THE SYNTHESIS AND TURNOVER OF RAT LIVER PEROXISOMES

I. Fractionation of Peroxisome Proteins

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ABSTRACT

Rat liver peroxisomes isolated by density gradient centrifugation were disrupted at pH 9, and subdivided into a soluble fraction containing 90% of their total proteins and virtually all of their catalase, D-amino acid oxidase, L-α-hydroxy acid oxidase and isocitrate dehydrogenase activities, and a core fraction containing urate oxidase and 10% of the total proteins. The soluble proteins were chromatographed on Sephadex G-200, diethylaminoethyl (DEAE)-cellulose, hydroxylapatite, and sulfoethyl (SE)-Sephadex. None of these methods provided complete separation of the protein components, but these could be distributed into peaks in which the specific activities of different enzymes were substantially increased. Catalase, D-amino acid oxidase, and L-α-hydroxy acid oxidase contribute a maximum of 16, 2, and 4%, respectively, of the protein of the peroxisome. The contribution of isocitrate dehydrogenase could be as much as 25%, but is probably much less. After dissolution of the cores at pH 11, no separation between their urate oxidase activity and their protein was achieved by Sephadex G-200 chromatography.

INTRODUCTION

In a previous publication, a method has been described for the isolation of rat liver peroxisomes in relatively large amounts and in an acceptable state of purity (13). The present paper reports the results of various attempts to separate and characterize the individual protein components of such preparations. Subsequent papers of this series deal with their rate of turnover and synthesis.

EXPERIMENTAL PROCEDURES

Fractionations

The separation of peroxisomes from the livers of female rats injected 3½ days previously with Triton WR-1339 (0.85 g per kg body weight, intraperitoneally) was carried out by the two-step technique described by Leighton et al. (13). The first step utilized differential sedimentation to yield a λ fraction, in which the peroxisomes were purified about 5-fold with a 40% yield. The λ fraction was then subfractionated by isopycnic centrifugation in a sucrose gradient containing 5% of dextran-10. The whole procedure was checked by means of enzyme assays, and the gradient fractions containing high levels of peroxisomal enzymes together with negligible amounts of cytochrome oxidase (mitochondria), acid phosphatase (lysosomes), and glucose 6-phosphatase (microsomes), were pooled and used for the subsequent protein fractionation. The methods used for

disrupting the particles and extracting their protein components will be described in the text. The solubilized proteins were fractionated by means of column chromatography, usually after buffer exchange by dialysis or by passage through a column of Sephadex G-25 previously equilibrated with the desired buffer. The conditions of these experiments will be given in detail below.

Assays

The measurements of enzymic activities and, in some cases, of protein were carried out by means of the automated methods described previously (13). In the experiments with diethylaminoethyl (DEAE)-cellulose and hydroxylapatite, the eluting solvents used interfered with the automated Lowry method (14), and protein was estimated spectrophotometrically at 280 nm. Glycolate was used as substrate in the assay of L-α-hydroxy acid oxidase.

Materials

Sephadex preparations were purchased from Pharmacia Fine Chemicals Inc. (Piscataway, N.J.), suspended in water, and allowed to swell for at least 4 days at 4° before use. DEAE-cellulose was obtained from the Schleicher & Schuell Co. (Keene, N.H.). A 100–230 mesh fraction was separated by sieving, treated, and equilibrated with buffer according to Peterson and Sober (19). Hydroxylapatite (Bio-Gel HT) was purchased from Bio-Rad Laboratories (Richmond, Calif.).

Purification of Catalase

Catalase was purified from rat liver by a modification of the methods described by Greenfield and Price (8), Price et al. (20) and Higashi and Peters (10). 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. (6), was isolated from rat liver by differential centrifugation, and resuspended in a solution (2 ml per g liver) consisting of 220 ml of ethanol and 44 ml of 1 M sodium acetate buffer pH 4.1 diluted to 1 liter. After 30 min, the suspension was mixed vigorously with one-tenth its volume of chloroform, and left to stand for 10 min, following which it was centrifuged for 15 min 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-min 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-min 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 and Fig. 1 are shown the results of a purification experiment. As will be shown below (see Fig. 8 and Table VI), our purified material showed the typical spectrum of catalase, with mass extinction coefficients 1.58, 0.30, and 1.47 at 407, 312, and 276 nm, respectively. Comparable values of 1.68, 0.29, and 1.55 can be calculated from the data given by Greenfield and Price (8) and Price et al. (20) for their best preparations. It appears from these results that the purity of our material is comparable to that of the catalase preparations isolated by these authors, and also that bovine serum albumin represents a reliable standard for the determination of catalase protein by the method of Lowry et al. (14). Expressed in *Kat.f.* (*Katalase-fähigkeit*) units, our purified catalase has a specific activity of 51,000, which compares favorably with the values given in the literature for

TABLE I

Purification of Rat Liver Catalase

Catalase units are those defined by Beaufay et al. (3). Protein measured by the method of Lowry et al. (14) is expressed in terms of a bovine serum albumin standard.

