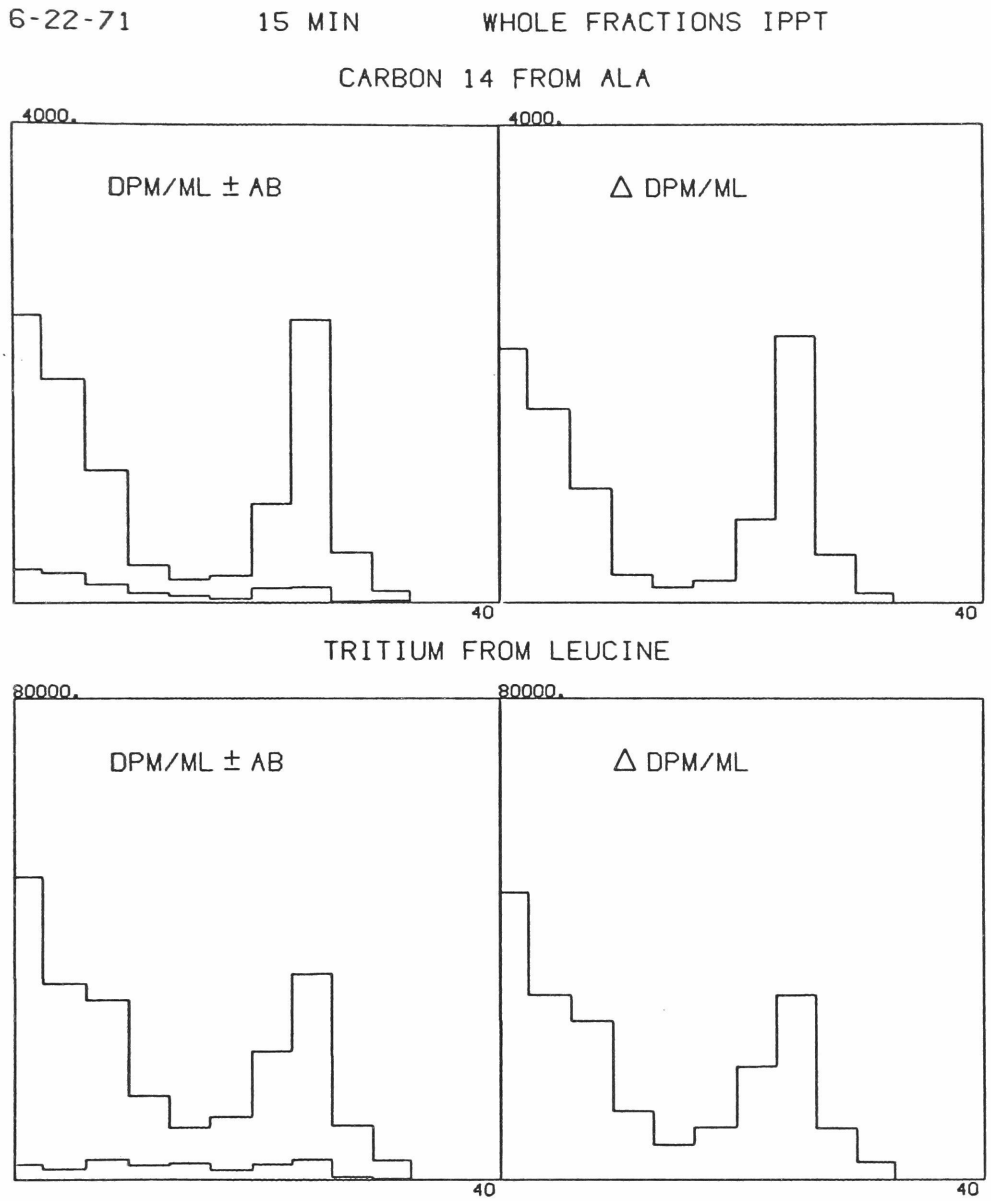


Figure 41. Distribution of label in the whole fraction immunoprecipitates - 15 minutes.

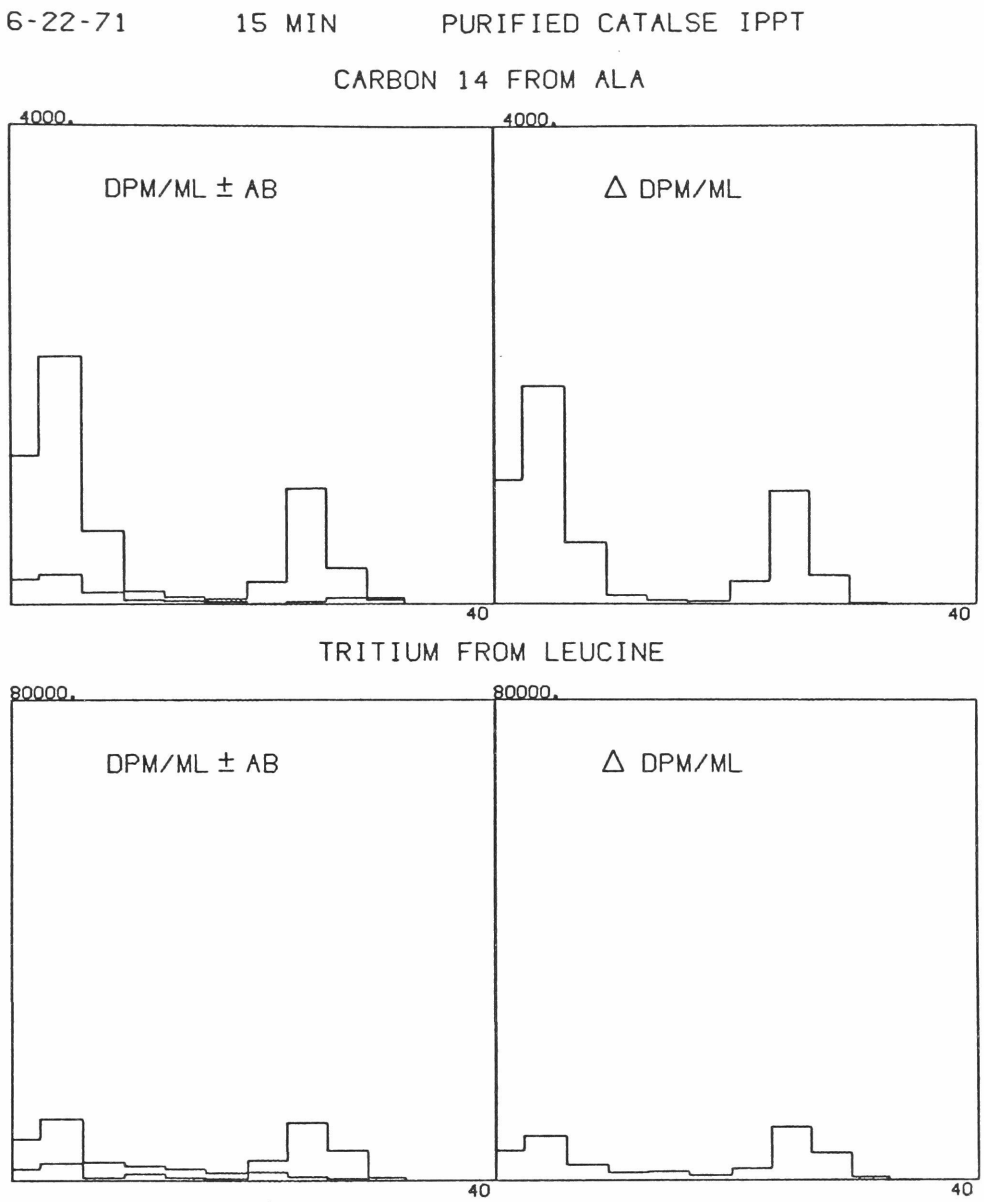


Figure 42. Distribution of label in the purified catalase immunoprecipitates - 15 minute rat.

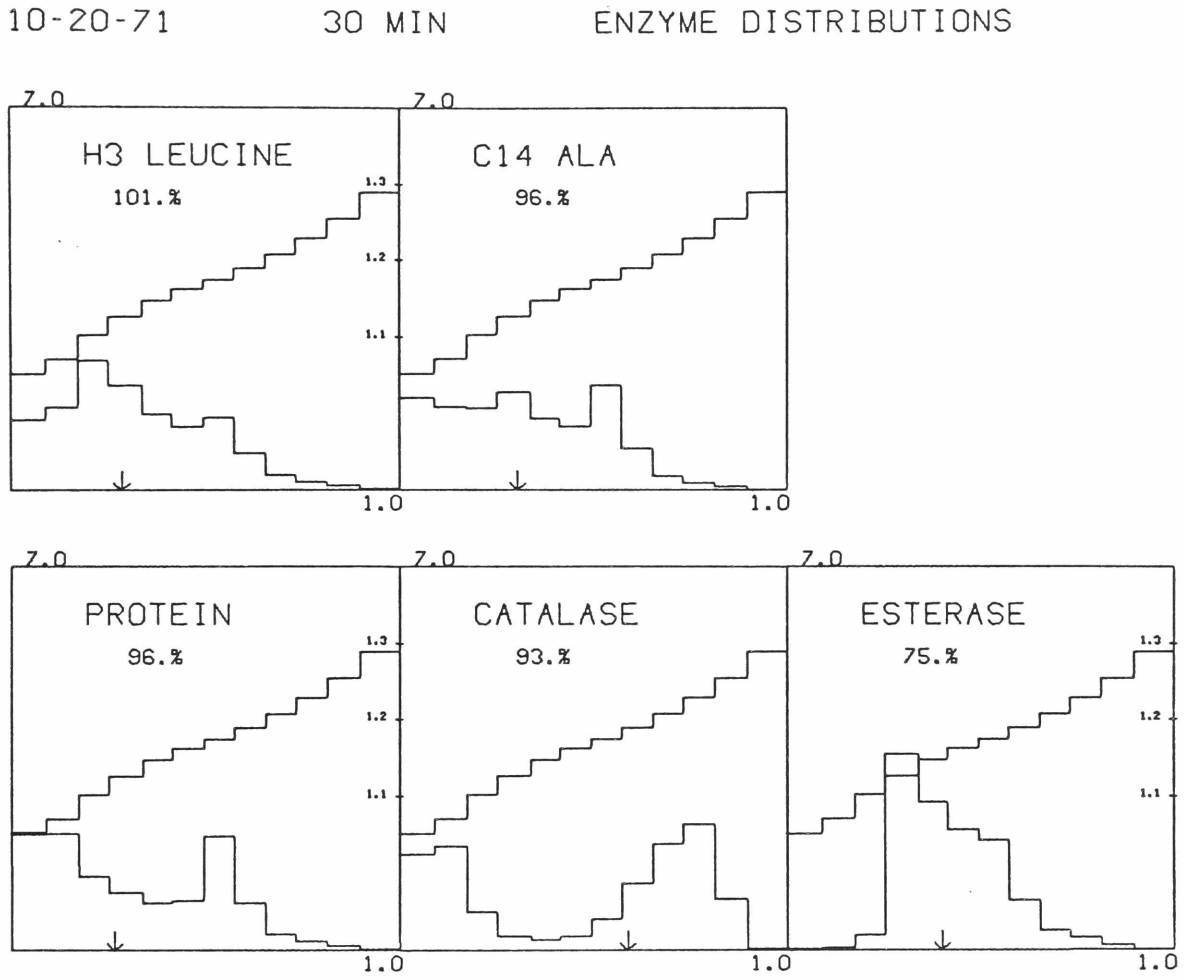


Figure 43. Distribution of marker enzymes and TCA precipitable radioactivity in the gradient - 30 minute rat.

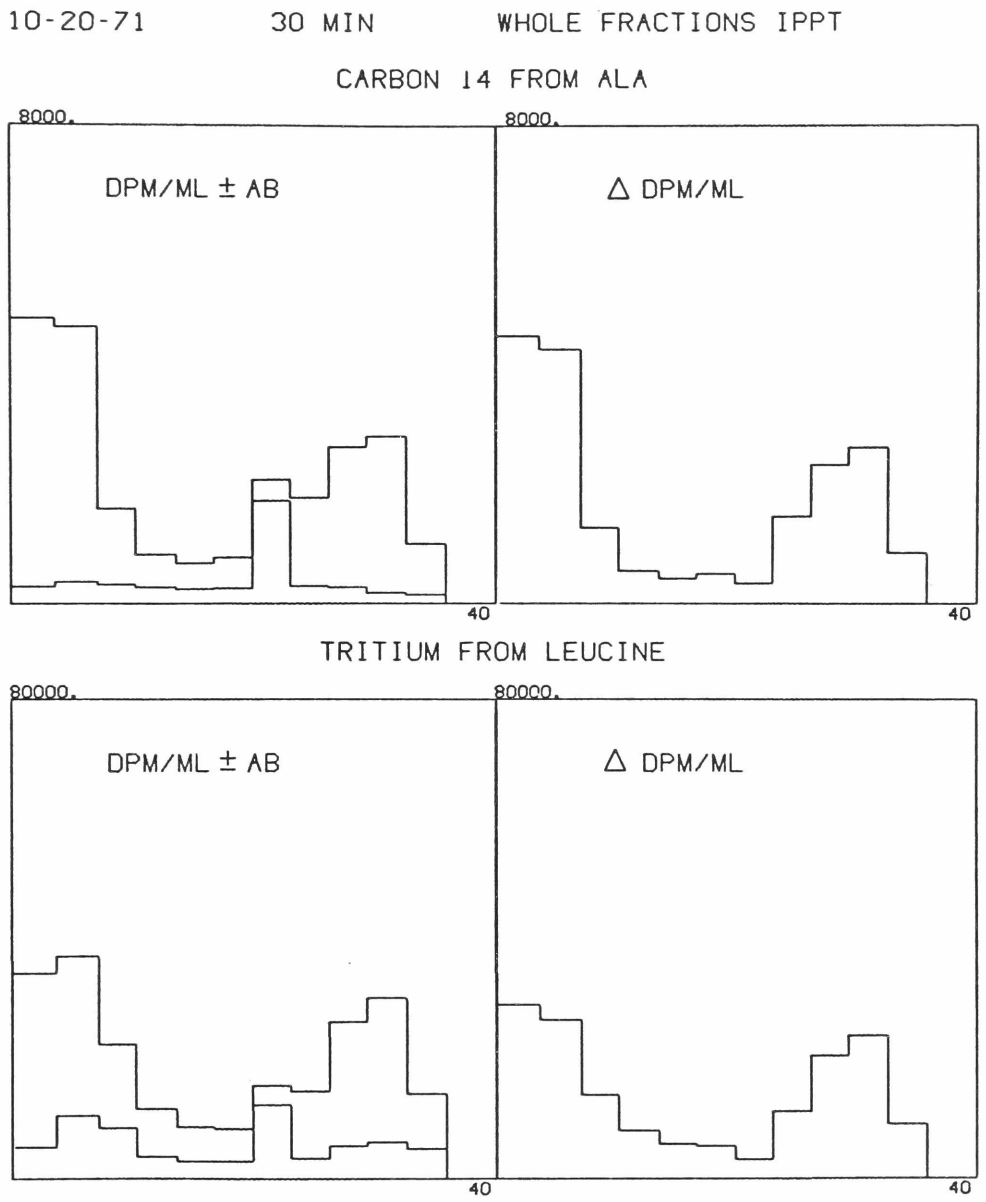


Figure 44. Distribution of label in the whole fraction immunoprecipitates - 30 minutes.

