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Tissue Fractionation Studies

17. INTRACELLULAR DISTRIBUTION OF MONOAMINE OXIDASE, ASPARTATE AMINOTRANSFERASE, ALANINE AMINOTRANSFERASE, D-AMINO ACID OXIDASE AND CATALASE IN RAT-LIVER TISSUE*

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Conflicting reports exist in the literature with respect to the intracellular localization of catalase, D-amino acid oxidase and monoamine oxidase in liver.

Studies performed by a number of different authors indicate that catalase is associated partly with cytoplasmic particles and partly with the supernatant fraction, the ratio of particulate to soluble activity depending on the species and sex of the animal and on the nature of the medium adopted for preparing the homogenate (Ludewig & Chanutin, 1950; Nyberg, Schuberth & Änggård, 1953; Thomson & Mikuta, 1954; Greenfield & Price, 1956; Thomson & Klipfel, 1957; Adams & Burgess, 1957; Adams, 1959; Datta & Shepard, 1959; Mason, Chin, Li & Ziffren, 1960; Miller, 1962; Higashi & Peters, 1962; Peters & Higashi, 1963). Most workers believe that the particles that contain catalase are mitochondria, but this view has been brought into question by the results of Thomson & Klipfel (1957), who have shown that catalase sediments closely together with urate oxidase in homogenates of mouse liver centrifuged through a stabilizing density gradient. In view of this observation and of the finding that the activity of particulate catalase is enhanced by Triton X-100 (Adams & Burgess, 1957), Feinstein (1959) has put forward the hypothesis that the enzyme may be a component of the lysosomes.

A mitochondrial localization has also been reported for D-amino acid oxidase in rat liver (Dianzani, 1954), but Paigen (1954) has found in subfractionation experiments that this enzyme comes down predominantly with the lighter mitochondrial fractions, in contrast with succinoxidase which is more abundant in the heavier fractions.

According to Cotzias & Dole (1951) and Zile & Lardy (1959), monoamine oxidase belongs essentially to the mitochondria in rat liver, whereas Hawkins (1952) and Zeller, Barsky & Berman (1955) have found the enzyme to be associated also partly with the microsomal fraction.

In view of these discrepancies, it was decided to re-investigate the distribution of these three enzymes, by using the centrifugation scheme specially developed by Appelmans, Wattiaux & de Duve (1955) for the purpose of unravelling complex enzyme distributions.

In two experiments, assays were also made of aspartate aminotransferase and of alanine aminotransferase. The former enzyme has been found to be unevenly distributed between the mitochondrial fraction and the final supernatant (Müller & Leuthardt, 1950; Hird & Rowsell, 1950; Rowsell, 1956; May, Miyazaki & Grenell, 1959; Gaull & Villee, 1960; Rosenthal, Thind & Conger, 1960;

* Part 16: Lejeune, Thinès-Sempoux & Hers (1963).

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Eichel & Bukovsky, 1961), whereas the latter has been shown to be present mainly in the soluble fraction, a small amount of activity being possibly associated with the mitochondria (Rowsell, 1956; Kafer & Pollak, 1961).

Some of the results described in the present paper have already been briefly reported (de Duve, 1960; de Duve *et al.* 1960; de Duve, Beaufay & Baudhuin, 1963).

EXPERIMENTAL

Tissue fractionations

The experiments were performed on adult albino rats of either sex, kept without food for a short period (12 hr.) to deplete the hepatic glycogen stores. The livers were homogenized in ice-cold 0·25 M-sucrose and fractionated according to the method described by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). This method differs from the classical one mainly by the fact that the large cytoplasmic granules are isolated in two successive steps, yielding a heavy mitochondrial fraction, M, containing 80–90% of the nitrogen and cytochrome-oxidase activity generally associated with the mitochondrial fraction in the conventional procedure, and a light mitochondrial fraction, L, containing the remainder of these components. The advantage of this scheme is that non-mitochondrial cytoplasmic particles, such as the lysosomes and the particles containing urate oxidase, which are brought down largely together with the mitochondria when the conventional scheme is followed, are partially resolved from the latter granules thanks to their somewhat slower sedimentation rate which causes them to be 5–10 times as concentrated in fraction L as in fraction M. Thus enzymes associated with such particles can be easily distinguished from those belonging to the true mitochondria. One drawback is the delicate decantation step whereby the supernatant is separated from the loosely packed M fraction. This step, which determines in a critical fashion the partition of components between the M and L fractions, is not easily reproducible, even by the same worker, and represents an important source of variability. Another factor responsible for differences between individual experiments is the variable contamination of the nuclear fraction with cytoplasmic components and of the mitochondrial fractions with microsomal elements. To obviate these disadvantages, the fractions were assayed systematically for cytochrome oxidase, acid phosphatase, urate oxidase and glucose 6-phosphatase, which served as reference enzymes for their respective host particles, mitochondria, lysosomes, 'microbodies' (Baudhuin & Beaufay, 1963) and microsomal membrane fragments, according to the general procedure adopted in previous publications from this Laboratory. The rationale behind this procedure has been analysed in detail by de Duve (1964).