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

* Practically equivalent to 10^{-3} *Kat. f.*

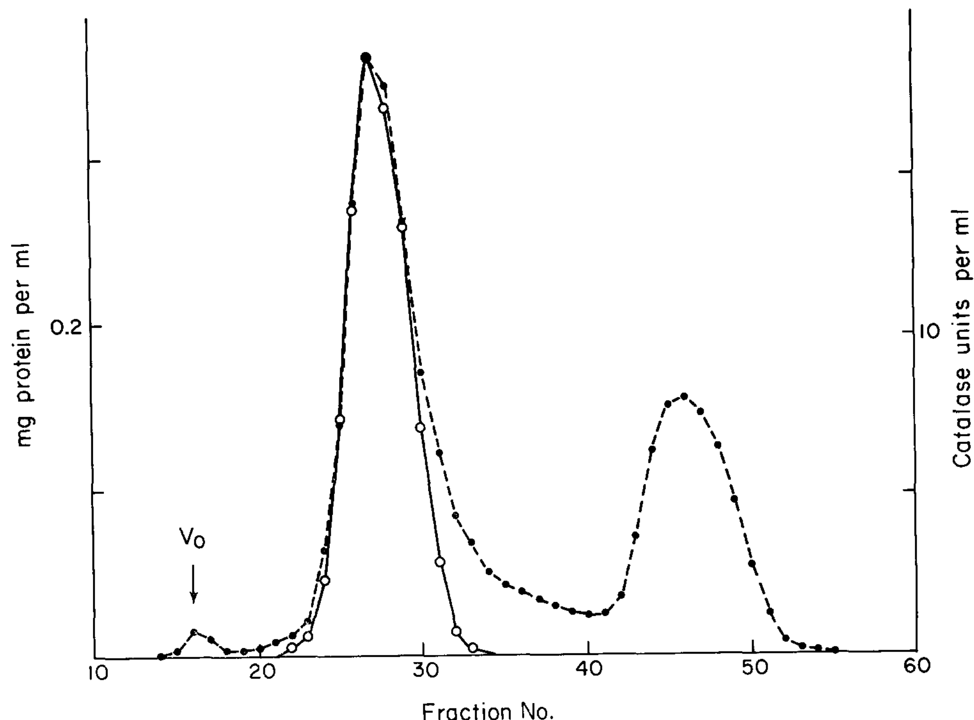

FIGURE 1 Final purification of catalase by gel filtration on Sephadex G-200 in a column 2.5 × 91 cm in 0.5 M NaCl, 0.1 M potassium phosphate pH 6.0, with upward flow at 20 ml/hr. A sample of 5 ml was applied to the column and 10 ml fractions were collected. Protein: ●------●. Catalase: ○——○. For definition of units see Table I.

crystalline hepatic catalase from other species (25), but which is 30% lower than the value of 73,000 reported by Price et al. (20) for rat liver catalase. These authors measured catalase at 22° and converted their results to *Kat. f.* units on the basis of an assumed Q_{10} of 1.1 (15). The discrepancy between their data and ours would be explainable if the Q_{10} of rat liver catalase were of the order of 1.3. However, preliminary tests run in our laboratory do not support this hypothesis. Nevertheless, some difference in assay procedure, or possibly in the strain of animals used, appears most likely to account for the discrepancy, since the range of activity reported by Price et al. (20) for the total catalase content of rat liver exceeds by 50–100% the average value found with our method both in Louvain (3) and in New York (13). The exclusive use in our work of female rats, which have less catalase in their livers than males (9), can account for only a small part of this difference. In any case, the results obtained in the two laboratories appear to be internally coherent, and on the basis of the spectral properties of our preparations, we feel that these represent relatively pure samples of the catalase present in the livers of our rats.

RESULTS

Properties of Purified Peroxisomes

In Table II are listed the enzymic properties of the purified peroxisome fractions used in this work. On the average, catalase, D-amino acid oxidase, and L-α-hydroxy acid oxidase, which are mainly or entirely associated with peroxisomes in the intact cells, are purified 35-fold with respect to the homogenate, with a 15% yield. A higher proportion of the total urate oxidase is recovered in the fractions, because isolated cores arising from damaged particles are separated together with intact peroxisomes. Isocitrate dehydrogenase, although present in the preparations at a 15-fold lower relative specific activity, is a true component of the particles; the larger part of the activity of this enzyme is located in the soluble fraction, another small but significant amount being associated with the mitochondria (13). The other enzymes measured are present in the preparations

TABLE II

Properties of Purified Rat Liver Peroxisomes

Values given are means ± SD.

Enzyme	No. of experiments	Liver content	Peroxisomes*		Over-all recovery‡
			Yield	RSA	
		units/g§	*% of liver*		%
Protein	10	238 ± 33	0.43 ± 0.11	1.00	96.7 ± 3.2
Catalase	10	47.7 ± 7.7	14.1 ± 3.9	32.6 ± 4.5	96.5 ± 3.8
D-amino acid oxidase	3	1.38 ± 0.29	12.1 ± 7.2	28.8 ± 8.6	105.4 ± 3.9
L-α-hydroxy acid oxidase	5	1.16 ± 0.32	16.9 ± 6.5	36.8 ± 8.2	98.8 ± 3.9
Urate oxidase	2	3.05 ± 0.35	22.2 ± 0.9	57.4 ± 19.9	74.8 ± 11.0
Isocitrate dehydrogenase	1	2.64	0.88	2.02	99.7
Cytochrome oxidase	10	25.9 ± 2.4	0.04 ± 0.03	0.09 ± 0.06	96.3 ± 8.7
Acid phosphatase	10	8.28 ± 1.3	0.22 ± 0.31	0.45 ± 0.56	94.0 ± 4.9
Glucose 6-phosphatase	8	15.2 ± 4.2	0.03 ± 0.03	0.07 ± 0.05	97.5 ± 4.6

* RSA = relative specific activity with respect to whole liver.

‡ Sum of activities of peroxisomes and of discarded fractions, as percentage of activity of homogenate.

§ Units = mg for proteins, units (13) for enzymes.

only in trace amounts, which indicates that the contamination by mitochondria, lysosomes, and microsomes is very small. These conclusions have been fully confirmed by quantitative morphological measurements (13).

Extraction of Peroxisomes

A number of procedures were tried for separating the soluble peroxisomal proteins from the insoluble crystalloid core. Detergents, which are known to be effective (26), were avoided since it was feared that they might interfere with the subsequent fractionation of the soluble proteins. Repeated freezing and thawing was found to cause a progressive release of catalase, D-amino acid oxidase and L-α-hydroxy acid oxidase, without any solubilization of urate oxidase (Fig. 2). However, when this treatment was applied in the absence of salts, it gave good results only with crude preparations, such as cytoplasmic extracts, but it was less effective with λ fractions. It released only a small proportion of the soluble enzyme activities from purified peroxisomes, and caused substantial inactivation of the oxidases if the preparations were too dilute. Searching for buffer solutions that would increase the efficacy of freezing and thawing, we found that the highest yield of soluble enzymes was obtained in relatively alkaline media, which already by themselves cause a considerable disruption of the peroxisomes. We finally adopted the procedure outlined in Fig. 3.