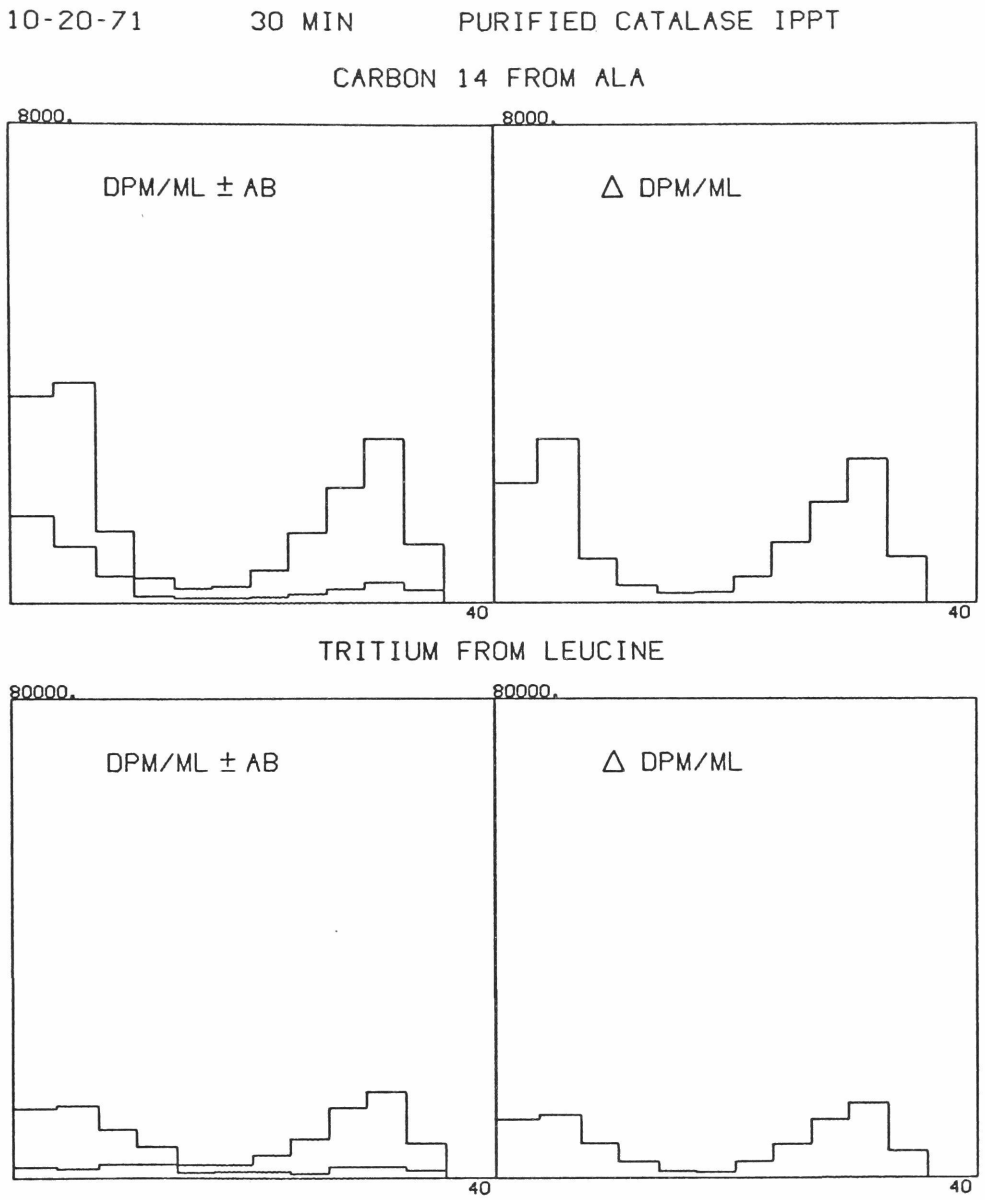


Figure 45. Distribution of label in the purified catalase immunoprecipitates - 30 minute rat.

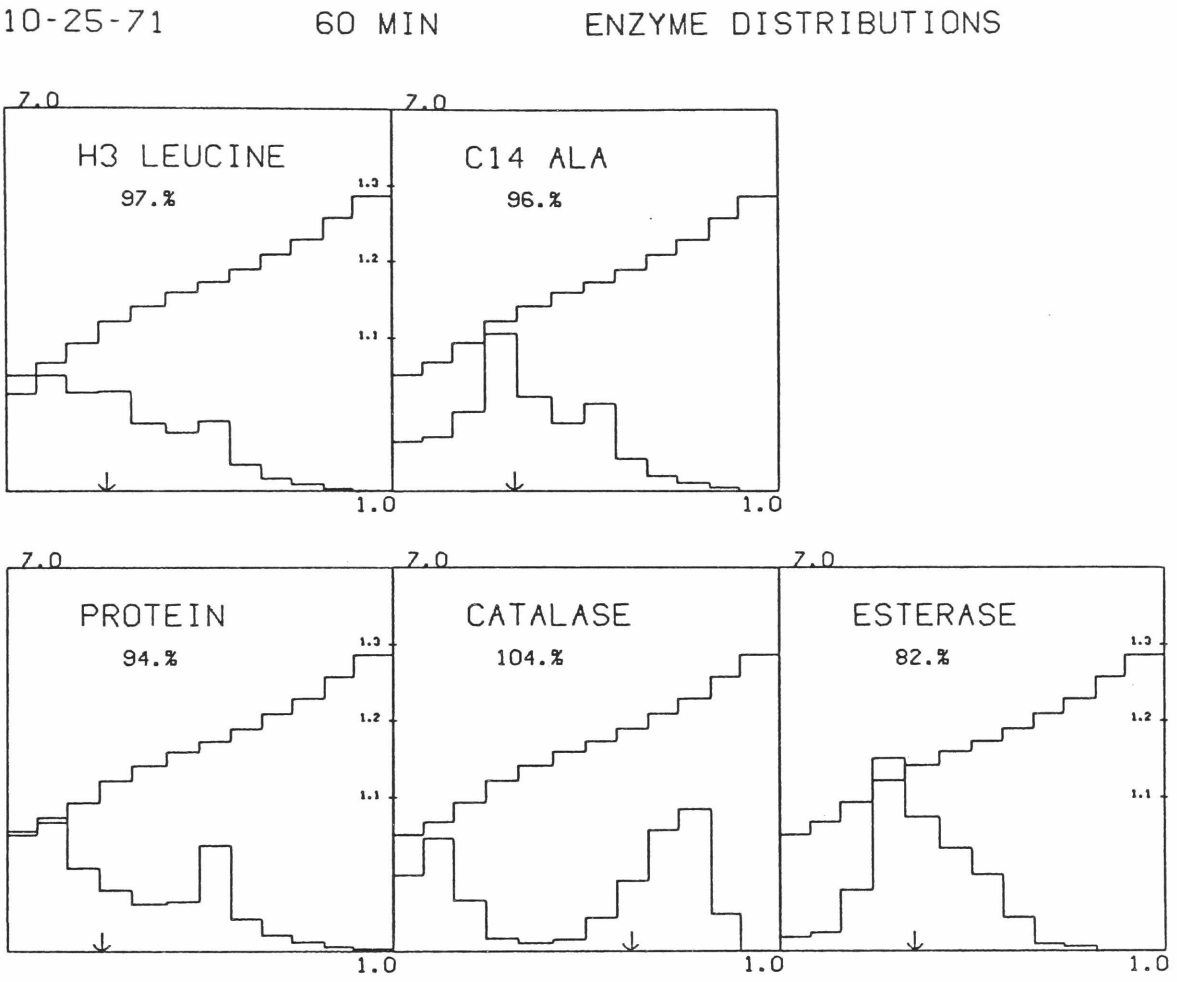


Figure 46. Distribution of marker enzymes and TCA precipitable radioactivity in the gradient - 60 minute rat.

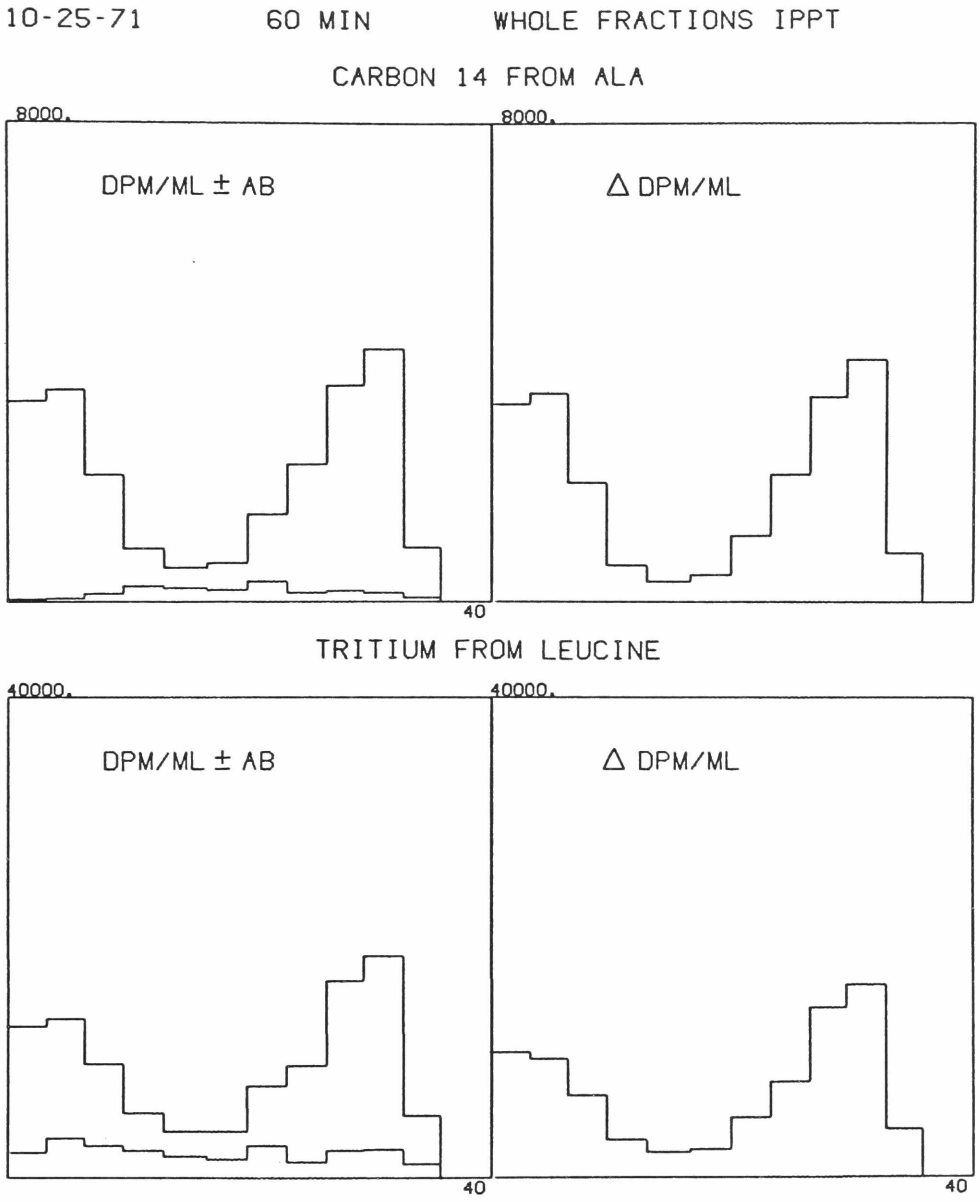


Figure 47. Distribution of label in the whole fraction immunoprecipitates - 60 minutes.

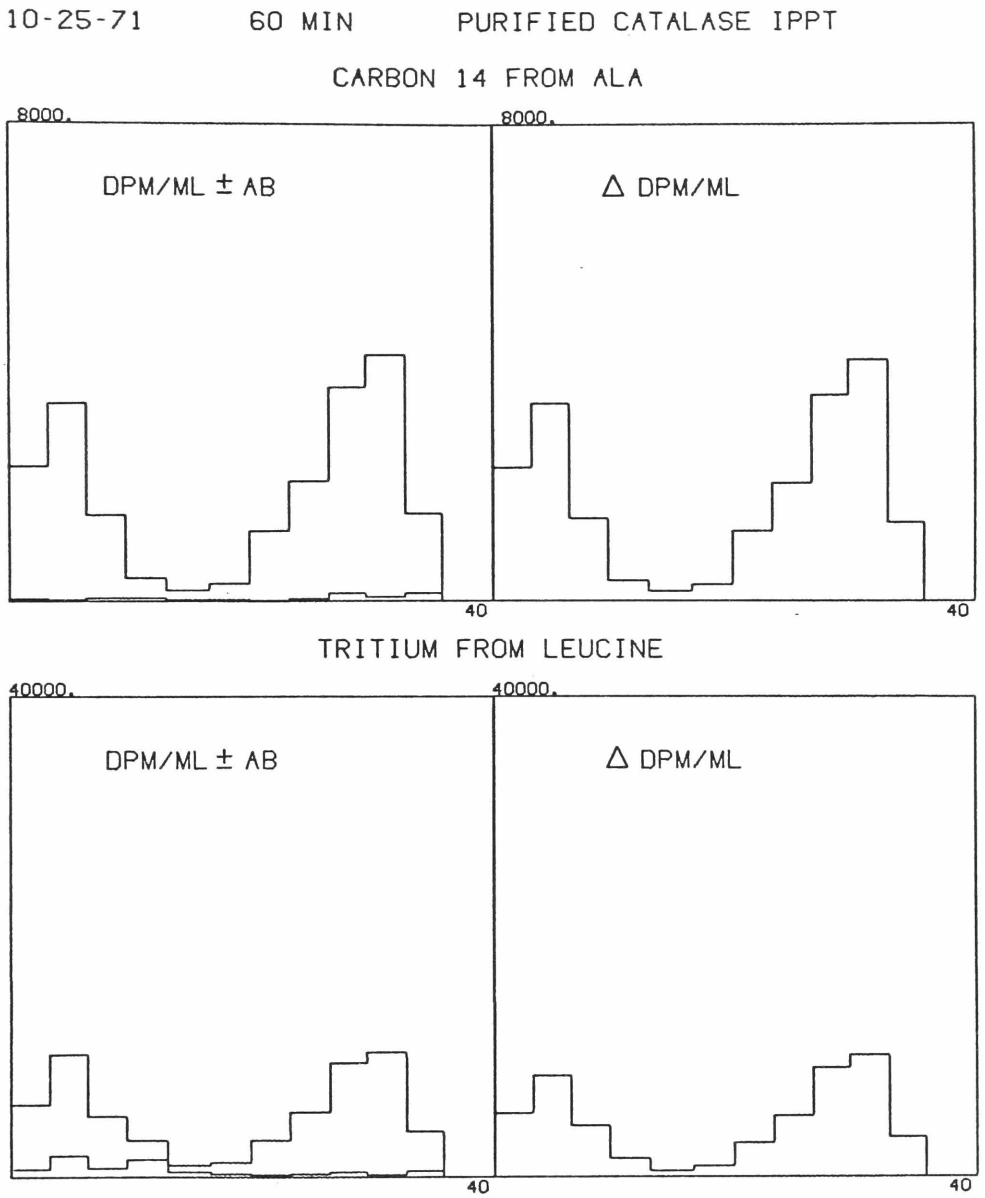


Figure 48. Distribution of label in the purified catalase immunoprecipitates - 60 minute rat.

Recovery values are summarized in Table XIV.

C. Discussion

1. Marker enzymes

Little need be said concerning the activities and distributions of marker enzymes, which have already been adequately discussed in the preceding chapter. It must simply be pointed out that the results of the 15 minute gradient are slightly abnormal, owing to a malfunction of the gradient maker which produced a non-linear gradient in this experiment. Nevertheless, sufficient, though less than optimal, resolution of peroxisomes and microsomes was achieved, with the catalase peak being displaced one fraction to the right of the esterase mode.