Enzyme assays

Reference enzymes. Cytochrome oxidase was assayed as described by Appelmans *et al.* (1955), but at 25°; glucose 6-phosphatase by the method of de Duve *et al.* (1955); urate oxidase by the technique of de Duve *et al.* (1955), but at 37° to increase the sensitivity of the assay; and total acid phosphatase by the procedure of Wattiaux & de Duve (1956), in the presence of 0·1% of Triton X-100 as releasing agent. Nitrogen was determined by a micro-Kjeldahl method. All results are expressed in the units defined by de Duve *et al.* (1955).

Monoamine oxidase. This enzyme was assayed by measuring the amount of ammonia released aerobically from tyramine, by using the procedure of Seligson & Hirahara (1957) to recover the ammonia and that of Russell (1944) for its colorimetric determination. Incubation was carried out for 16–18 hr. at 30° in penicillin flasks. The incubation mixture contained, in a total volume of 1 ml.: tyramine (5 mM), EDTA (mM) and potassium phosphate buffer, pH 7·3 (80 mM). The reaction was stopped by the addition of 1 ml. of saturated K_2CO_3, previously boiled to remove traces of ammonia. The flasks were quickly stoppered with a rubber stopper fitted with a glass rod protruding partly inside the flask, but without touching the surface of the fluid. The flame-thickened tip of this rod had first been dipped into N-H_2SO_4 which served to collect the ammonia released by addition of the alkali. To speed up distillation, the flasks were rotated horizontally on a specially constructed drum. After 15 min., which sufficed for the complete transfer of the ammonia, the flasks were unstoppered and the ammonia was recovered by washing the rods with 1·5 ml. of distilled water. It was then measured colorimetrically according to the method of Russell (1944). A blank was run by incubating the enzyme preparation in a similar way, but without tyramine, the latter being added just before introducing K_2CO_3. The blank value was subtracted from the test reading. Under these conditions, the reaction proceeded linearly with time and with enzyme concentration up to an ammonia production of approx. 0·15 μmole, but tended to slow down above this value. Readings falling beyond the linear range were corrected by means of a reference curve. One unit of activity is defined as the amount of enzyme releasing 1 μmole of ammonia/min. under the conditions described.

Aminotransferases. Aspartate aminotransferase was assayed under the general conditions set out by Tonhazy, White & Umbreit (1950), incubation being carried out for 20 min. at 37° and the final extraction and measurement of the dinitrophenylhydrazone of pyruvate being performed as described below for the determination of D-amino acid oxidase. Alanine aminotransferase was assayed in a similar fashion, according to the method of Caldwell & McHenry (1953). The reactions were found to fulfil the necessary linearity requirements. One unit of aminotransferase activity is defined as the amount of enzyme forming 1 μmole of oxaloacetate (measured as pyruvate) or of pyruvate/min. under the conditions of the assays.

D-Amino acid oxidase. This enzyme was assayed by measuring, as the dinitrophenylhydrazone, according to a modification of the method of Tonhazy *et al.* (1950), the amount of pyruvate formed aerobically from D-alanine. Linearity with time and with enzyme concentration was achieved by incubating the preparations at 25° in a total volume of 1 ml. containing: D-alanine (50 mM), sodium pyrophosphate–HCl buffer, pH 8·6 (37·5 mM), and FAD (25 μM). The reaction was stopped, generally after 2 hr., by the addition of 1 ml. of 2 M-sodium acetate–acetic acid buffer, pH 5, followed by the addition of 0·8 ml. of a 0·1% solution of dinitrophenylhydrazine in 2 N-HCl. After

5 min., the mixture was shaken with 2 ml. of water-saturated toluene and centrifuged to separate the two phases. The lower water phase was sucked off with a thin pipette, care being taken not to lose any of the toluene phase, and the latter was washed twice with 1 ml. of distilled water according to the same procedure. The dinitrophenylhydrazone of pyruvate was then re-extracted from the toluene solution with 4 ml. of M-Na_2CO_3. After centrifugation, 3 ml. of the water phase was removed and diluted with 3 ml. of 1·5 N-NaOH, to serve for the colorimetric measurement of the hydrazone which was done with a purple light-filter (filter 42, Klett–Summerson photocolorimeter). An unincubated mixture of the same composition was treated similarly for measurement of the blank value, which was subtracted from the test reading. One unit of activity is defined as the amount of enzyme forming 1 μmole of pyruvate/min. under the conditions described. The molar extinction coefficient of the hydrazone was measured by treating in an identical manner known quantities of pyruvic acid, freshly redistilled under vacuum.