The particles, either fresh from a gradient or thawed after storage in the freezer, were extracted overnight in the cold with gentle stirring in 0.01 M sodium pyrophosphate buffer at pH 9.0. The pellet separated after this first extraction was then resuspended for several hours in the same buffer. The two supernatants were pooled and constituted the starting material for column chromatography. The pellet was washed once more and was then extracted further with 0.5 M NaCl containing 0.1 M sodium phosphate buffer pH 6.0, and 0.09% of Triton X-100, as a means of removing nonenzymic, probably membrane, protein from the urate oxidase-bearing cores.

Table III gives the recoveries of protein and of enzymic activities in the various subfractions. Those designated S_3 and S_4 contain very little protein and they were not subjected to any further study after the initial biochemical measurements. The fraction S_2 contains 85–90% of the soluble enzymes and about 70% of the total protein, and is entirely devoid of urate oxidase activity. All the recovered activity of this enzyme is found in the final pellet P_4, together with about 15% of the total protein and with negligible amounts of the

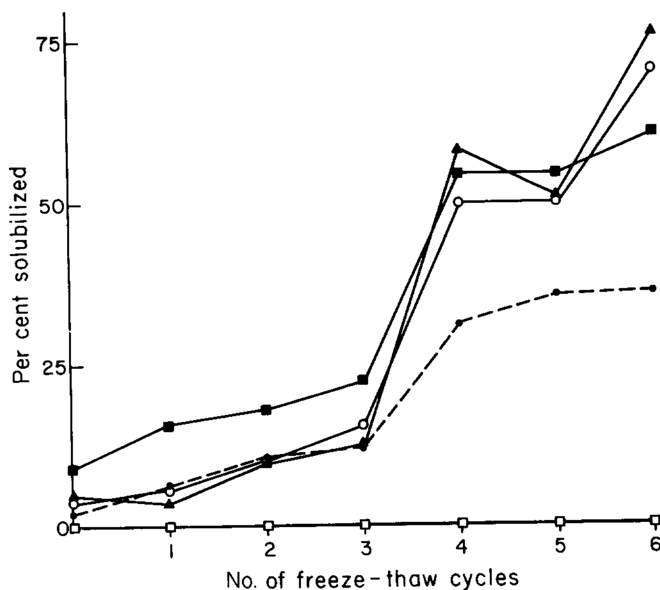

FIGURE 2 Effect of freezing and thawing of the λ fraction in 0.25 M sucrose on the solubility of the enzymes. Freezing in dry ice—ethoxyethanol and thawing in water bath at 37° with gentle agitation. Protein. ●-----●. Catalase: ○——○. L-α-hydroxy acid oxidase: ▲——▲. D-amino acid oxidase: ■——■. Urate oxidase: □——□.

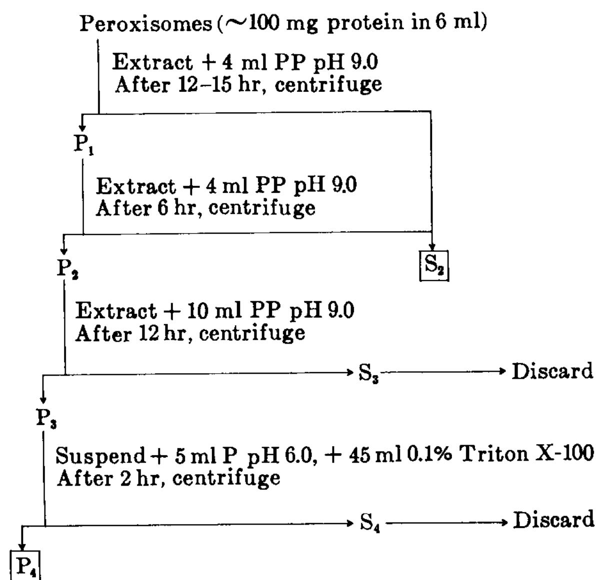

FIGURE 3 Scheme for peroxisome subfractionation. PP: 0.01 M sodium pyrophosphate buffer pH 9.0. P: 0.5 M NaCl containing 0.1 M sodium phosphate buffer pH 6.0.

soluble enzymes. Unfortunately, the procedure leads to a substantial inactivation of urate oxidase.

The results of Table III cannot be taken to reflect precisely the partition of the peroxisomal proteins between core and matrix, since our preparations contain isolated cores arising from damaged particles, in addition to intact peroxisomes (13). From the excess of urate oxidase over the other enzymes (Table II), we may estimate that about one-third of the proteins of the P_4 fraction must originate from isolated cores. It follows that in intact peroxisomes, the core contains about 10%, and the matrix 90%, of the total proteins.

Fractionation of the Soluble Matrix Proteins

The elution pattern of S_2 proteins recorded with four different fractionation systems are shown in Figs. 4–7. In Table IV are listed the amounts of protein and of enzymes recovered from the columns, together with the highest specific enzyme

TABLE III

Fractionation of Peroxisomes

Results are given as percentage of the content of the peroxisome fraction ± SD.

Enzyme	No. of experiments	S_2	S_3	S_4	P_4	Final recovery
Protein	7	71.0 ± 5.7	3.8 ± 2.0	7.8 ± 4.7	14.6 ± 1.9	97.2 ± 5.0
Catalase	7	89.1 ± 11.7	7.4 ± 5.2	5.8 ± 4.6	0.2 ± 0.3	102.5 ± 4.1
D-amino acid oxidase	7	91.8 ± 13.4	10.6 ± 5.3	9.9 ± 7.7	1.3 ± 0.9	113.8 ± 9.7
L-α-hydroxy acid oxidase	7	84.7 ± 13.4	10.6 ± 3.8	12.6 ± 11.6	1.2 ± 0.7	109.0 ± 10.2
Isocitrate dehydrogenase	1	106.1	0.1	0.0	0.0	106.2
Urate oxidase	1	0.0	0.0	0.0	35.2	35.2