2. Overall incorporation kinetics

The incorporation of ^{3}H-leucine into TCA precipitable proteins and into the whole liver immunoprecipitate falls within the general picture already described in Chapter VI: the triangles in Figures 18, 19, and 21 represent the values found in the present experiments.

The incorporation of ^{14}C-ALA into TCA precipitable material (Table XIII) shows a lag followed by a slow progressive increase, in sharp contrast with the rapid rise to a plateau seen previously with ^{3}H-ALA (see Fig. 18b). This difference is probably due to the fact that we inject much larger amounts of chemical ALA when the precursor is labeled with ^{14}C (2.5 to 12 μmoles) than when it is labeled with ^{3}H (11 nmoles). Even if only one percent of the injected ALA reaches each gram of liver, this still amounts to a concentration of 0.13 mM at the higher dosage. This could well expand the ALA and subsequent intermediate pool sizes, resulting in the situation depicted in Figure 23, model 1 (Chapter VI), and accounting for the observed lag in incorporation.

This interpretation receives some support from the fact that a similar lag is observed in the appearance of label in the whole liver

Table XIV

Recoveries of Immunoprecipitable Radioactivities in the Gradients

Time	Whole liver ippt		Purified catalase ippt	
	^{3}H-leucine	^{14}C-ALA	^{3}H-leucine	^{14}C-ALA
min	%	%	%	%
8	not determined			
15	98	50	84	60
30	111	66	111	77
60	130	99	86	86

immunoprecipitate (Fig. 49), indicating that the specific activity of the free heme pool continues to rise for a long time after administration of the precursor.

3. Kinetics of conversion of biosynthetic intermediates to purifiable catalase

Table XV shows that the percentage of label found in non-purifiable intermediates decreases with time, and is consistently higher with leucine than with ALA as precursor, indicating that in each individual animal, the pool of intermediates labeled with ALA is smaller than the pool of intermediates labeled with leucine. Thus these dual-label experiments confirm the earlier results obtained with separate groups of animals: there must be two biosynthetic intermediates, one without and one containing heme.

4. Distributions of TCA precipitable radioactivity

At 8 minutes the distribution of total TCA precipitable ^{3}H-leucine is similar to that of esterase, which indicates that most of the newly synthesized protein is associated with the microsomes. There is more label than esterase at the top of the gradient, which may be accounted for by the protein-synthetic efforts of the free ribosomes. With time there is a redistribution of label towards the top of the gradient and the label at 60 minutes appears to be distributed somewhat like total protein, although there is relatively more label in the microsomes and less in the mitochondria (in agreement with their known relative rates of turnover, or possibly because some secretory proteins still remain in the microsomes). It is perhaps noteworthy that many gradients (not illustrated) have shown at 15 to 30 minutes a considerable amount of label in the third fraction from the top. This can be accounted for neither by the microsomes nor by the soluble proteins. Components that could conceivably be concentrated in this fraction are free polysomes and Golgi elements.

With ALA as precursor, the highest concentrations of TCA-precipitable radioactivity at 8 and 15 minutes are in the mitochondria and

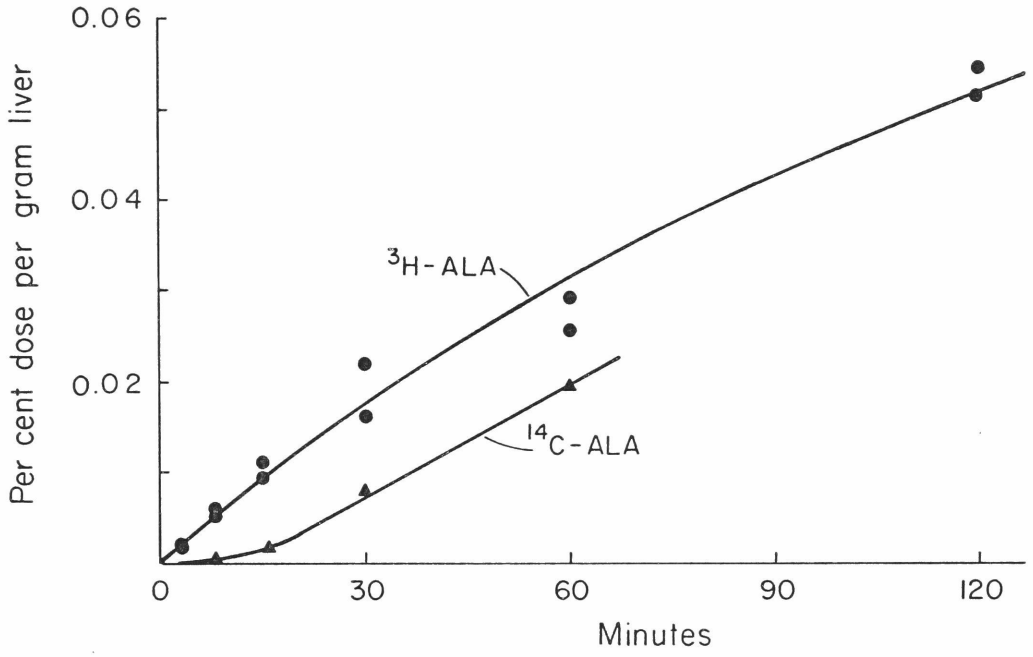


Figure 49. Kinetics of incorporation of label from ^{14}C-ALA into material precipitated by anticatalase (whole liver immunoprecipitate).

The results obtained previously with ^{3}H-ALA (Chapter VI, Figure 22) are included for the sake of comparison.

Table XV

Label in Catalase Biosynthetic Intermediates

in Double-labeled Rats Used for Gradients

The difference between the total radioactivity of the whole liver immunoprecipitate histogram and that of the purified catalase immunoprecipitate histogram is expressed as a percentage of the former.

Time min	^{3}H-leucine %	^{14}C-ALA %
8	91	55
15	81	42
30	58	35
60	36	8

supernatant. This result agrees with the fact that the last enzyme in heme synthesis, ferrochelatase, is mitochondrial (Sano and Granick, 1961). With time there is a redistribution of label toward the microsomes, probably reflecting the incorporation of new heme into cytochromes b_5 and P_{450}.

5. Distributions of immunoprecipitable radioactivity

To facilitate an overall view of the results, we have summarized the results in two composite graphs showing the superimposed distributions of label recovered in the unpurified and purified catalase immunoprecipitates at the four time points (Figs. 50 and 51). The ^{3}H-leucine graphs have all been normalized to the same total surface area for the unpurified immunoprecipitate, since it is clear that the differences in incorporation between the four animals (Table XIII) must be due mostly to individual variability, as incorporation is already more than 80% complete at the earliest time point (see Fig. 19, Chapter VI). This is not true in the case of ^{14}C-ALA, for which precursor the surface areas of the graphs are proportional to the amount of incorporation, as depicted in Figure 49.

Figure 50 shows clearly that labeled catalase appears rapidly in the peroxisomes. A substantial fraction of the total label is apparent at the bottom of the gradient as early as 15 minutes, and by 60 minutes about two-thirds of the labeled catalase is there. Figure 50 also shows that at 15 minutes most of the immunoprecipitable radioactivity at the bottom of the gradient is nonpurifiable. As time progresses, the fraction of the label that is purifiable (shaded histogram) increases. Therefore we see, in a qualitative way, that catalase biosynthetic intermediates are transported to the peroxisomes and completed there.

A striking characteristic of the distributions of both Figures 50 and 51 is that they tend to approach that of catalase more and more closely as time goes on. After 60 minutes, the distribution patterns of labeled material precipitated by anticatalase, whether derived from whole fractions or after partial purification of the enzyme, and with

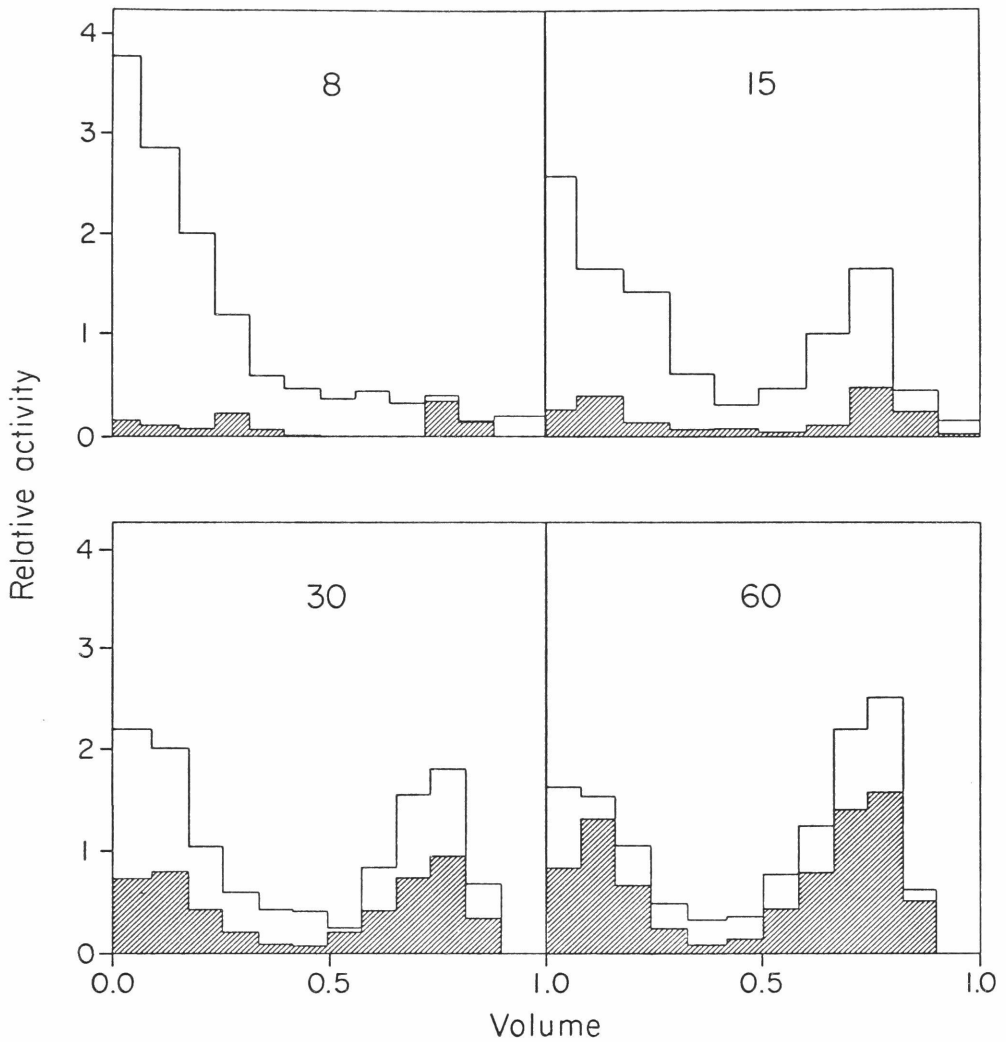


Figure 50. Distributions of catalase labeled with ^{3}H-leucine in the polypeptide.

The upper histograms represent the whole liver immunoprecipitates and the lower shaded histograms the purified catalase immunoprecipitates.

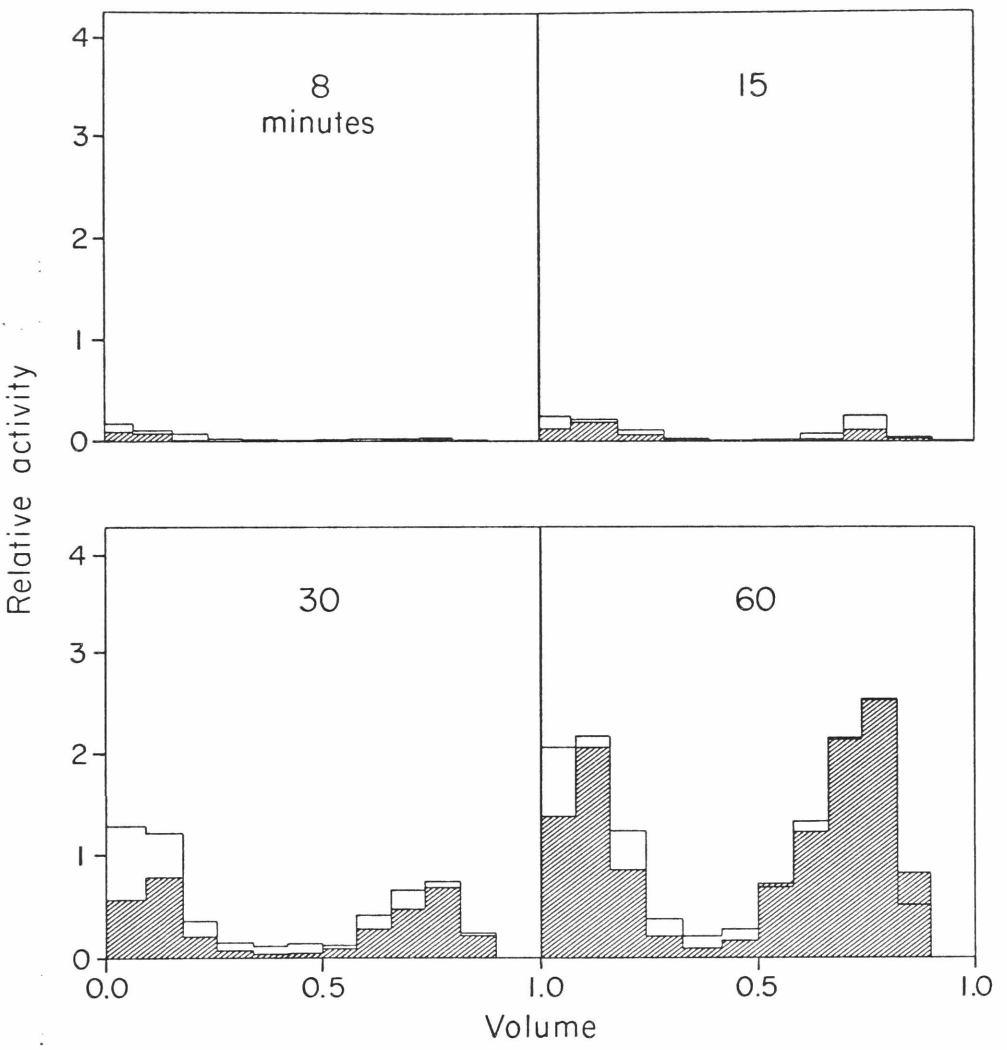


Figure 51. Distributions of catalase labeled with ^{14}C in the heme.

The upper histograms represent the whole liver immunoprecipitates and the lower shaded histograms the purified catalase immunoprecipitates.

either ALA or leucine as precursor, are almost indistinguishable from that of catalase activity. By that time, therefore, the newly-made catalase and the bulk of its biosynthetic intermediates are distributed essentially like pre-existing catalase, with about two-thirds associated with the peroxisome-rich fractions, and the remainder with the starting layer. In earlier publications of this laboratory, strong evidence has been presented in support of the contention that catalase occupies a single pool confined entirely to peroxisomes, at least in female rat liver (Beaufay et al., 1964; Leighton et al., 1968). According to this theory, the amounts of catalase and of peroxisomal oxidases recovered in soluble form in the final supernatant have been released from peroxisomes as a result of a homogenization artifact. Biochemical homogeneity of the peroxisomes has also been emphasized in the quoted publications. The present results provide additional support to these views. It should also be noted that the small amount of catalase activity found in microsomal fractions actually belongs to small peroxisomes, which equilibrate in sucrose gradients just the way larger peroxisomes do, if they are centrifuged long enough to reach their equilibrium position.