In a few preliminary experiments the reaction was run similarly but without FAD. After addition of the acetate buffer, the mixture was heated for 10 min. in a boiling-water bath and filtered. Pyruvic acid was measured in the filtrate as the dinitrophenylhydrazone in the manner described above, but without toluene extraction. When measured in this manner the reaction did not proceed linearly. To overcome this defect each fraction was assayed at three concentrations of enzyme and its relative activity was obtained by fitting its kinetic curve to that of the cytoplasmic extract.

Catalase. In preliminary experiments this enzyme was assayed spectrophotometrically at 25° by following the decrease in extinction at 230 mμ, essentially as described by Beers & Sizer (1952). In later work a procedure based on the method of Chantrenne (1955) was adopted. The preparations were pretreated with 1% (v/v) Triton X-100 to release the enzyme and were then incubated at 0° for 10 min. or less in a total volume of 5·2 ml. containing: imidazole–HCl buffer, pH 7·2 (10 mM), bovine serum albumin (0·1%) and hydrogen peroxide (1·5 mM). The reaction was stopped by the addition of 3 ml. of a threefold dilution of a saturated solution of titanium sulphate [titanium (IV) oxysulphate; Merck] in 2 N-H_2SO_4, and the remaining hydrogen peroxide was determined colorimetrically in this mixture as the yellow 'peroxy titanium sulphate' (Patel & Mohan, 1960). The initial hydrogen peroxide concentration was measured similarly on an unincubated mixture of the same composition. With both assay methods, the breakdown of hydrogen peroxide followed first-order kinetics. One unit of activity is defined as the amount of enzyme causing the destruction of 90% of the substrate in 1 min. in a volume of 50 ml. under the assay conditions.

RESULTS

In Table 1 are listed the means ± S.D. of the distribution patterns recorded in the present experiments. Fig. 1 shows the corrected distribution patterns represented in the manner proposed by de Duve *et al.* (1955). To allow a closer comparison with the reference enzymes, the patterns for catalase and D-amino acid oxidase are shown

Table 1. *Intracellular distribution of enzymes*

Experimental details are given in the text. Absolute values are given in mg./g. for nitrogen, and in units/g. fresh wt. of liver for enzymes. The results are given as means ± S.D. E, Cytoplasmic extract; N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatant. Absolute values for catalase refer to activity at 0°; in assays at room temperature a mean value of 70·9 ± 12·5 units/g. was observed.

Enzyme	No. of expts.	Absolute values, E + N	Percentage values					
			E + N	N	M	L	P	S	Recovery
Nitrogen	10	32·9 ± 5·3	100	19·4 ± 4·4	20·8 ± 3·9	3·3 ± 1·3	21·5 ± 2·2	35·2 ± 6·2	100·2 ± 2·9
Cytochrome oxidase	10	28·7 ± 4·7	100	12·0 ± 7·4	60·9 ± 8·6	8·4 ± 3·8	6·3 ± 3·2	—	87·6 ± 8·6
Monoamine oxidase	4	0·132 ± 0·062	100	17·1 ± 5·4	48·4 ± 4·3	5·6 ± 1·2	17·0 ± 1·6	5·3 ± 1·2	93·4 ± 5·9
Glucose 6-phosphatase	10	18·7 ± 6·3	100	10·2 ± 5·0	5·0 ± 2·1	3·8 ± 1·9	74·3 ± 8·7	4·6 ± 2·5	97·9 ± 6·6
Aspartate aminotransferase	2	30·0 ± 0·2	100	5·3 ± 0·8	19·8 ± 1·9	11·0 ± 5·5	2·3 ± 0·1	61·0 ± 2·0	99·4 ± 2·4
Alanine aminotransferase	2	34·6 ± 1·6	100	3·1 ± 0·5	4·2 ± 2·5	3·7 ± 3·5	1·5 ± 0·7	86·2 ± 3·8	98·7 ± 9·7
Urate oxidase	6	3·42 ± 1·0	100	5·6 ± 3·7	24·3 ± 12·0	28·4 ± 9·2	21·6 ± 4·7	1·3 ± 1·1	81·2 ± 11·7
D-Amino acid oxidase	6	0·66 ± 0·11	100	9·8 ± 3·8	29·9 ± 11·5	18·1 ± 8·1	16·2 ± 8·6	16·1 ± 9·3	90·1 ± 12·6
Catalase	7	46·1 ± 12·0	100	7·4 ± 5·6	21·7 ± 10·6	21·7 ± 9·5	9·0 ± 6·1	31·2 ± 6·8	91·0 ± 6·0
Acid phosphatase	10	5·5 ± 2·0	100	7·0 ± 2·9	26·9 ± 3·5	31·8 ± 8·3	26·5 ± 8·8	12·1 ± 3·0	104·3 ± 7·8