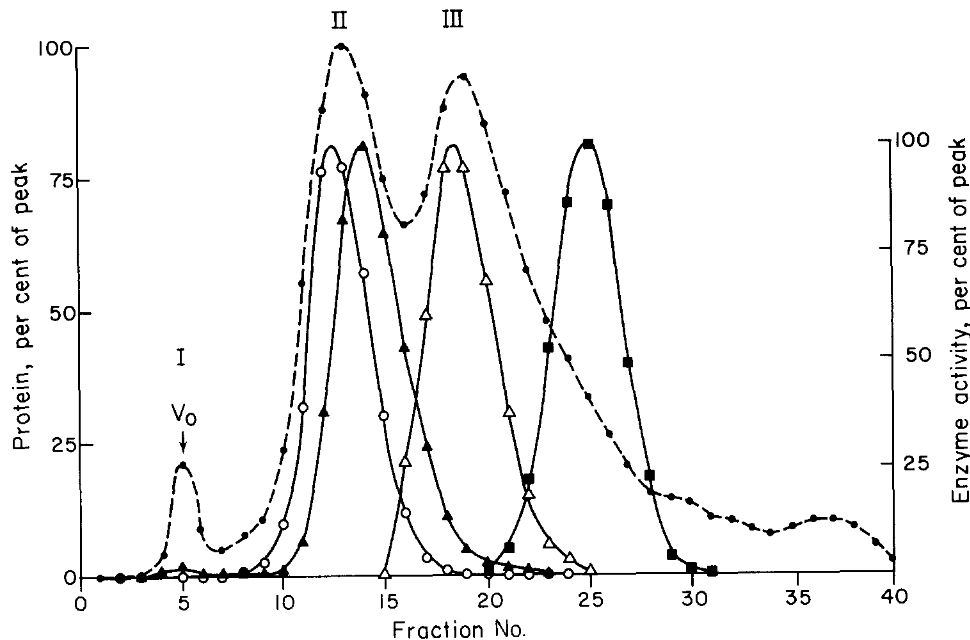

FIGURE 4 Gel filtration of S_2 fraction on Sephadex G-200 in a column 2.5 × 91 cm in 0.1 M potassium phosphate, 0.5 M NaCl, 0.1% ethanol, pH 6.0, with upward flow at 20 ml/hr. A sample of 5 ml was applied to the column and 10 ml fractions were collected. Protein: ●------●. Catalase: ○——○. L-α-hydroxy acid oxidase: ▲——▲. D-amino acid oxidase: ■——■. Isocitrate dehydrogenase: △——△.

activities found in any fraction. The recoveries were satisfactory only with the Sephadex G-200 system, which was adopted for our subsequent turnover experiments and will be examined in greater detail. The other three systems proved unsuitable for routine work, but provided partial information relevant to the interpretation of the results. A number of other systems were tried, but with no additional advantage, and the results obtained with them will not be mentioned.

With Sephadex G-200 (Fig. 4), a small amount of protein with no detectable enzymic activity emerged from the column at the void volume (peak I). The rest of the protein came out in two overlapping peaks, the second of which had a long tail. The catalase activity emerged with the first

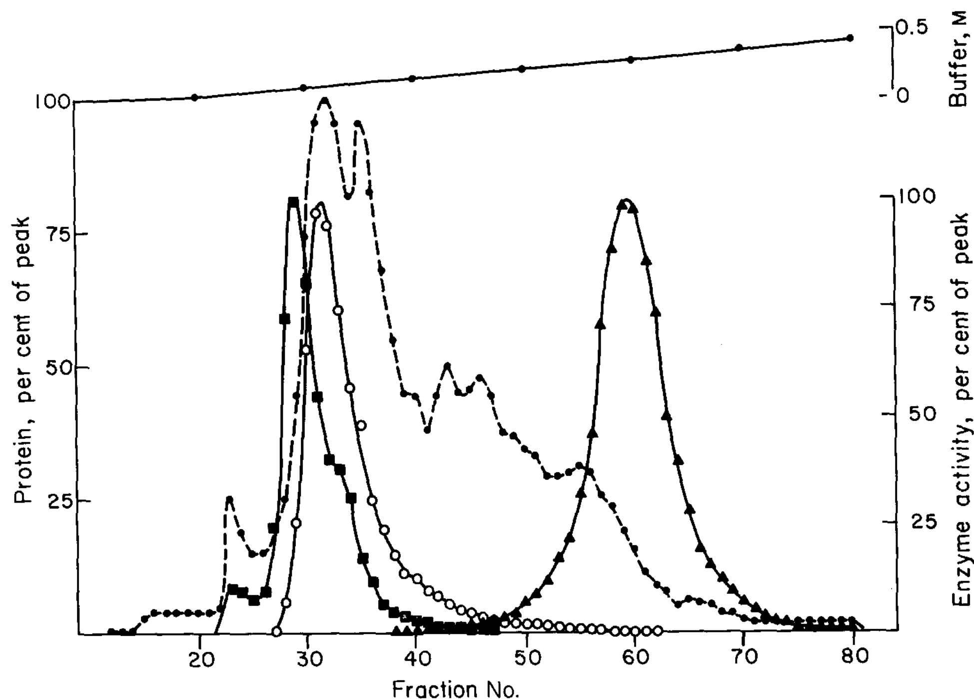

FIGURE 5 Chromatography of S_2 fraction on hydroxylapatite in a column 1.7 × 20 cm equilibrated with 0.001 M K_2HPO_4-NaH_2PO_4, 0.1 M NaCl, pH 6.8. Elution with a gradient of phosphate concentration of 1 mM/ml at a rate of 20 ml/hr, collecting fractions of 10 ml. Effluent molarity was determined from electrical conductance. Protein: ●------●. Catalase: ○——○. L-α-hydroxy acid oxidase: ▲——▲. D-amino acid oxidase: ■——■.

main peak of protein (peak II) and had a high, rather constant, specific activity on the leading edge of its peak. L-α-hydroxy acid oxidase followed closely after the catalase. The second broader peak of protein (peak III) almost coincided with the activity of isocitrate dehydrogenase though the protein shows much more trailing. D-amino acid oxidase emerged later on the tail of peak III.