In order to analyze quantitatively the early events in catalase biosynthesis and transport, we made the assumption that the homogeneity of peroxisomes observed after 60 minutes obtains also at the earlier times, and therefore that any label contained in the peroxisomes in the intact liver would become distributed like catalase in the gradient. We could then, on the basis of this assumption, estimate the amount of peroxisomal label, and by difference the amount and distribution of non-peroxisomal label. We considered for this purpose the four next to bottom gradient fractions, which contain a large part of the peroxisomes relatively uncontaminated by other subcellular components. The ratio of the cumulative percentage of label to that of catalase activity in these four fractions was taken as a measure of the fraction of the label associated with peroxisomes in the liver. By multiplying the distribution of catalase by this fraction, and subtracting the resulting attenuated catalase distribution from the radioactivity distribution,

we could obtain the distribution of non-peroxisomal immunoprecipitable label. The graphs computed in this manner for the two immunoprecipitates are shown in Figures 52 and 53.

Looking first at the results obtained with ^{3}H-leucine, we see that the amount of non-peroxisomal purifiable radioactivity precipitated by anticatalase is very small at all times, and so randomly distributed as to be without significance. Therefore, the completion of the catalase molecule appears to take place exclusively in the peroxisomes. In contrast, the labeled immunoprecipitable intermediates that do not accompany catalase through purification are largely non-peroxisomal at the beginning, showing a strange distribution pattern which will be discussed in greater detail below. The proportion of non-peroxisomal label decreases progressively with time, and becomes very small one hour after injection of the labeled leucine.

The results of Figure 52 are summarized in Figure 54. It is clear that labeled leucine is incorporated into catalase, in the form of non-purifiable intermediate(s), outside the peroxisomes. The intermediates are transferred as such to the peroxisomes and the enzyme molecules are completed there. It is particularly noteworthy that there seems to be no significant lag or transit time between biosynthesis and transfer to the peroxisomes. Apparently, finished polypeptide chains are released into what looks like a homogeneous extra-peroxisomal pool of intermediates, which empties into the peroxisomes in a strictly exponential fashion, with a half-clearance time of 12 minutes. Conversion of the intermediates to a purifiable form, presumably completed catalase, occurs within the peroxisomes with an estimated rate constant corresponding to a half-clearance time of the order of 25 minutes. Thus, since the half-life of catalase itself is 36 hours (Poole et al., 1969; Poole, 1971), we estimate the extra-peroxisomal pool of catalase biosynthetic intermediates to be 0.55% of the total catalase, or about 5 μg/g liver, and the peroxisomal pool of intermediates to be about twice this amount. These estimates are consistent with the total pool

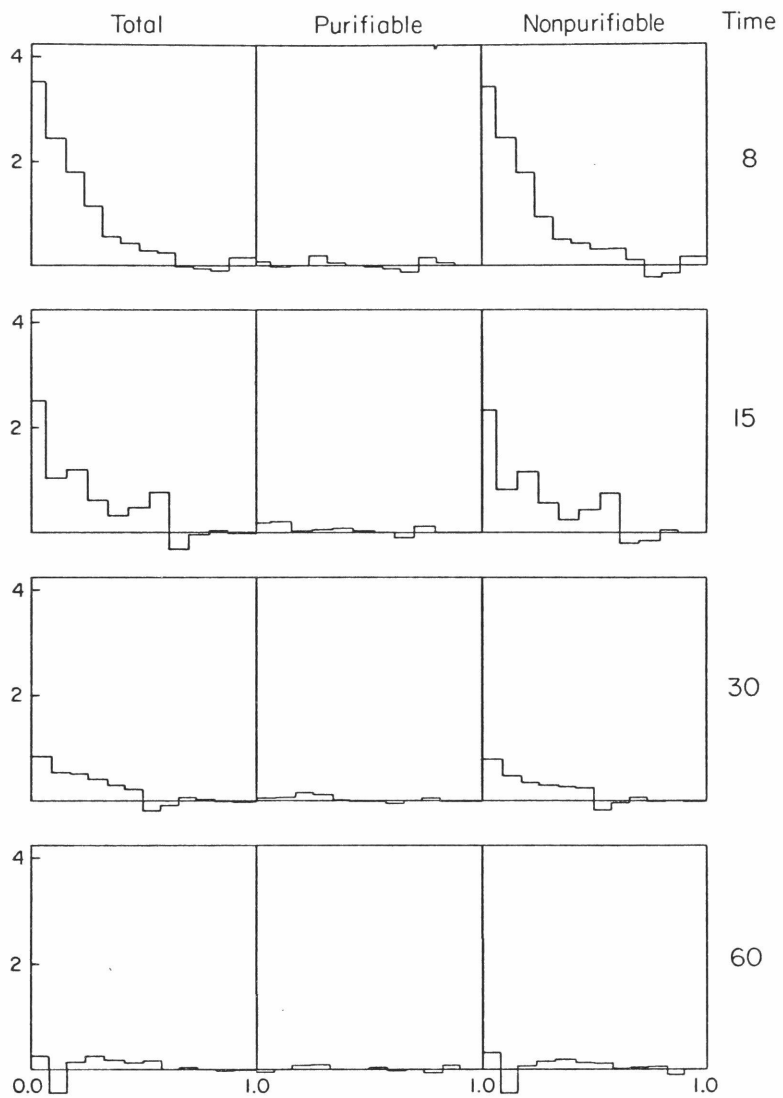


Figure 52. Distribution of non-peroxisomal catalase labeled with ^{3}H-leucine in the polypeptide.

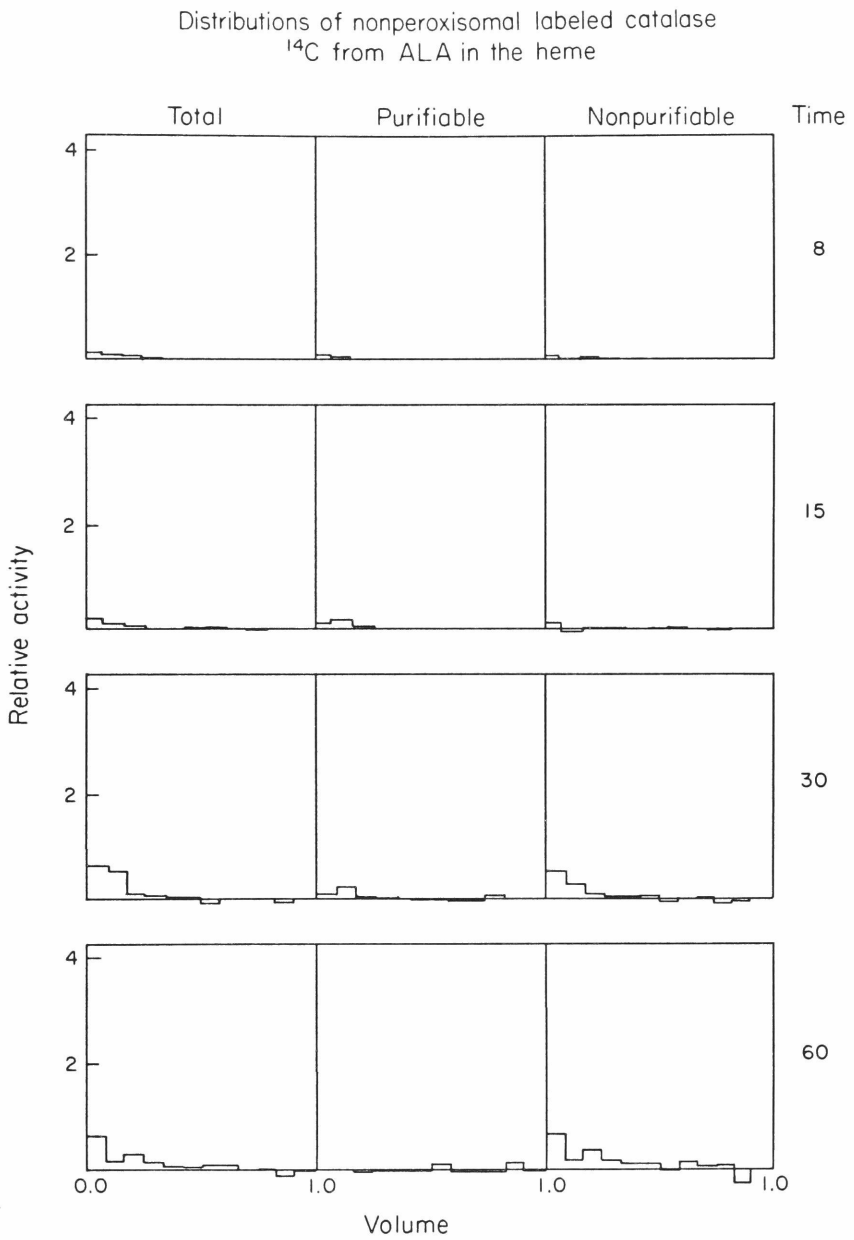


Figure 53. Distributions of non-peroxisomal catalase labeled with ^{14}C in the heme.

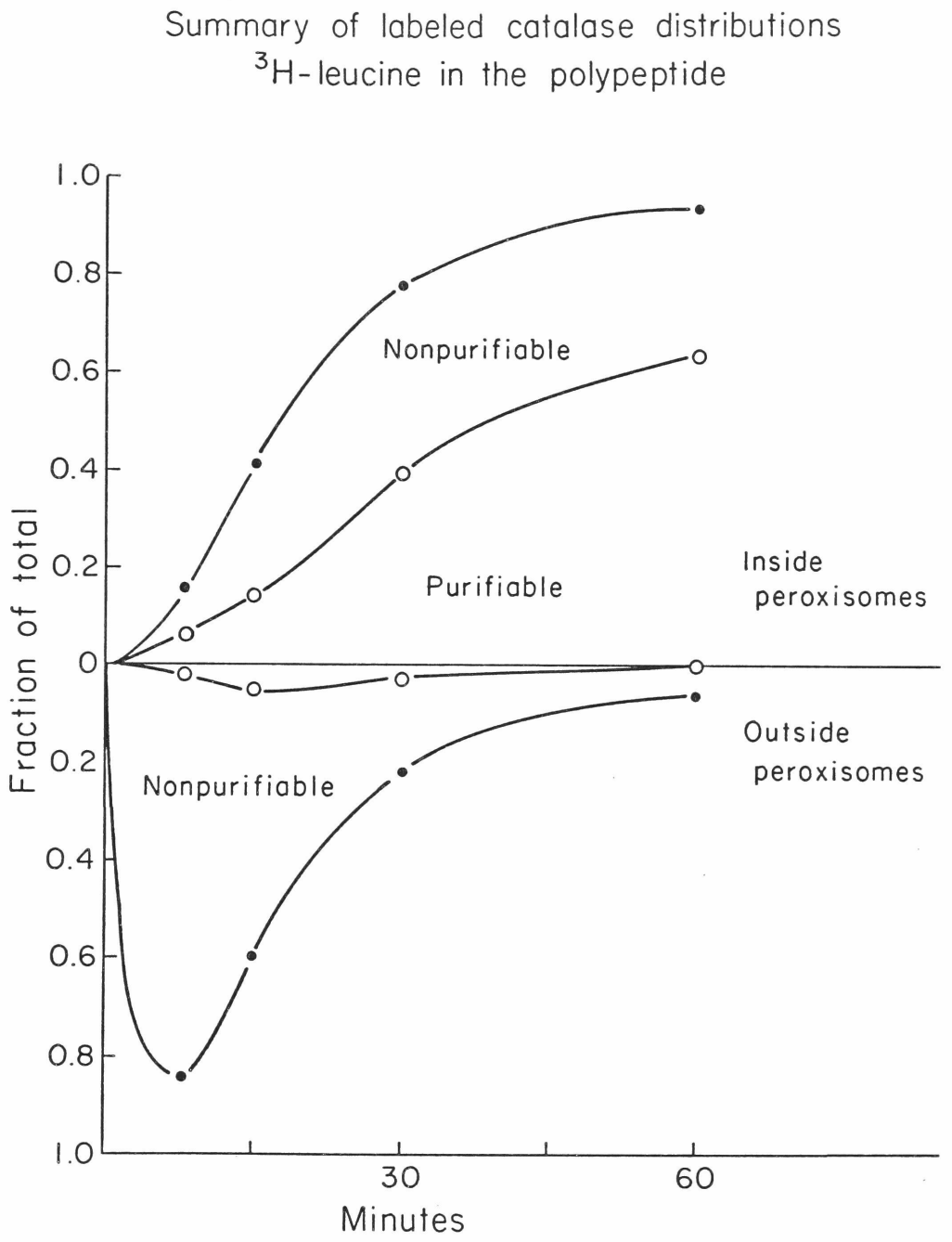


Figure 54. Summary of the distributions of catalase labeled with ^{3}H-leucine in the polypeptide.

size of 1.85% which can be evaluated from a regression line drawn through the triangles of Figure 21. Apparently these 4 rats have somewhat smaller pools than do the other rats, represented by circles in Figure 21. The difference in slope raises the possibility that availability of heme may be rate-limiting in the completion of catalase. As will be pointed out below, the only known difference between these experiments (done with ^{14}C-ALA) and the others (done with ^{3}H-ALA) is that they involved the injection of non-negligible amounts of chemical ALA, apparently sufficient to increase considerably the intracellular concentration of porphyrin intermediates.

The kinetics of incorporation of ^{14}C-ALA is more complex and more difficult to interpret. The main complication arises from our use of ^{14}C-labeled precursor, in order to carry out a double-labeling experiment in the same animals. As already mentioned, we probably expanded the pool sizes of porphyrin intermediates considerably with the amounts of chemical ALA that were injected, and this rules out the assumption of a steady state for the interpretation of the results. Nevertheless some conclusions may be drawn. First of all, a comparison of Figures 50 and 51 shows clearly that the fraction of non-purifiable, peroxisomal catalase labeled with ALA, is always lower than the corresponding fraction with leucine as label. This is consistent with our results on these same 4 animals for the total liver catalase (Table XV) and is also consistent with the earlier results of Chapter VI. It indicates that both intermediates are present in peroxisomes and therefore that heme is added to apocatalase in peroxisomes. A crude estimate of the time constants indicates that at least 80% of the intermediates in the peroxisomes lack heme.

When the data of Figure 51 are replotted after subtracting that label which can be attributed to peroxisomes (Fig. 53), we see that some non-peroxisomal ALA-labeled material remains. This indicates that some heme can be added to apocatalase before it reaches the peroxisomes. What is surprising, however, is that the supernatant fraction seems to

contain some purifiable ALA-labeled catalase. This is in contrast to the results obtained with leucine, which show negligible non-peroxisomal, purifiable label.

A second discrepancy appears when the ALA labeling data are summarized in Figure 55. The amount of non-purifiable label outside the peroxisomes is distinctly greater than that inside, especially at later time points. This is despite the fact that the intra-peroxisomal pool of intermediates is about twice as big as that outside (as determined with leucine). There are several possible explanations for this. The steady-state concentration of heme-containing intermediate could be lower inside than outside the peroxisomes as a result of its conversion to catalase. Alternatively, the peroxisomes could contain a sizeable free heme pool, which would cause the specific radioactivity of the labeled heme molecules to decrease as they enter these particles from the extra-peroxisomal region. In the latter event, Figures 53 and 55 could be grossly misleading since the non-peroxisomal radioactivity would be much greater than the actual amount of non-peroxisomal intermediates. Artifactual attachment of hot heme to apocatalase released from peroxisomes during homogenization is also possible. We can envision other explanations, but the available data do not warrant further speculation.