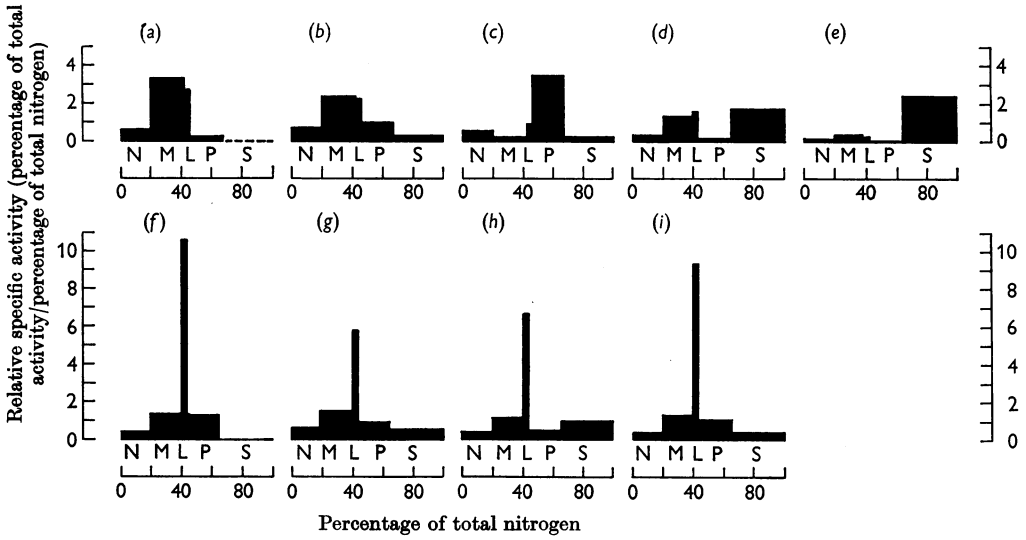

Fig. 1. Distribution pattern of enzymes: (*a*) cytochrome oxidase (4) ; (*b*) monoamine oxidase (4); (*c*) glucose 6-phosphatase (4); (*d*) aspartate aminotransferase (2); (*e*) alanine aminotransferase (2); (*f*) urate oxidase (6); (*g*) D-amino acid oxidase (6); (*h*) catalase (7); (*i*) acid phosphatase (7). Fractions are represented in the order of their isolation, i.e. (from left to right) N, M, L, P and S (see Table 1). Each fraction is represented separately in the ordinate scale by its own relative specific activity (percentage of total recovered activity/percentage of total recovered nitrogen). In the abscissa scale, each fraction is represented (cumulatively from left to right) by its nitrogen content, expressed as a percentage of total recovered nitrogen. The numbers in parentheses refer to numbers of experiments.

together with those observed for urate oxidase and acid phosphatase in the same experiments, and those for monoamine oxidase with the corresponding patterns for cytochrome oxidase and glucose 6-phosphatase.

COMPLEMENTARY DATA AND DISCUSSION

Reference enzymes. The activities and distribution patterns observed for the reference enzymes resemble closely those found previously in similar experiments, except for the absolute activity of urate oxidase, which is more than 10 times as high as the mean value of $0{\cdot}29 \pm 0{\cdot}06$ (S.D.) unit/g. fresh wt. of liver reported by de Duve *et al.* (1955). This difference cannot be accounted for only by the change in assay temperature, since the ratio of the activity of urate oxidase at 37° to that at 25° is only 2·05, according to our determinations. Neither are the present values in any way exceptional, since 29 assays carried out during the last 4 years have yielded a mean urate-oxidase activity of $2{\cdot}6 \pm 1{\cdot}3$ (S.D.) units/g. It was commented by de Duve *et al.* (1955) that their values were 4–5-fold lower than those found by Schneider & Hogeboom (1952). The present values agree with those of the latter authors, taking into account the difference in assay temperature. Whatever the reason for the low activities observed previously, it did not affect the distribution pattern of the enzyme, which is identical in the two groups of experiments.