In Table V are shown the amino acid compositions of pooled catalase-rich fractions from peak II and of the top fractions from peak III, together with that reported by Higashi and Shibata (11) for purified rat liver catalase. Some spectral properties of the catalase peak and of our purified catalase sample are illustrated in Figs. 8 and 9. Taken together, our data allow the following conclusions: Catalase, which in the homogenate has a specific activity of 0.2 unit per mg protein (Table II), is purified 40-fold in the S_2 fraction (Tables II and III), to a specific activity of 8 units per mg. This is 15% of the specific activity of our purified material (Table I), indicating that catalase can represent no more than 15% of the total proteins of the S_2 fraction. As shown in Table IV, in none of our fractionation systems do we reach complete purification of the enzyme. The best fractions recovered from the Sephadex G-200 column were 40–70% pure, depending on the experiment. The enzyme in these fractions was recovered with little loss, and was obviously in the native, fully active state. Its activity per unit absorbance at 407 nm was essentially the same as that of our purified sample (Fig. 8), and the iron in it suffered no appreciable reduction upon addition of dithionite (Fig. 9), a property characteristic of native catalase (7). Furthermore, its activity per tyrosine residue was also the same as that of pure catalase, as shown by the data of Table V. The sample of peak II for which analytical results are given in this Table had a specific activity of 21.2 units per mg and was therefore 40% pure. It had 40% of the tyrosine content of pure catalase.

Our results give some information on the properties of the main contaminant accompanying cata-

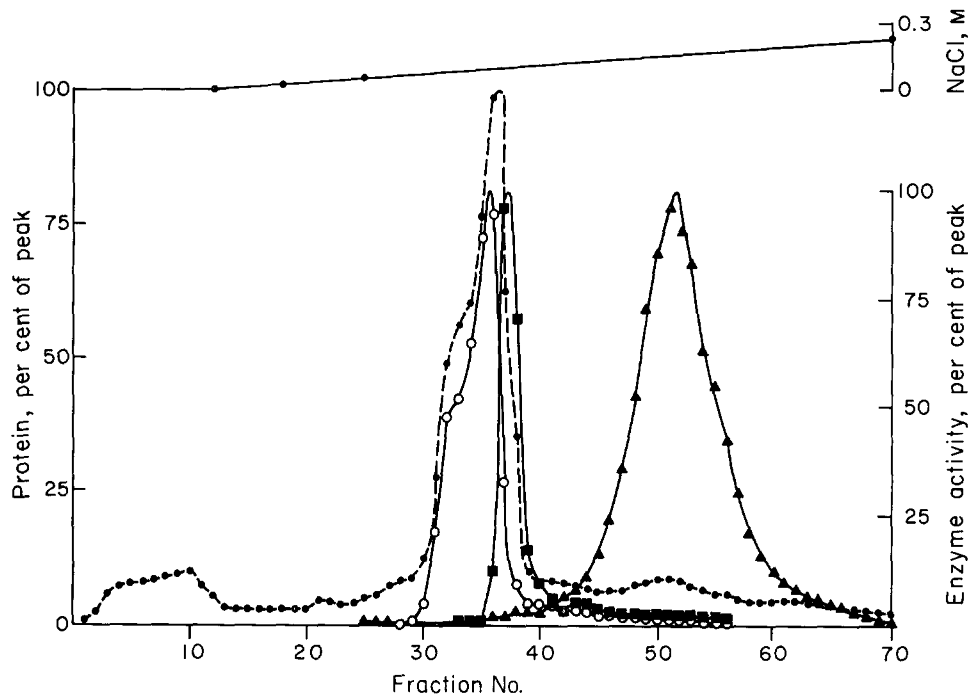

FIGURE 6 Chromatography of S_2 fraction on DEAE-cellulose in a column 1.5 × 36 cm in 0.01 M Veronal buffer, pH 7.8. Elution at 50 ml/hr with a gradient of NaCl concentration, collecting fractions of 10 ml. Effluent molarity was determined from electrical conductance. Protein: ●------●. Catalase: ○——○. L-α-hydroxy acid oxidase: ▲——▲. D-amino acid oxidase: ■——■.

lase through the Sephadex G-200 column. It must, like catalase, have a molecular weight of the order of 250,000; it is practically devoid of tyrosine, and has a low methionine content, of the order of 0.3 mole per cent (Table V). It cannot be a hemoprotein, since essentially all the absorbance at 407 nm of the catalase-rich fractions is accounted for by catalase itself (Fig. 8), and it must have a low absorbance at 276 nm relative to its ability to react with the Lowry reagent. Taking the absorbance at 407 nm as reference, we calculate from the excess absorbance at 276 nm of the catalase-rich sample referred to in Fig. 8 and Table VI that this sample contains only 18% of noncatalase protein, as compared to the value of 30% in terms of Lowry protein derived from the specific activity. An even lower value was obtained in another experiment, in which the estimated contamination was 18% in terms of absorbance at 276 nm, against 60% in terms of Lowry protein. Thus, the absorbance at 276 nm per unit Lowry protein of the contaminant is 2 to 3 times lower than that of catalase. This property suggests that in addition to having essentially no tyrosine, the contaminant is likely to be poor also in tryptophan. Since aromatic amino acids enhance the color given by the Lowry reagent, the true degree of contamination of the catalase peak may actually be higher than has been estimated from the specific activity of the enzyme.

L-α-hydroxy acid oxidase, which is poorly resolved from catalase, could be responsible for the decrease in specific activity of catalase on the descending side of peak II. However, the other data make this unlikely. Both on hydroxylapatite (Fig. 5) and on DEAE-cellulose (Fig. 6), the emergence of this enzyme is associated with only a slight hump on the protein curve. From Fig. 6 we may estimate that L-α-hydroxy acid oxidase is at best 50% pure in its peak fraction, which is purified 14-fold over the S_2 fraction (Table IV). Thus, L-α-hydroxy acid oxidase can represent little more than 3% of the total proteins of the S_2 fraction.