The main conclusions we can draw from the above data are:

1) Catalase intermediates are synthesized outside the peroxisomes and transported into them with a half-time of about 12 minutes.

2) Inside the peroxisomes the apocatalase is equipped with heme and converted to purifiable, presumably authentic catalase.

3) Some heme may be added to apocatalase before it reaches the peroxisomes.

6. Additional gradients with reversed isotopic labeling

In addition to the four gradient fractionation experiments just described, additional experiments were performed with less refined immunochemical methods. These include more rats double-labeled with

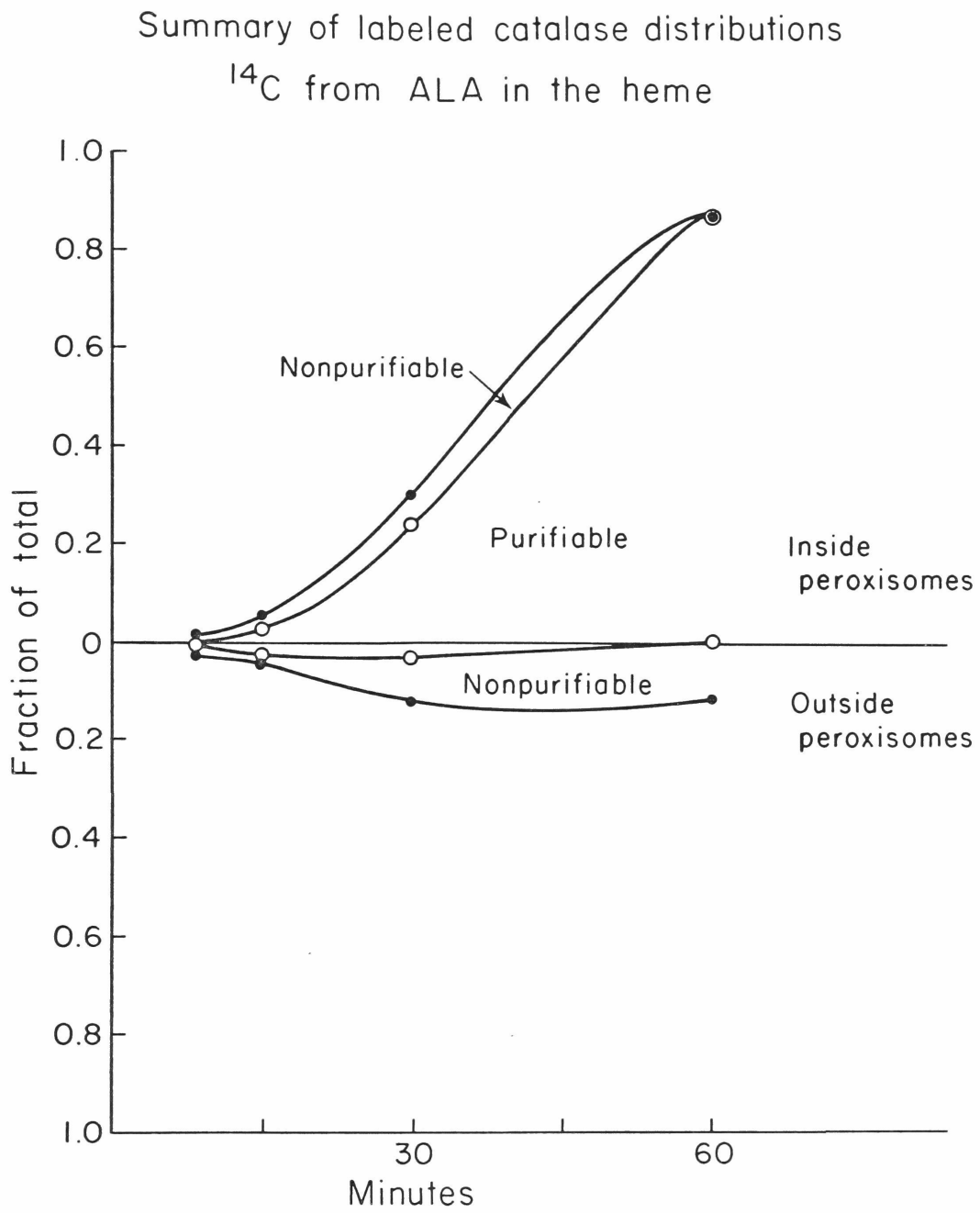


Figure 55. Summary of the distributions of catalase labeled with ^{14}C in the heme.

ALA and leucine, but with the isotopes reversed. After correction for a substantial amount of non-specific immunoprecipitation, the results were found to agree with those reported above. There is a similar rapid transport of labeled catalase intermediates to the peroxisomes. Furthermore the ratio of non-purifiable to total immunoprecipitable label is always smaller with ALA than with leucine, both in the whole gradient and in peroxisomes, just as described above in Table XV. Therefore, regardless of which precursor is labeled with which isotope, we reach the same conclusion: both catalase biosynthetic intermediates are present in the peroxisomes.

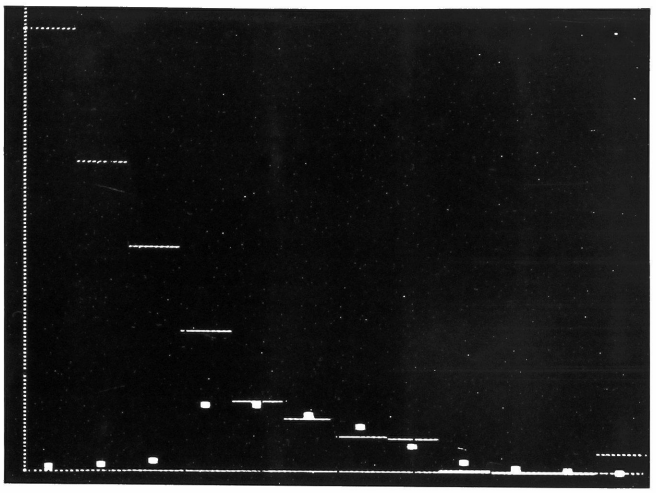
IX. ATTEMPTS TO DETERMINE THE EARLY EVENTS IN CATALASE BIOGENESIS

A. Distribution of Non-peroxisomal Label

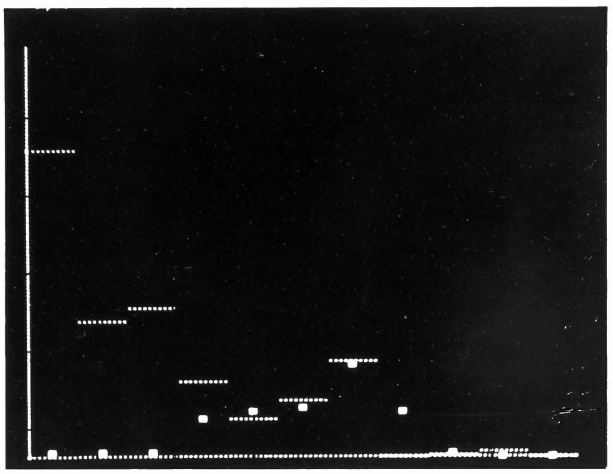
One major question that remains unanswered by the results of the previous chapter is the nature of the extra-peroxisomal site or sites of biosynthesis and temporary storage of the catalase biosynthetic intermediates. The distributions of non-peroxisomal label shown in Figures 52 and 53 suggest that the major part of the label remains in the starting layer, presumably in soluble form. Some binding to lipid, significant or artifactual, is indicated by the systematic finding of the highest radioactivity in the top fraction. In addition, with leucine, but not with ALA as precursor, a significant part of the label is seen invading the region mostly occupied by microsomes.

We have estimated the amount of label associated with microsomes by fitting the esterase distribution to that of non-peroxisomal label. This was done by means of a computer program which allowed simultaneous visual display of the two distributions on an oscilloscope. The esterase distribution was then reduced in size to fit the right hand end of the distribution of non-peroxisomal label as closely as could be evaluated visually. The reduction factor arrived at in this manner for esterase distribution gave the microsomal component of the label. Figure 56 illustrates the method and the goodness of fit for the 8 and 15 minute gradients. In Table XVI are listed the computed distributions, including the peroxisomal component as calculated before by a similar fitting of the catalase distribution. It will be noted that the microsomal component does not exceed 22% of the total label.

In contrast to this, more than half of the label appears to be present in soluble form at 8 minutes. There can be little doubt, however, about the catalase nature of this material because it migrates on SDS gels exactly as does catalase (Fig. 57).



A



B

Figure 56. Use of computer with on-line oscilloscope display to estimate the contribution of microsomes to the distribution of labeled catalase. A. 8 minute experiment. B. 15 minute experiment.

The solid lines indicate the distribution of the label in the whole fraction immunoprecipitates, after subtraction of that label which is attributable to peroxisomes. The dots represent the attenuated distributions of esterase. The fits in the 30 and 60 minute experiments (not shown) were as good or better.

Table XVI

Distribution of ^{3}H-leucine Labeled Immunoprecipitable Material in Gradients

Time min	% of total ^{3}H-leucine in whole liver immunoprecipitate in		
	Peroxisomes	Microsomes	Soluble fraction
8	16	20	64
15	41	22	37
30	78	10	12
60	94	6	0

SDS gel electrophoresis of catalase biosynthetic intermediates from the supernatant fractions of a rat labeled with ^{3}H-leucine for 8 minutes

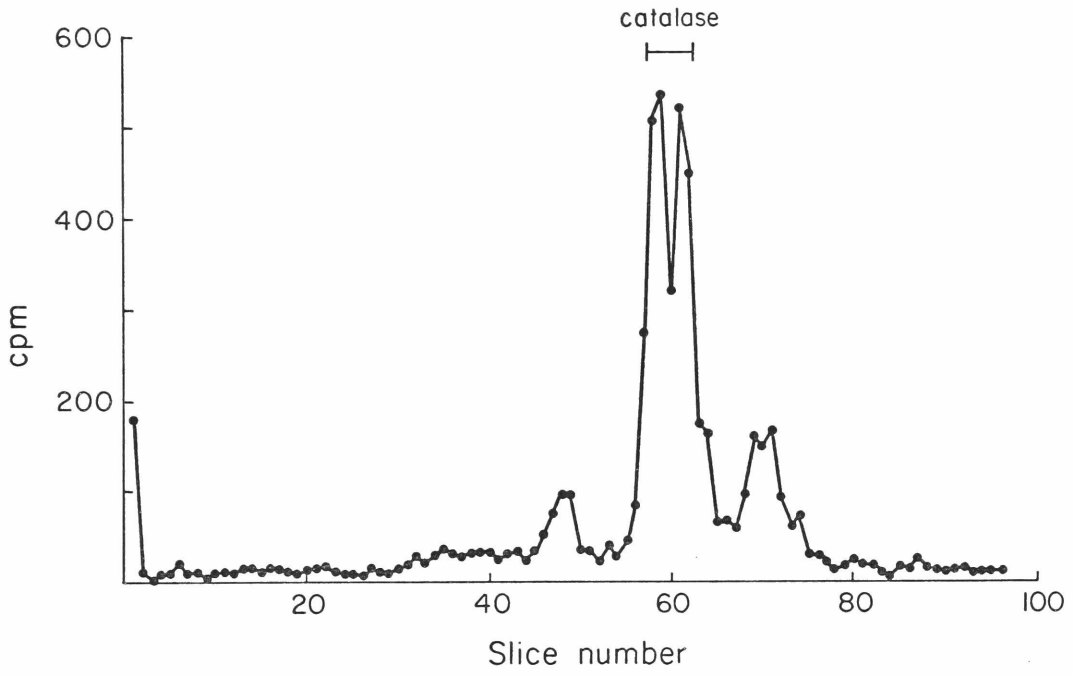


Figure 57. SDS gel electrophoresis of catalase biosynthetic intermediates labeled with ^{3}H-leucine.

The sample was a whole liver immunoprecipitate prepared from a mixture of the two next to top gradient fractions from the 8 minute rat (Figures 37-39). About 29% of the radioactivity is nonspecific (Figure 38). Of the label specifically precipitated by anticatalase, only 4% is in the form of purifiable authentic catalase (Figures 38 and 39). The cpm shown are due to tritium.

B. Use of a Shallow Gradient

One possible explanation for the puzzling distribution of non-peroxisomal label at early time points is that, instead of being partitioned between microsomes and the soluble fraction, as indicated in Table XVI, the label is actually attached to a special population of particles having a low median density, together with a broad density distribution. To test this possibility, we labeled a rat for 8 minutes (^{3}H-leucine only), and then fractionated the liver extract on a shallow gradient extending between density limits of 1.05 and 1.10. Everything else was kept the same as in other fractionation experiments, including the use of 1.32 density cushion and W equal to 1.4×10^{10} sec^{-1}. The results are shown in Figures 58 and 59. Most of the peroxisomes and mitochondria are piled up on the cushion at the bottom of the gradient. So are many of the microsomes, but there is a long trail of esterase back through the gradient. The distribution of TCA precipitable ^{3}H-leucine resembles closely that of esterase, except for somewhat more radioactivity remaining in the layer. This confirms the results seen in the previous 8 minute gradient: most of the newly synthesized proteins are in the microsomes at 8 minutes.

The distribution of immunoprecipitable label shows that about 40% of this material is located in the lower half of the gradient, in association with microsomes and peroxisomes. Comparison with the distributions of marker enzymes indicates that the larger part of this particle-bound label is microsomal, in confirmation of the results obtained in the previous 8 minute gradient. The rest of the label shows a distribution strikingly similar to that seen in the previous 8 minute gradient, in spite of the difference in gradient shape. The highest value is again found in the top fraction. But at the same time we find significant amounts of label in fractions 4 and 5, which are virtually free of both catalase and esterase activities. Again we are confronted with some peculiar features. But, at least, this experiment appears to rule out the existence of a particle population of low density, and suggests that the microsomal localization of part of the label is real.