Peak III from the Sephadex G-200 column coincides fairly well with the isocitrate dehydrogenase peak. Unfortunately we have no measurements of this enzyme in other systems which could help us

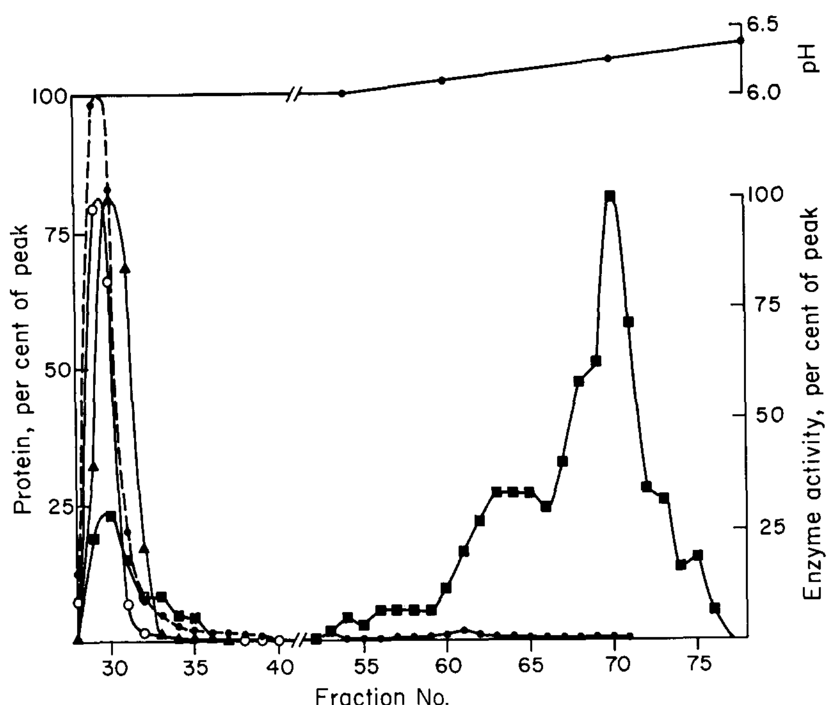

FIGURE 7 Chromatography of S_2 fraction on SE-Sephadex C-25 in a column 2.5 × 90 cm equilibrated with 0.1 M sodium phosphate buffer, pH 6.0, then eluted with a gradient of pH at constant phosphate concentration. The flow rate was 13.2 ml/hr downward and fractions of 4.4 ml were collected. Protein: ●------●. Catalase: ○——○. L-α-hydroxy acid oxidase: ▲— —▲. D-amino acid oxidase: ■——■.

TABLE IV

Chromatography of S_2 Fraction

Chromatographic system		Protein	Catalase	D-amino acid oxidase	L-α-hydroxy acid oxidase	Isocitrate dehydrogenase
Sephadex G-200* (Fig. 4)	Purification‡	—	4.14 ± 0.74 (12)	9.95 ± 2.56 (26)	4.00 ± 0.45 (14)	3.81 (18)
	% Recovery	97 ± 6	80 ± 12	76 ± 25	84 ± 4	94
Hydroxylapatite (Fig. 5)	Purification‡	—	3.74 (31)	5.65 (28)	5.89 (62)	–
	% Recovery	130	75	78	41	–
DEAE-cellulose (Fig. 6)	Purification‡	—	2.73 (35)	7.90 (39)	14.0 (51)	–
	% Recovery	77	89	75	68	–
SE-Sephadex (Fig. 7)	Purification‡	—	4.92 (29)	51.2 (70)	1.46 (32)	–
	% Recovery	48	112	17	43	–

* Average of 7 experiments ± SD.

‡ Highest purification achieved in any given fraction; the number of this fraction is given between parentheses.

TABLE V

Amino Acid Composition of Fractions from Sephadex G-200 Filtration

Fractions obtained as in Fig. 4 were pooled, hydrolyzed in 6.0 N HCl for 20 hr at 110° in sealed evacuated tubes, and analyzed for amino acids with a Beckman Model 120 C amino acid analyzer by the method of Spackman et al. (24). Peak III is the sum of fractions 18–20; the leading edge of catalase peak is the sum of fractions 10–12 and had a specific activity of 21.2 units per mg protein. Results are expressed in moles per cent. Analyses kindly performed by Mr. E. Gall.

	Peak III	Leading edge of catalase peak	Purified catalase*
Lysine	6.13	6.14	5.83
Histidine	2.59	2.78	3.61
Arginine	5.03	6.56	5.62
Aspartic Acid	8.48	12.50	12.75
Threonine	4.95	4.15	4.81
Serine	6.54	6.18	4.67
Glutamic Acid	15.40	10.42	10.28
Proline	5.65	6.37	7.84
Glycine	9.39	8.97	6.64
Alanine	8.39	8.95	7.53
Valine	7.28	6.27	6.59
Methionine	0.30	1.26	2.32
Isoleucine	3.72	4.32	3.74
Leucine	9.90	7.85	6.10
Tyrosine	1.70	1.42	3.56
Phenylalanine	4.68	4.96	5.70

* Data taken from Higashi and Shibata (11).

to determine whether isocitrate dehydrogenase activity belongs to a major or a minor component of peak III. According to the maximum degree of purification achieved (Table IV), it can account for no more than 25% of the total proteins of S_2, but could of course represent much less.

Attempts to characterize further the components of peak III were thwarted by solubility problems. Most of the protein recovered in the peak III fractions showed a strong tendency to precipitate out of solution, and its concentration or further subfractionation could not be accomplished. We suspect that the properties of this component may be responsible for the deficient protein recoveries from DEAE-cellulose, and especially from SE-Sephadex (Table IV), and also for the irregular trailing of the proteins eluted from the hydroxylapatite column (Fig. 5). As shown in Table V, peak III has an amino acid composition not very different from that of catalase, except that it contains more glutamic and correspondingly fewer aspartic acid residues, and has a relatively low content in methionine and tyrosine.

D-amino acid oxidase emerged last from the Sephadex G-200 column (Fig. 4), forming a clean symmetrical peak associated with only a slight decrease in the protein slope. Thus, this enzyme probably represents only a small fraction of the S_2 proteins. This conclusion is confirmed by the results obtained on SE-Sephadex (Fig. 7). This system gave very poor resolution, except that it allowed a more than 50-fold purification of D-amino acid oxidase over the S_2 fraction. Although the yield was very low, we may take it from these results that D-amino acid oxidase accounts for 2% or less of the S_2 proteins.

Fractionation of the Core Proteins

This fraction was found to be soluble in 0.1 M sodium carbonate buffer at pH 11.0. The solution was chromatographed on a Sephadex G-200 column equilibrated with the same buffer, and the elution pattern obtained is illustrated in Fig. 10. This procedure effects very little increase in the specific activity of urate oxidase. Except for a small amount of protein which left the column at the void volume, the elution patterns of protein and urate oxidase activity coincided very closely.