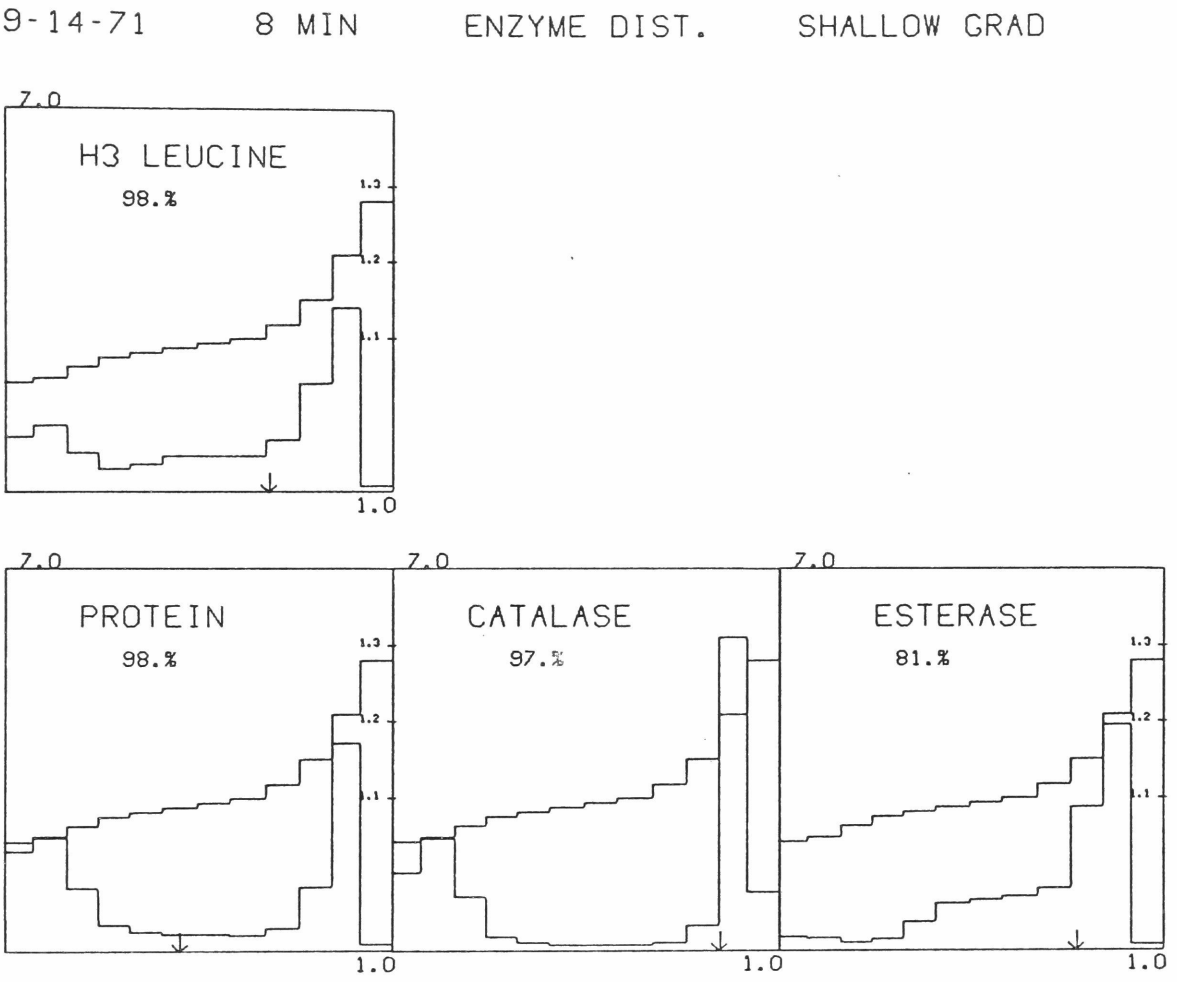


Figure 58. Distribution of marker enzymes and TCA precipitable radioactivity in the gradient - 8 minute rat.

A shallow sucrose gradient was used.

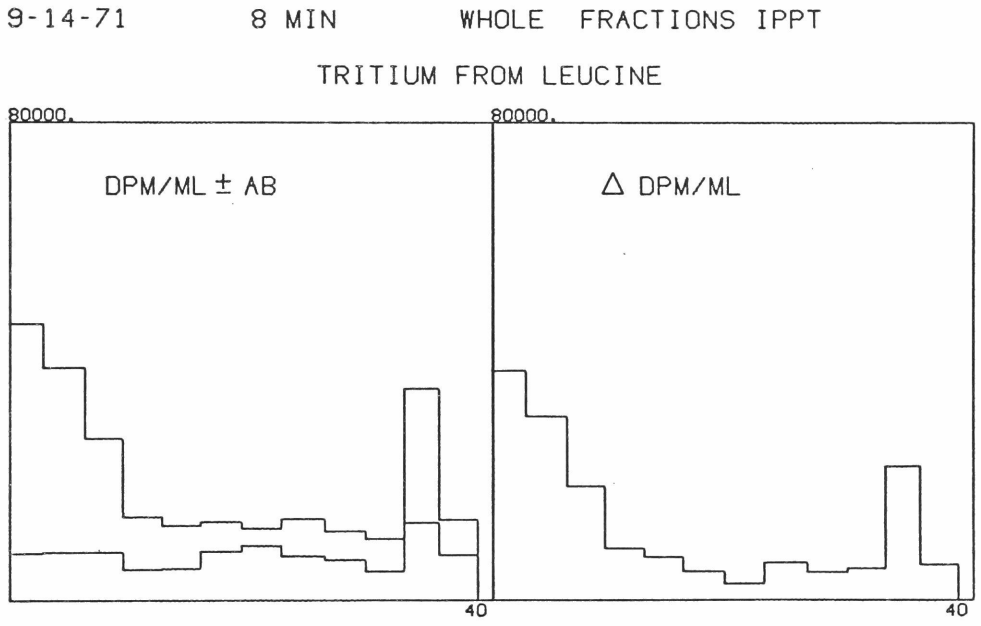


Figure 59. Distribution of label in the whole fraction immunoprecipitates - 8 minutes.

A shallow sucrose density gradient was used.

C. Early Events in Catalase Biosynthesis

In Figure 60 are shown the results of an experiment in which a number of rats were given ^{3}H-leucine by intraportal injection, and killed at various times afterwards. Liver homogenates were prepared in the usual manner, and were then centrifuged for 2 hours at 40,000 rpm. About 30 to 35% of the catalase and L-α-hydroxy acid oxidase activities appeared in the supernatants and less than 6% of the soluble proteins (as determined with phosphoglucomutase) appeared in the high speed pellet. Immunoprecipitable label was measured in the pellet and in the supernatant, without prior purification of the catalase. The data show considerable scatter, due to individual variation, but some conclusions may be drawn.

The results obtained between 8 and 60 minutes after injection of the labeled leucine agree entirely with those of the gradient experiments: unsedimentable radioactivity is essentially equal to that recovered in the top gradient fractions, and sedimentable radioactivity to that which has penetrated significantly into the gradient. The time course of the redistribution of label argues against the existence of a precursor-product relationship between microsomes and supernatant fraction. Whether we estimate the amount of microsome-bound radioactivity from the results of the gradient experiments (Table XVI), or by subtracting from the total sedimentable radioactivities shown in Figure 50 what we know from the gradient results to be associated with intact peroxisomes, we end up with the same picture of the microsomes losing their label with a half-clearance time of the order of 10 to 15 minutes, not faster therefore than the whole extra-peroxisomal pool, or than the supernatant fraction corrected for the contribution of broken peroxisomes. Kinetically, one cannot arise from the other.

At earlier time points, the relationship is different. At 1 and 2 minutes after injection of the labeled amino acid, there is more radioactivity in the pellet than in the supernatant. However, at this time the majority of total TCA precipitable label is in nascent

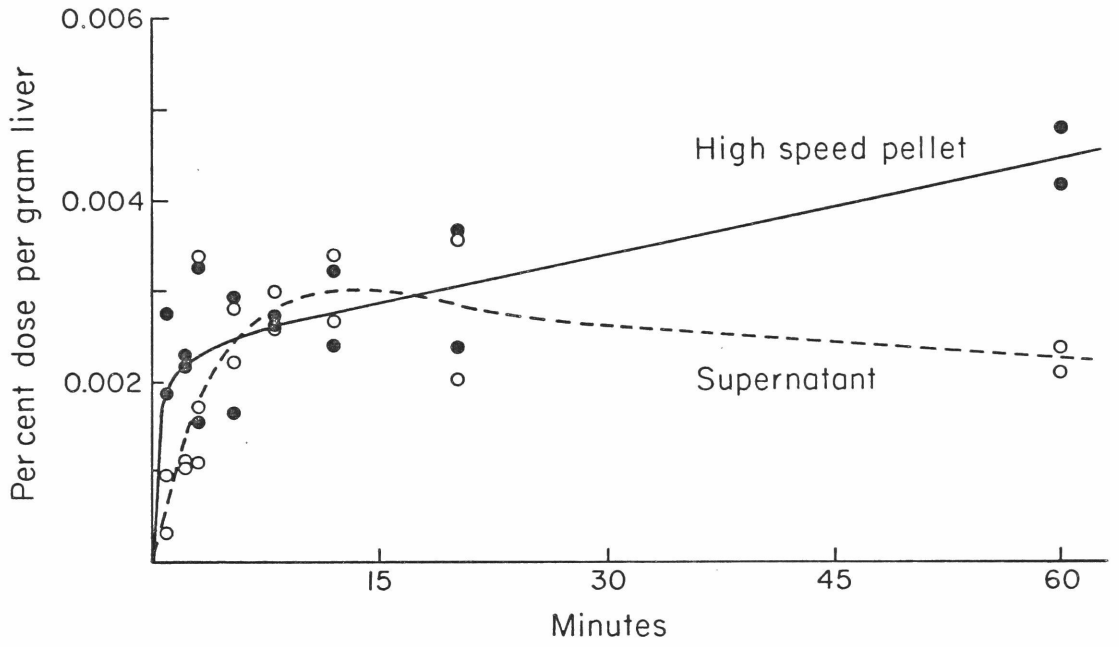


Figure 60. Kinetics of appearance of ^{3}H-leucine in whole liver immunoprecipitates from high speed supernatants and pellets.

polypeptide chains (see Section F). Therefore it is reasonable to assume that the large proportion of sedimentable label precipitated by anticatalase also reflects the presence of incomplete, ribosome-bound catalase polypeptides. This interpretation receives some support from the claim of Higashi and Kudo (1971) of the specific precipitation of catalase-synthesizing ribosomes by anticatalase. Our centrifugation conditions are such that most polysomes would be sedimented, whether they are free or bound. Therefore the above results do not distinguish between these two localizations.

We also fractionated on a gradient the liver extract of a rat labeled for 3 minutes with ^{3}H-leucine only, and we prepared only whole liver immunoprecipitates. The radioactivity data are shown in Figure 61. The distributions of marker enzymes were normal (Fig. 34), and most of the TCA-precipitable radioactivity (not shown) was associated with the microsome-rich fractions. In contrast, there was relatively little immunoprecipitable label in these fractions. Much of it was in the top fractions, and some transfer of radioactivity to the peroxisomes already seemed to have occurred. These results argue against microsomes being the main bearers of immunoprecipitable radioactivity present in the high speed pellet at 3 minutes, raising the possibility that soluble polysomes may play a role in the synthesis of catalase. Note that partly sedimented soluble polysomes might also account for the label observed in fractions 4 and 5 in the shallow gradient experiment.

D. A Search for Nascent Catalase Chains on Free and Bound Ribosomes

A rat was injected with 5 mC of ^{3}H-leucine under Nembutal anesthesia and killed 3 minutes later. Bound and free ribosomes were prepared as described by Redman (1969). Nascent polypeptide chains were released according to the puromycin/high salt method of Sabatini (personal communication) and desalted by passage through a column of Sephadex G-25 equilibrated in PBS. The label in nascent catalase, serum proteins and ferritin was determined by immunoprecipitation. Purified

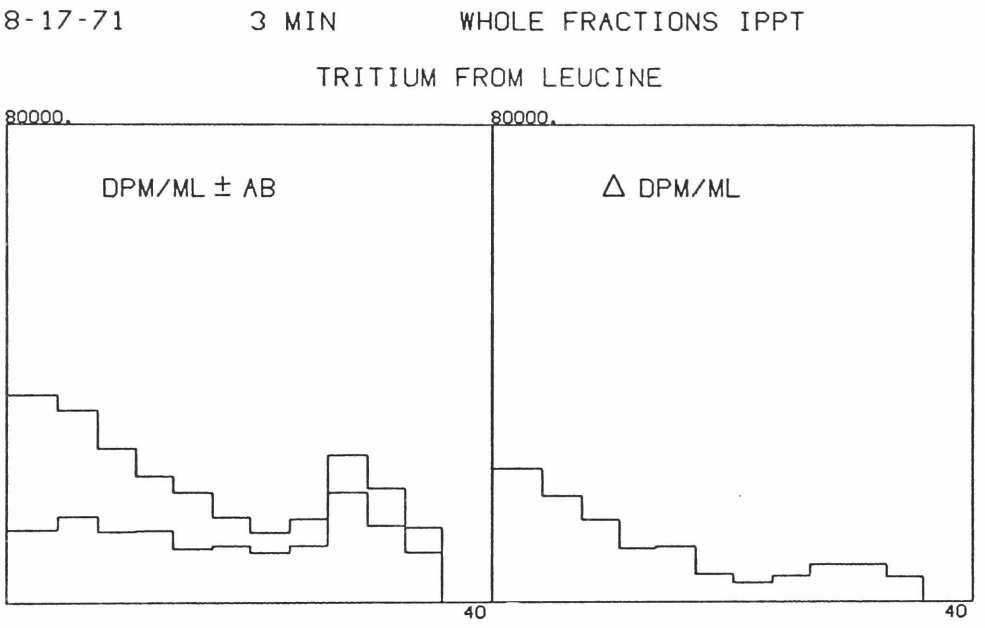


Figure 61. Distribution of label in the whole fraction immunoprecipitates - 3 minutes.

rabbit anticatalase and carrier purified rat liver catalase were used. The other antisera were commercial goat preparations and the appropriate carrier proteins were included. Two types of controls were run: one with control goat globulins and one with PBSLA only. Everything was run in duplicate. Incubation was for 1 hour at 37° and 23 hours at 0°. The results for the two fractions of nascent chains released from bound ribosomes, prepared as described by Redman (1969), are shown in Table XVII. They indicate that catalase is synthesized on bound ribosomes. Unfortunately, high and variable control radioactivities found with the nascent chains released from free ribosomes prevented us from drawing any conclusion about whether or not catalase might also be synthesized on free ribosomes.

This experiment was later repeated with several modifications, including the omission of the 37° incubation step, but no more reliable results were obtained. The indication was that catalase is synthesized on both bound and free ribosomes in similar amounts.

Experiments of this type suffer from several drawbacks. First, neither the isolation of ribosomes nor the release of nascent polypeptides is quantitative. Second, since the polypeptides represent all stages of synthesis, their physical properties differ by varying amounts from the corresponding finished proteins. The nascent chains from free ribosomes in particular show a great tendency to denature, resulting in high blanks during immunoprecipitation.

Thirdly, there is no way to measure the extent of the separation of ribosome classes that has been effected, except to show a dissociation of the synthesis of two proteins of known distribution. We attempted to use serum proteins and ferritin for this purpose (Redman, 1968, 1969), but the high control radioactivities combined with the low levels of radioactivity incorporated into ferritin made this unsuccessful.

Some methodological improvements will be required before the distribution of nascent chains of minor proteins on the two populations of liver ribosomes can be accurately determined.

Table XVII

CPM from ^{3}H-leucine in Nascent Proteins Released from Bound Ribosomes

In addition to an excess of one antiserum, each tube received the corresponding carrier antigen, and the tubes were incubated for 1 hour at 37° and 23 hours at 0°. The two fractions of bound ribosomes were prepared according to Redman (1969).

Antiserum added	Fraction I		Fraction II	
	Total	Mean less control*	Total	Mean less control*
Anti-rat serum proteins	33,500	27,800	12,600	9,890
	32,700		14,400	
Anticatalase	13,100	7,610	7,720	4,730
	12,800		8,960	
Antiferritin	6,880	1,260	2,773	-250
	6,330		3,959	
Control goat globulins	5,480		2,710	
	4,940		3,400	
PBSLA	5,130		3,520	
	5,810		4,830	
TCA-precipitable	200,000		110,000	

* Mean of four controls subtracted.

E. Synthesis of Catalase in vitro by Isolated Microsomes

Another approach to the study of the site of catalase synthesis is to isolate microsomes and then test whether or not they have the capacity to synthesize catalase in vitro. If they do, then where does this catalase go when synthesis is complete: into the cisternae of the microsomes or into the equivalent of the cell sap, the suspension medium? The following experiments were performed in collaboration with Dr. Gert Kreibich and Dr. David Sabatini.