DISCUSSION

The information provided by our results with respect to the protein composition of peroxisomes is summarized in the first column of Table VII. This information is transposed to the whole liver in the second column of Table VII, on the basis of the value of 2.5% given by Leighton et al. (13) for the proportion of total liver protein associated with peroxisomes. Combining these data with the absolute enzyme levels listed in Table II, we estimate minimum values for the specific activities of the pure enzymes, for comparison with available data from the literature (3rd and 4th columns of Table VII). Unfortunately, catalase is the only peroxisomal enzyme that has been isolated in pure form from rat liver, and the other comparisons have to be made with enzymes from other sources.

According to our data, catalase accounts for about 16% of the total peroxisomal proteins, or 0.4% of the liver proteins. This is considerably less

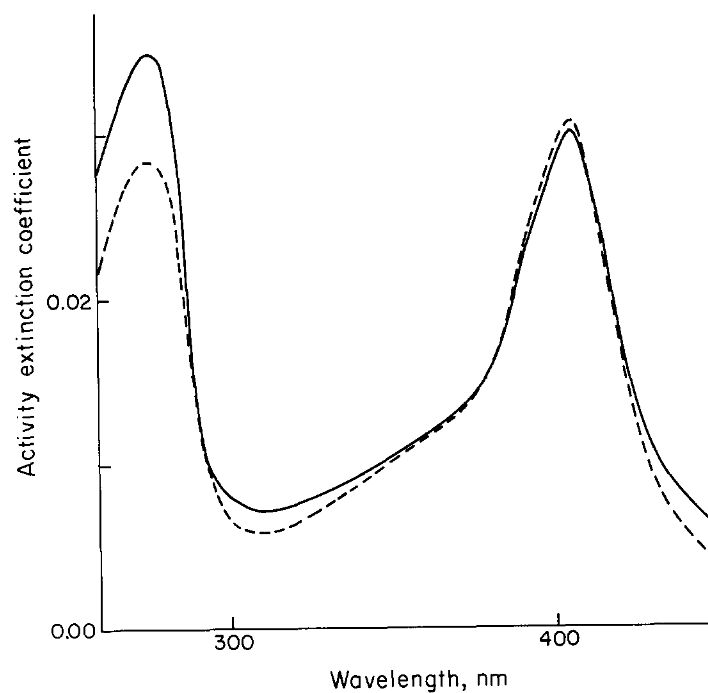

FIGURE 8 Spectrum of protein in the leading edge of the catalase peak after Sephadex G-200 chromatography of soluble peroxisomal protein. Solid line: catalase peak from chromatography. Dotted line: reference spectrum of purified catalase. Activity extinction coefficient = absorbance per centimeter of a solution containing 1 unit per ml of catalase activity. The chromatographic sample had a specific activity of 35 units of catalase activity per mg protein (see Table VI).

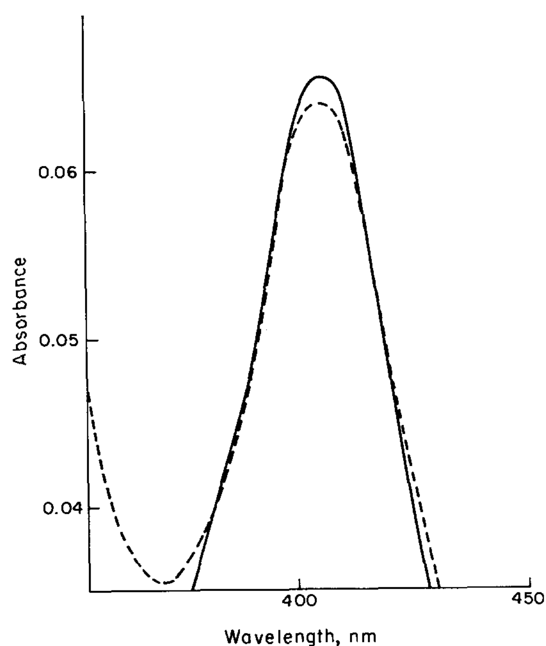

FIGURE 9 Soret band of catalase separated from peroxisomal proteins on Sephadex G-200. Solid line: initial spectrum. Dotted line: spectrum after the addition of a few small crystals of dithionite.

TABLE VI

Comparison of Catalase Peak from Sephadex G-200 Chromatography with Purified Catalase

Catalase preparation	Specific activity	Activity extinction coefficient* 407 *nm*	Mass extinction coefficients‡ 407 *nm*	312 *nm*	276 *nm*	$\frac{\epsilon_{407}}{\epsilon_{276}}$
	units/mg					
Purified (Table I)	52	0.0306	1.58	0.30	1.47	1.075
Fraction 12 (Fig. 4)	35	0.0300	1.05	0.25	1.22	0.86

* Absorbance per cm of a solution containing 1 unit of catalase activity per ml.
‡ Absorbance per cm of a solution containing 1 mg (Lowry) protein per ml (ϵ).

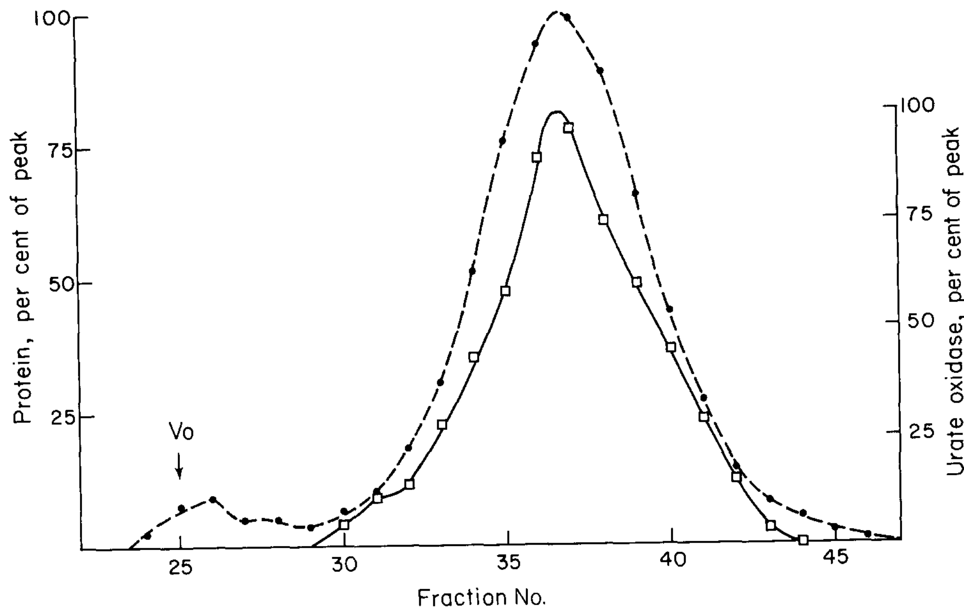

FIGURE 10 Gel filtration of P_4 fraction on Sephadex G-200 in a column 2.5 × 73 cm in 0.1 M sodium carbonate buffer pH 11.0 with upward flow at 17 ml/hr. A sample of 4 ml was applied and 5.6 ml fractions were collected. Protein: ●------●. Urate oxidase: □——□.