A rough microsome fraction was prepared from the livers of 4 female rats as described by Adelmann, Blobel and Sabatini (1972). It was incubated with ^{3}H-leucine (1 mC, 40 C/mmole), ATP, an ATP generating system, unlabeled amino acids except leucine, and desalted rat liver high speed supernatant for 7 minutes at 37° according to Blobel and Sabatini (1970). 18.3 million cpm were incorporated into 100 mg of protein. This is 1.8% of the added cpm. The microsomes were pelleted by centrifugation, washed and resuspended in a solution of 0.25 M sucrose, 50 mM Tris, 175 mM KCl, and 5 mM $MgCl_2$, pH 7.6, and treated with puromycin (1 mM, final concentration) to release the nascent chains. The microsomes were again pelleted and then resuspended in phosphate-buffered saline containing leucine and ALA (PBSLA) and subfractionated by treatment with 0.1% Triton X-100, a procedure that has been shown by Kreibich and Sabatini (1972) to make the membranes leaky, allowing some of the contents to diffuse out, while maintaining basic membrane integrity.

The effectiveness of the various procedures was checked by sucrose gradient centrifugation, as described by Kreibich and Sabatini (1972). Total TCA soluble and insoluble radioactivities were determined in the various fractions. Catalase and serum proteins were isolated immunochemically and their radioactivities determined. Only the results directly relevant to our problem are shown here, in Table XVIII.

We notice first that a small but significant incorporation into catalase antigen has taken place, amounting to 2.4% of the total

Table XVIII

Release of Catalase and Other Proteins from Rough Microsomes after *in vitro* Labeling

		Total TCA precipitate	Immunoprecipitates					
			Serum proteins			Catalase		
			+Ab	-Ab	Δ	+Ab	-Ab	Δ
		(in thousands of cpm)						
Microsomes before puromycin		1,700	-	-	-	-	-	-
After puromycin:	Supernatant*	105	9	1	8	2	1	1
	Microsomes	1,230	275	9	265	43	12	31
	Recovery	1,335 (79%)			273			32
Puromycin-treated microsomes		1,230	275	9	265	43	12	31
After 0.1% Triton X-100:	Supernatant†	653 (53-55%)	188	4	184 (69-67%)	21	13	8 (26-16%)
	Microsomes	536	98	9	89	50	9	41
	Recovery	1,189 (97%)			273 (103%)			49 (158%)

*Before performing the immunoprecipitations, the supernatant was passed through a Sephadex G-25 column to remove a substantial amount of TCA soluble radioactivity. The amount of radioactivity appearing in the void volume was approximately the same as the amount of TCA precipitable label.

†The first percentage in parentheses is relative to the radioactivity in the starting material; the second is relative to the sum of the recovered radioactivities.

incorporation in TCA-insoluble material. Incorporation into serum proteins is 9-fold greater. Puromycin treatment leaves most of the radioactivity in the microsomes, from which it can be partly released by 0.1% Triton X-100. Such behavior is interpreted as a demonstration that the chains detached from the ribosomes by the action of puromycin are released into the lumen of the microsomal vesicles. The findings of Table XVIII therefore tend to support the idea that catalase chains made by rough microsomes are discharged intracisternally, as are those of serum proteins and, in fact, most other polypeptide chains synthesized in this experiment. Unfortunately, the catalase chains are released very incompletely by Triton X-100, and we cannot exclude adsorption as an alternative explanation for the lack of release by puromycin alone.

F. Localization of Labeled Catalase in Microsomal Cisternae after in vivo Labeling

We also performed a similar experiment to see whether catalase could be detected in the cisternae of microsomes after in vivo labeling and natural chain termination. This experiment, also, was performed in collaboration with Dr. Gert Kreibich and Dr. David Sabatini.

A female rat was fasted overnight and injected with 5 mC of ^{3}H-leucine (6 C/mmole) in the portal vein under Nembutal anesthesia. 2.5 minutes after the beginning of the injection (which took 30 seconds) the liver was excised, chilled, weighed and minced. Rough microsomes were prepared as described by Adelmann, Blobel and Sabatini (1972) and suspended in 0.15 M NaCl buffered to pH 7.6 with 10 mM Tris, containing 0.1 mM leucine and 5 mM $MgCl_2$ (TBSLMg).

The microsomes were solubilized with 1% Triton X-100 and then centrifuged to pellet the ribosomes. 46% of the radioactivity appeared in the supernatant, indicating that about half of the label was in completed chains, released from the ribosomes. These completed chains were assayed immunochemically and it was found that 0.25% of the label was in catalase and 19% was in serum proteins.

We then applied the low detergent method of Kreibich and Sabatini (1972) to determine whether this label was associated with the microsomal contents. Another aliquot of microsomes was treated with 0.1% Triton X-100 and then sedimented to pellet the microsomes, less the contents which had leaked out. The pellet was resuspended in more TBSLMg, solubilized with 1% Triton X-100 and recentrifuged. The second supernatant contained those completed chains which had not leaked out of the microsomes. The two supernatants were assayed immunochemically for labeled catalase and serum proteins, as well as for total radioactivity. The results appear in Table XIX. We see that a large proportion of the finished catalase chains--as of serum and total proteins--is released by the low Triton X-100 concentration. This indicates that catalase is present in the cisternae of the endoplasmic reticulum.

G. Summary

What can we conclude from these various experiments about the biosynthesis of catalase and the first steps in its transport to peroxisomes?

First of all, are the microsomes involved? The distributions of leucine-labeled catalase of the previous chapter indicated that as much as 20 to 22% of the labeled biosynthetic intermediates are microsomal at 8 to 15 minutes after isotope administration. The shallow gradient experiment with a second rat labeled for 8 minutes also indicates that the microsomes are involved, as does the detection of nascent catalase on bound ribosomes. The *in vitro* labeling experiment suggested that rough microsomes have the capability to synthesize catalase and that nascent catalase is not released into the suspension medium when detached from ribosomes by puromycin treatment. Finally, microsomes isolated from a rat labeled *in vivo* for 2.5 minutes contained completed catalase polypeptides, and these were released by dilute detergent treatment, as were serum proteins, indicating that the catalase is present in the microsomal cisternae. Opposed to a large-scale involvement of microsomes is the observation that in the 3 minute gradient the

Table XIX
Release of Catalase and Other Proteins from Rough Microsomes after *in vivo* Labeling

Supernatant after	Total TCA precipitate	Immunoprecipitates					
		Serum proteins			Catalase		
		+AB	-Ab	Δ	+Ab	-Ab	Δ
		(in thousands of cpm)					
1.0% Triton X-100	1,040*	197	4	193	4.5	1.8	2.7
0.1% Triton X-100†	553 (53-63%)	160	2	158 (81-88%)	2.7	1.1	1.6 (59-76%)
0.1-1.0% Triton X-100	330	21	1	20	0.7	0.2	0.5
Recovery	883 (85%)			178 (92%)			2.1 (78%)

* The label, solubilized by 1.0% Triton X-100, represented half of the total TCA-precipitable radioactivity of the microsomes. The other half belonged to ribosome-bound unfinished chains, as shown by sucrose gradient centrifugation (Kreibich and Sabatini, 1972).

† The first percentage in parentheses is relative to the radioactivity in the starting material; the second is relative to the sum of the recovered radioactivities.

distribution of label was distinctly unlike that of esterase (or RNA). The limited effectiveness of 0.1% Triton X-100 in releasing puromycin detached chains from microsomes must also be recalled.

Are free polysomes involved? This possibility is definitely not excluded by, and in fact received some weak support from, the search for nascent catalase on free and bound ribosomes.

With what cell structure is the label remaining at the top of the gradients associated? It could be the cell sap. The excess label in the top fraction suggests a physiological or artifactual attachment to lipid. Alternatively, the label might be present in some fragile structure which we break when we homogenize the liver. Association with a particle of very low density is ruled out by our results.

Finally, what is the kinetic relationship between the sedimentable and the soluble label? As discussed above, we tend to discount the existence of a precursor-product relationship between the two and to attribute the more intense labeling of sedimentable components at very early times to the presence of ribosome-bound incomplete chains.

These various questions will be examined in more detail in the following chapter, against the background of information provided by the results and theories of other investigators.

X. GENERAL DISCUSSION

A. The Biochemical Pathway of Catalase Synthesis

Our results demonstrate that catalase is synthesized by way of at least two distinct intermediates, one without heme and the other containing heme, which are precipitated by anticatalase, but do not survive the early steps in the purification of catalase. These results are not particularly surprising, since it is to be expected that hemoproteins could be synthesized in the form of apoproteins and acquire heme subsequently, and this has indeed been found to be the case for both cytochrome c (Kadenbach, 1970) and cytochrome b_5 (Negishi and Omura, 1970). In the case of a tetrameric protein such as catalase, one can easily visualize the additional involvement of one or more heme-containing intermediates, in the form of either free subunits or tetramers incompletely supplied with heme, as discussed in Chapter VI.

In an earlier paper, Higashi and Peters (1963a) have reported the presence in rat liver of a material which precipitated with anticatalase, but was enzymatically inactive, and also was found to separate from catalase in Ouchterlony diffusion tests. They found the highest concentration of this material in rough-surfaced microsomes, the apparent site of catalase biosynthesis according to these authors (Higashi and Peters, 1963b), and concluded that it is probably a biosynthetic precursor of catalase. Despite the apparent similarity in results, there is in our opinion some doubt as to the identity of the material isolated by Higashi and Peters with the intermediates detected in our work. There is too much of it (12% of the total catalase content, against about 2%, as estimated kinetically in our experiments). Also, we have not seen a second precipitation line in immunodiffusion tests with microsomes and our anticatalase. As will be pointed out below, there are serious reasons to believe that the anticatalase preparation of Higashi and Peters is not pure, and therefore that the alleged catalase precursor is an unrelated contaminant, most probably

a plasma protein. The authors actually mention that their antisera "gave a few weak lines against undiluted rat serum." They claim to have removed the contaminating antibodies by adsorption with "a slight excess of combined α_2- and γ-globulin fraction," but it would seem that this step was not entirely effective.

Except in showing that the catalase biosynthetic intermediates migrate like catalase upon SDS gel electrophoresis, our experiments give no information concerning the nature of these intermediates. Their characterization is likely to be a difficult task, since they can be detected only by immunoprecipitation, appear to be relatively fragile, and occur in very small amounts, of the order of 10 to 20 micrograms per gram of liver.

B. The Cytological Pathway of Peroxisomal Catalase Biogenesis

The conclusions that can be drawn from our density gradient fractionation results are summarized as follows: the polypeptide chains of catalase are synthesized outside the peroxisomes, and there enter a small pool which represents about 0.6% of the total catalase and empties into the peroxisomes at the rate of about 6% per minute. While in this extra-peroxisomal pool, apocatalase can acquire heme, but it cannot to an appreciable extent be converted to purifiable catalase. The intra-peroxisomal pool of intermediates is about twice the size of their extra-peroxisomal pool. In it, heme is added and authentic purifiable catalase is made, at the rate of about 3% of the pool contents per minute. Catalase itself, as is known from earlier papers (Price et al., 1962; Poole et al., 1969; Poole, 1971), is destroyed at the rate of 0.032% per minute; we find that it represents about 98% of the material reacting with anticatalase.

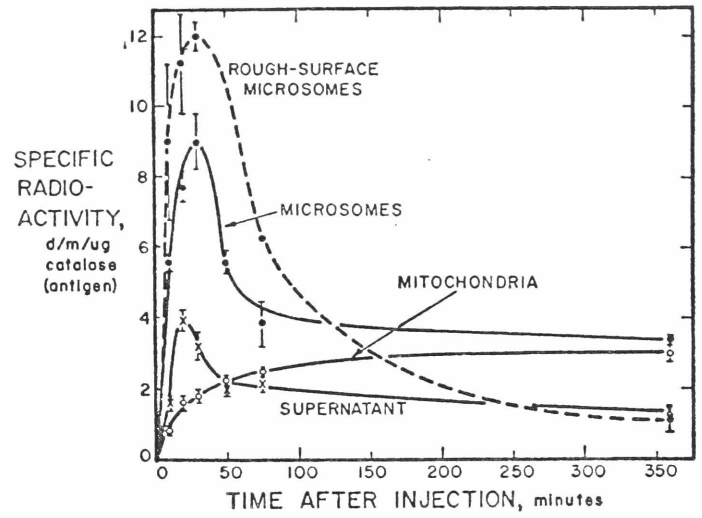
We have not been able to follow as clearly the pathway followed by heme. Our evidence indicates that heme addition occurs both outside and inside the peroxisomes, but from what appear to be two distinct pools. A comparison of the kinetics of transfer of label from non-

purifiable to purifiable catalase within the peroxisomes, using leucine and using ALA as precursor, indicated that a minimum of 80% of the intra-peroxisomal intermediates lack heme. The significance of the extra-peroxisomal heme-containing intermediate was difficult to assess, and we cannot even rule out the possibility that extra-peroxisomal heme addition represents a side reaction or an experimental artifact, of minor or no biological significance. In the remainder of this discussion, we will consider only the pathway of the protein part of catalase, as traced down with the help of ^{3}H-leucine.

An important feature of the system revealed by the present results, as well as by earlier observations made in this laboratory, is that all the pools involved behave kinetically as homogeneous pools, from which molecules are removed at random, irrespective of their age. It must also be emphasized that newly formed finished catalase seemed, as early as it could be detected, to be distributed exactly like the bulk of the pre-existing catalase. There was no indication that it first appeared in peroxisomes differing in size, density or fragility from the general peroxisome population of the liver. It is true that our methods were not designed to reveal small differences in such physical properties. However, it is worth remembering that Poole et al. (1970) looked for exactly such differences with a high resolution technique, and were unable to detect any difference in distribution between newly synthesized catalase and total catalase in peroxisomes of different sizes 3 hours or later after injection of labeled leucine, or even in less complete observations, as early as one hour after administration of the precursor. These results do not, of course, prove that the newly made catalase is distributed homogeneously throughout the whole catalase pool of the liver. It could just as well be located in a very small number of newly made peroxisomes, provided the statistical distribution of the physical properties of the new and old particles is the same.

To date, our results do not provide an unequivocal identification of the intracellular site of catalase biosynthesis, nor of the mechanism of transfer of the newly made polypeptide chains to peroxisomes. We have strong hints that membrane-bound ribosomes are involved, and that their product is delivered into the lumen of the ER cisternae to which they are attached. But we cannot exclude the participation of free ribosomes. Also, we are faced with the formidable problem of explaining the association of a large fraction of the extra-peroxisomal pool of intermediates with the soluble fraction. This amount is much larger than could be expected to be released from ruptured microsomal vesicles, as indicated by the results of Peters (1962b) on serum albumin, and also by our own observations on the distribution of total TCA-precipitable ^{3}H-leucine: at the early time points, the bulk of the labeled proteins accompanies the microsomes, in contrast with labeled catalase biosynthetic intermediates which are largely soluble.

Before discussing the results in further detail, it is of interest to consider first the observations and opinions of the workers who have studied this problem before us. The only biochemical investigation of catalase biosynthesis is that, already mentioned above, of Higashi and Peters (1963a, b). Their results, which are reproduced in Figure 62a, were taken to indicate that catalase is synthesized by ribosomes bound to rough-surfaced elements of the endoplasmic reticulum and is then transferred to the peroxisomes (believed at that time by the authors to be mitochondria) by an unknown mechanism, which could possibly involve passage through the cell sap. These data, which are expressed in the figure in terms of the specific radioactivity of the immunoprecipitates isolated from each fraction, are more easily compared with ours after conversion to total dpm per gram of liver. This was done using the authors' own catalase antigen distribution data (Higashi and Peters, 1963a), and the resulting values are shown in Figure 62b. We now see a clear similarity between the two groups of results. Most of the immunoprecipitable label is extra-peroxisomal at the early time points, and there is actually more radioactivity in the



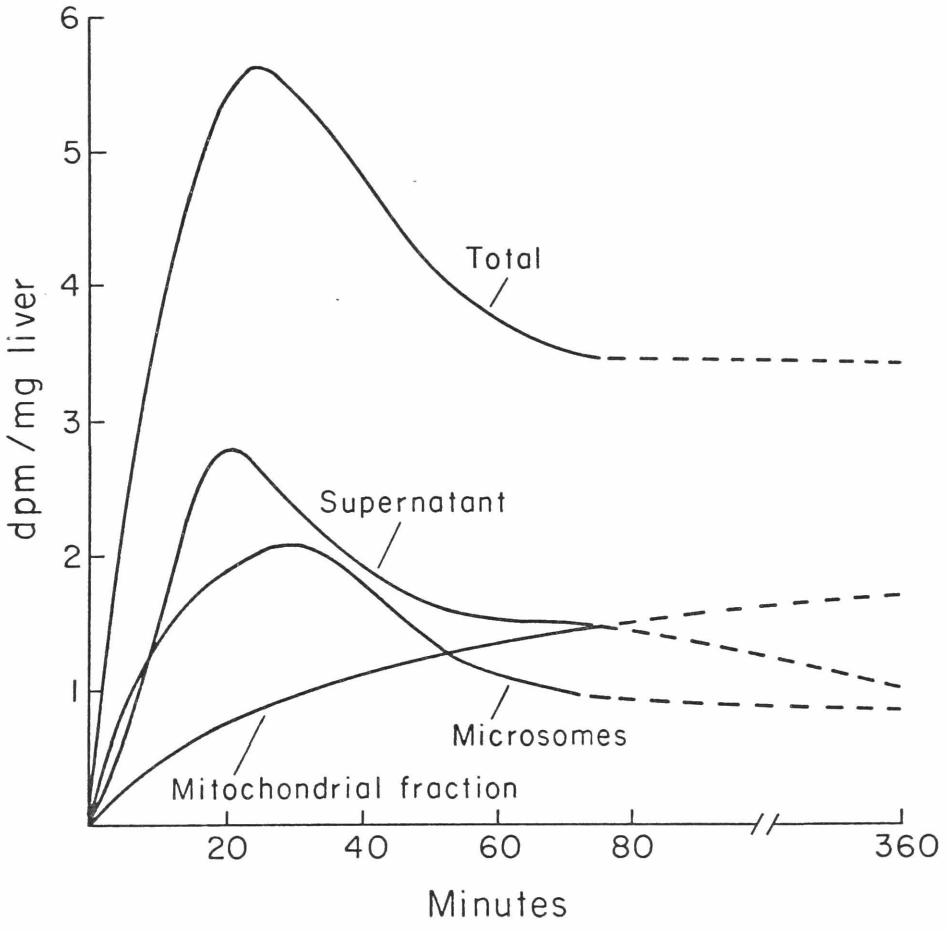


Figure 62. Data of Higashi and Peters (1963b).
A. Their own figure representing the data as specific activities.
B. Their data replotted by us as total activities per mg of liver.

supernatant fraction than in the microsomes at most times, except during the first minutes after administration of the precursor amino acid. The process is more protracted in the case of the Higashi and Peters experiments, but this is understandable since they injected the radioactive leucine in the tail vein and did not achieve as good pulse-chase conditions as did we. One important difference between the results of Higashi and Peters and ours does, however, become manifest when the evolution of the sum of the radioactivities in the three fractions is considered. It is seen to reach a maximum after 20 minutes and then to decrease by 40% during the following hour. Or, to put it differently, a considerable part of the radioactivity that is seen to accumulate in the microsomes and supernatant fraction during the first 20 minutes does not find its way into peroxisomes, but is lost. We do not see this phenomenon in our experiments, and it is not easily explainable, since there is no evidence that the liver secretes or otherwise loses a substantial part of the catalase it synthesizes. The liver does so, however, for serum albumin and other plasma proteins, following exactly the time course shown by the loss of "catalase" in the experiments of Higashi and Peters, and since there is evidence (see above) that their antiserum contained antibodies against plasma proteins, we are inclined to conclude that some 40% of the total immunoprecipitable label which they recover in their fractions 20 minutes after injection of the labeled leucine belong to plasma proteins, and not to catalase antigen. Should this amount remain entirely microsome-bound until it is secreted, it could account for practically all the radioactivity found at early times in the microsomal fraction. Incomplete depletion of this fraction after one hour or later probably reflects largely its content in small peroxisomes. In conclusion, the results of Higashi and Peters afford no undisputable proof of the participation of microsomes in catalase biosynthesis. They are, however, not incompatible with the possibility that, at early times after injection of labeled leucine, a modest amount of authentic labeled catalase antigen is associated with microsomes, and a distinctly larger amount with the supernatant fraction, which is exactly what we find.

Reference must further be made to a recent paper by Kashiwaga, Tobe and Higashi (1971). These authors claim that both free polysomes, and polysomes released from microsomes by detergent treatment, are capable of synthesizing catalase in vitro. Unfortunately, the adequacy of their immunochemical controls is dubious, since the authors present no evidence that the immunoprecipitation with an unrelated antigen and antibody which they perform prior to the specific immunoprecipitation with anticatalase, suffices to eliminate nonspecific precipitation in the latter step. Also, the specificity of the anticatalase is obviously crucial. Furthermore, there is no control on the quality of the separation of free and bound ribosomes. It is disturbing that these authors report that their free ribosomes, obtained by centrifugation through 2.0 M sucrose, were "further purified by 1.3% deoxycholate treatment as described above, followed by centrifugation at 244,500 x g for 1 hour to remove the contaminating membrane fraction." This suggests that the final preparation of "free" ribosomes may be contaminated with bound ribosomes.

The question of the site of catalase synthesis was also investigated by Takagi, Tanaka and Ogata (1970) by similar methods. However, these latter authors made careful immunochemical controls and also measured the labeling of serum albumin in their preparations. Unfortunately their anticatalase was made against commercial beef liver catalase and neither the purity of the catalase nor the specificity of the anticatalase was determined. Both free and bound polysomes appeared to incorporate some labeled leucine into catalase.

Thus three independent groups have implicated both free and bound ribosomes in catalase biosynthesis. However, none of these results (our own included) are compelling. We must simply defer judgement on this matter until better data become available.

It is remarkable that morphological observations confront us with a dilemma very similar to that posed by the biochemical results. Until recently, there was a fair consensus of opinion favoring the view that

peroxisomes arise as projections of the endoplasmic reticulum. First suggested by Rhodin (1963) and by Hruban et al. (1963), this theory received considerable support from careful observations by Novikoff and Shin (1964), who noted numerous images suggestive of connections between microbodies and endoplasmic reticulum, and even went so far as to consider the possibility that all microbodies are so attached. Several other workers have made similar observations (Svoboda and Azarnoff, 1966, 1967; Essner, 1967; Tsukada, 1968; Reddy and Svoboda, 1971), and it has been generally assumed on the basis of such images that the peroxisomal proteins, including catalase, are synthesized by membrane-bound ribosomes and delivered directly into the lumen of ergastoplasmic cisternae, from which they are then channeled through smooth-surfaced elements to the protruding peroxisomes. An involvement of the Golgi apparatus in this process has been suggested by Rouiller and Jézéquel (1963) and Bruni and Porter (1965), but has been denied by Novikoff and Shin (1964), and Tsukada et al. (1968). This postulated process thus resembles in its early stages the mechanism of synthesis and transport of secretory proteins, first unravelled by Palade and his coworkers for the exocrine pancreas (Siekevitz and Palade, 1958a, b, c, 1959, 1960a, b; Caro and Palade, 1964; Siekevitz and Palade, 1966; Redman, Siekevitz and Palade, 1966; Jamieson and Palade, 1967a, b, 1968a, b), and since shown to be of fairly general significance in both exocrine and endocrine secretion, and also in the formation of primary lysosomes.

Most proponents of the above theory have quoted the results of Higashi and Peters in support of it, discounting the apparent involvement of the supernatant fraction as a minor leakage artifact, and not realizing its actual quantitative importance. They have also drawn attention to the fact that, coincident with the period of maximum peroxisome formation in fetal liver at the end of the gestation period, the cisternae of the rough-surfaced endoplasmic reticulum are greatly dilated and filled with a material of about the same opacity as the microbody matrix (see the discussion of this problem and Fig. 9 of Palade, in de Duve and Baudhuin, 1966).

These arguments have come under strong criticism in a recent paper by Legg and Wood (1970), who have pointed out the weaknesses in them. These authors claim never to have seen the connections between microbodies and endoplasmic reticulum described by other workers. Furthermore, using the Graham and Karnovsky (1966) DAB staining technique for the visualization of peroxidase, as modified by Novikoff and Goldfischer (1969) for the visualization of catalase, they were unable to demonstrate reaction product within cisternae of the endoplasmic reticulum. On the other hand, when the method was applied to the livers of rats in which microbody proliferation had been stimulated by the administration of CPIB (p-chlorophenoxyisobutyrate), staining was observed on or near ribosomes attached to strands of endoplasmic reticulum closely apposed to microbodies. On the basis of these observations, the authors suggest that catalase synthesized by bound ribosomes may be released at the cytoplasmic side of the membrane, and may then enter peroxisomes by diffusion. In support of this new theory, they bring forth the biochemical results of Higashi and Peters!

At a recent meeting, Fahimi (1971) has proposed yet another theory, also based on DAB staining results, but obtained on regenerating liver. According to this theory, catalase is synthesized on bound ribosomes, released to the cell sap, and forms local aggregates around which a membrane is subsequently built de novo.

Unfortunately, we have not yet been able to devise an experiment capable of providing an unequivocal answer to the problem of the site of the biosynthesis of catalase. In our opinion, a participation of ER-linked ribosomes in the process, although not established beyond doubt, appears nevertheless very probable since it is supported by four distinct types of experiments: density gradient fractionation, high speed sedimentation, and subfractionation of microsomes after both in vitro and in vivo synthesis. We have obtained evidence that release of the biosynthetic product from the microsomes requires a mild detergent treatment, of the kind known to allow the contents of microsomal vesicles to leak out.

Since we have shown that the catalase intermediates associated with microsomes are transferred to peroxisomes, it seems not unreasonable to assume, in agreement with what might be termed the "classical" theory of microbody formation, that this transit takes place essentially intracisternally, either through the connections that many workers believe to exist between the endoplasmic reticulum and the peroxisomes or through intermittent connections. If such a process takes place, it follows from the kinetics of depletion of the microsomes that the system must be such as to allow the delivery of newly synthesized molecules to the peroxisomes to take place without systematic delay, and in a purely random fashion, irrespective of the age of the molecules. Two distinct models could theoretically account for these properties. In one, the intracisternal pool of intermediates is truly homogeneous, and connected to peroxisomes by short channels. In the other, there is no true intracisternal pool, and the randomness of transfer to peroxisomes reflects the statistical distribution of transit times or flux rates through the connections between endoplasmic reticulum and peroxisomes.

The alternative possibility that the catalase chains made by bound ribosomes are delivered into the cell sap, and then either diffuse into peroxisomes (Legg and Wood, 1970), or condense and later become enveloped by a membrane (Fahimi, 1971), has the virtue of providing an explanation for the high degree of labeling of the supernatant at the early time points. The theory contradicts the evidence of Sabatini and coworkers that most liver proteins that are synthesized on bound ribosomes are released intracisternally, and is not easily reconciled with the generally accepted assumption that cytomembranes are impermeable to proteins. But it would be unwarranted to reject the theory for these reasons in view of our ignorance concerning the site of synthesis of most cellular proteins and the manner in which they reach their final destination.

There are, however, experimental reasons for not accepting the theory. First, our results suggest that catalase is indeed released intracisternally. Second, our results are not compatible kinetically with the existence of a precursor-product relationship between the microsomes and the supernatant fraction (nor are those of Higashi and Peters [1963b], especially after correction for the alleged contamination by plasma proteins). As discussed in Chapter IX, the half-clearance time of the microsomes must be of the order of 10 to 15 minutes, close to the value of 12 minutes derived for the entire extra-peroxisomal pool. In other words, microsomes and supernatant behave as two parallel pools with about the same turnover rate, or as parts of the same pool, but not as two pools in series.

Another reason why we tend to reject the theory of Legg and Wood (1970) is that the evidence on which it is based is likely to be an artifact. According to our results, and to what is known of the structure of catalase, it is very unlikely that ribosome-bound, nascent catalase molecules contain heme and are enzymatically active. To attribute reaction product seen on or near ribosomes to the enzymatic action of such molecules is therefore almost certainly erroneous, and since such images were seen only in the immediate neighborhood of strongly stained peroxisomes, some sort of diffusion artifact seems most probable. As to the images of stained matrices incompletely surrounded by a membrane, interpreted by Fahimi (1971) as evidence of condensation of matrix proteins followed by membrane formation, they could as readily depict peroxisomes that were damaged upon fixation, and more probably do so.

If not the reflection of a soluble transit pool between microsomes and peroxisomes, what then is the significance of the early appearance in the supernatant fraction of a large part of the immunoprecipitable radioactivity derived from ^{3}H-leucine? Two possibilities deserve consideration. According to the first one, the label is located in the cell sap in the intact cell, and arises most probably from the synthetic activity of free polysomes. We have seen that

several authors have implicated free polysomes in catalase biosynthesis but that none of the evidence is convincing. Should confirmation of this theory be obtained, we would then be faced with the problem, already discussed above, of explaining how a protein present in the cell sap manages to become concentrated within a membrane-bounded structure. Note that this concentration would be considerable. We have seen that the intraperoxisomal pool of intermediates is twice the size of the extraperoxisomal pool, and we know that the volume of peroxisomes is only about one-twentieth the volume of the cell sap. The concentration factor would therefore be of the order of 40-fold. Furthermore, there is evidence that catalase, at least beef liver catalase, is made of four identical subunits (Schroeder, 1964). What then are the parts of the molecule made by free and those made by bound polysomes? Or, if they are the same, why isn't the catalase molecule completed in the cell sap, especially since it appears that heme addition can take place there? The objections are not overwhelming, in view of our ignorance of many of the factors involved, but they are serious.

The other possibility is that we are dealing with an artifact, and that the material in the supernatant fraction arises from ruptured microsomal vesicles. Were it so, we would understand the fact, emphasized in Chapter VIII, that the extraperoxisomal pool of intermediates behaves kinetically as a single homogeneous precursor pool of the intraperoxisomal pool. The excess of bound over soluble label observed during the first minutes after injection of the precursor amino acid does not contradict this hypothesis, since, as already mentioned, it can be explained by the binding of nascent chains to the ribosomes. The main objection against the explanation is that it presupposes an extreme mechanical fragility of the microsomes involved in the synthesis of catalase, leading to the release of as much as two-thirds of their contents upon homogenization. The microsomes that make serum albumin do not behave in this manner. On the other hand, the microsomes we would be dealing with must be different in some way, since

their products are not discharged extracellularly, but are segregated within peroxisomes.

It is obvious that our first concern in the future ought to be to try and distinguish between these two possibilities. Should our efforts prove fruitless, other approaches will have to be considered. Unfortunately, catalase synthesis is of minor quantitative importance in the protein economy of the liver cell. Its investigation by the autoradiographic techniques that have proved so successful in the unravelling of other similar processes does not appear feasible. Recourse to cytochemistry is not likely to be very useful either, since the catalase molecule probably has to be completed in order to be enzymatically active and cytochemically demonstrable. By that time, according to our results, it has reached the peroxisomes.

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