than the estimate of 40% of the peroxisome proteins given in earlier publications (2, 5). However, the previous calculations rested on very indirect data. The values given in Table VII for catalase are based on our observed value of 52 units per mg protein for the specific activity of the pure enzyme. If we accept the *Kat. f.* value of 73,000 (about 75 of our units per mg protein) reported by Price et al. (20) for their purest catalase preparations, then catalase makes up little more than 11% of the peroxisome proteins, or 0.25% of the total liver proteins (about 0.6 mg per g liver). As discussed above, it is not certain that the lower specific activity measured on our preparation reflects incomplete purity.

Our estimate of 12 units per mg protein for the specific activity of D-amino acid oxidase gives only the order of magnitude, since it is derived from measurements on samples with a very low protein content which, in addition, were separated with considerable over-all losses of both protein and enzyme activity. Even so, our value is comparable to that observed by Massey et al. (17) on the crystalline enzyme from hog kidney, indicating clearly that D-amino acid oxidase represents only a very small fraction of the peroxisome proteins. The

TABLE VII

Protein Composition of Peroxisomes

Component	Per cent of total proteins of		Specific activity of pure enzyme	
	Peroxisomes	Liver	Minimum	From literature
			units/mg protein	
Matrix	90	2.25		
Catalase	≤16	≤0.4	52	73*
D-amino acid oxidase	≤2	≤0.05	12	25‡
L-α-hydroxy acid oxidase	≤3	≤0.07	7	0.6§
Isocitrate dehydrogenase	≤25	≤0.6	0.1	61‖
Others	≥44	≥1.1		
Core	10	0.25		
Urate oxidase	≤10	≤0.25	5	20¶
Others	≥0	≥0		

* Purified enzyme from rat liver (20).
‡ Crystalline enzyme from hog kidney (17).
§ Crystalline enzyme from rat kidney (18).
‖ Partly crystalline enzyme from hog heart (23).
¶ Purified enzyme from hog liver (16).

remarkable resolution of this enzyme on SE-Sephadex under the conditions chosen deserves to be underlined (Fig. 7). Even uncorrected for losses, the purification ratio exceeds 50. It would reach a value of 300 if inactivation of the enzyme in the dilute fractions obtained from the column were responsible for its low recovery.

According to our evaluation, the specific activity of rat liver L-α-hydroxy acid oxidase is at least 10 times greater than that of the crystalline enzyme isolated from rat kidney by Nakano et al. (18). It is also much greater than the values of 0.4 and 1.1 units per mg protein which can be derived from the data obtained by Robinson et al. (21) on two comparable enzymes from hog kidney. However, there can be little doubt about our estimate. The specific activity of the enzyme in the purest fractions from the DEAE-cellulose column was 3.6 units per mg protein, and it is obvious from the elution pattern of the proteins (Fig. 6) that the enzyme was no more than about 50% pure in these fractions. Since, in addition, the recovery of enzyme activity is only 68% (Table IV), the specific activity of the pure enzyme could be as high as 10 units per mg, comparable to that of D-amino acid oxidase. Therefore, it must be concluded that the L-α-hydroxy acid oxidase of liver is much more active than the kidney enzyme, and represents a trace component in the peroxisomes.

Isocitrate dehydrogenase poses a more serious problem. According to the limited data that we have available, the enzyme could make up as much as 25% of the total peroxisome proteins. However, the corresponding specific activity is 600 times lower than the value reported by Siebert et al. (23) for the purified enzyme from hog heart, and isocitrate dehydrogenase may well account for a much smaller proportion of the total peroxisome proteins.

In any event, our results make it clear that a considerable fraction of the soluble peroxisome proteins remain unaccounted for after allowance is made for their known enzyme components. These unknown proteins make up between one-half and two-thirds of the total peroxisome proteins.

The relation of urate oxidase to the peroxisome core has been discussed in a number of publications. Except for one report of a possibly aberrant case (4), comparative studies have indicated that the peroxisomes lack a crystalloid core whenever urate oxidase is absent (1, 22). The suggestion has even been made that the peroxisome core may consist exclusively of urate oxidase, in some kind of crystalline form (12). This opinion has been challenged by Baudhuin et al. (2), who have pointed out that isolated cores have only one-tenth the specific urate oxidase activity of the purified prepa-

rations of this enzyme obtained by Mahler et al. (16) from hog liver.

Our present results do not settle the argument, but they weaken to some extent the objection formulated by Baudhuin et al. (2). In the first place, they show that if the cores contain other protein components besides urate oxidase, these components cannot be dissociated from the enzyme by Sephadex G-200 chromatography (Fig. 10). They indicate further that the low specific activity of isolated cores is due in part to losses of enzyme activity incurred in the course of isolation. As shown in Table VII, if such losses were avoided, the enzyme would be purified 400-fold in the cores, with a specific activity of 5 units per mg. Owing to inactivation of the enzyme, we reach only about one-fourth this value. Similar losses presumably occur with the procedure of Tsukada et al. (26, 27) since their best core preparations are purified only 140-fold over the homogenate.

However, our corrected value of the specific activity of urate oxidase in the peroxisome core is still well below that observed by Mahler et al. (16) on the purified hog liver enzyme. Unless a species difference is involved, it would appear that urate oxidase contributes no more than 25% of the protein of the cores and that the cores contain other components having about the same molecular weight as urate oxidase. Also, it is a little difficult to visualize how a single protein could form the complex polytubular structure characteristic of the peroxisome core.

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