Monoamine oxidase. Except for a small amount of activity occurring in the final supernatant, this enzyme was found to be almost entirely particle-bound, in confirmation of the results obtained by other workers. Its distribution appears to be bimodal and resembles closely that observed for NADPH–cytochrome *c* oxidoreductase (de Duve *et al.* 1955). The results are adequately explained by assuming that, on the average, the mitochondria contain about 70 % and the microsomal elements 24 % of the total activity, the remainder being present in soluble form, possibly as a result of leakage from damaged particles. In particular, the L fraction does not contain a significant excess of activity over that expected from its content in mitochondria and microsomes, and there is no reason to believe that either the lysosomes or the urate-oxidase-bearing microbodies have significant monoamine-oxidase activity.

Aminotransferases. The distribution of aspartate aminotransferase indicates that about one-third of the activity is associated with the true mitochondria and the remainder with the cell sap. The mitochondrial localization of the particulate

enzyme was further confirmed by density equilibration in a sucrose–D_2O gradient. In an experiment of this sort, performed according to the method of Beaufay, Bendall, Baudhuin, Wattiaux & de Duve (1959), the distribution of aspartate aminotransferase was found to follow closely that of cytochrome oxidase. These results are in full agreement with the observations of other workers. Evidence has been obtained that two distinct enzymic species may occupy the two locations (Rosenthal *et al.* 1960; Eichel & Bukovsky, 1961).

Alanine aminotransferase shows a much larger proportion of soluble activity than aspartate aminotransferase, but includes a small particulate component with a typical mitochondrial distribution. More experiments are needed to assess the true significance of this component, which was of variable magnitude, amounting to more than 15% of the total activity in one experiment, and to only 4% in the other. Rowsell (1956) mentions an experiment in which two additional washings of a mitochondrial fraction removed most of its alanine-aminotransferase activity, though not the aspartate-aminotransferase activity. On the basis of these results, adsorption of the enzyme to the surface of the mitochondria cannot be ruled out. However, such a phenomenon would have to involve a somewhat specific bonding, in view of the low activities associated with the microsomal fraction.

D-*Amino acid oxidase.* In confirmation of the results of Paigen (1954), D-amino acid oxidase was found to be preferentially located in the light mitochondrial fraction. The shape of the distribution curve does not authorize the statement that the enzyme is absent from the mitochondria, but suggests strongly that a considerable portion of it, at least, is associated either with the lysosomes or with the urate-oxidase-containing microbodies. As shown by Beaufay *et al.* (1964), density-gradient centrifugation experiments point to the latter particles as the bearers of the enzyme.

The origin of the activity found in the supernatant fraction is unknown. Experiments performed on particulate fractions showed that the enzyme can be partially released into solution by subjecting the particles to Triton X-100 or to prolonged agitation in a Waring Blendor. Repeated freezing and thawing or exposure to a hypo-osmotic medium were much less effective. These experiments were complicated by enzymic inactivations which made it impracticable to investigate the solubilization of D-amino acid oxidase in a quantitative fashion.

Attempts to demonstrate latency properties comparable with those observed for the lysosomal hydrolases or for catalase gave negative results. In short-term assays performed on freshly isolated particles in the presence of 0·25M-sucrose, the addition of 0·1% of digitonin or Triton X-100 to the incubation medium did not cause any increase in the measured activity. D-Amino acid oxidase resembles urate oxidase in this respect.

Catalase. Except for a larger proportion of soluble activity, the distribution pattern of catalase resembles that of D-amino acid oxidase and should be similarly interpreted as a strong indication that the larger part at least of the enzyme must be associated with non-mitochondrial particles. These are shown by Beaufay *et al.* (1964) to be represented by the microbodies, which also contain urate oxidase and D-amino acid oxidase.

In contrast with these two enzymes, catalase displays considerable latency in intact particles. This property has been investigated in some detail by P. Baudhuin & C. de Duve (unpublished work).

SUMMARY

1. The intracellular distribution of several enzymes has been investigated on homogenates of rat liver, fractionated according to a scheme in which the mitochondrial fraction is divided into two subfractions. Cytochrome oxidase, acid phosphatase, urate oxidase and glucose 6-phosphatase were assayed in all the fractions to serve as reference enzymes for their respective host particles.

2. Monoamine-oxidase activity was found in two main locations, mitochondria and microsomal elements, which contain about 70 and 24% of the total activity respectively.

3. The mitochondria were found to contain one-third of the total aspartate-aminotransferase activity, and a very small and variable part of the alanine-aminotransferase activity. The remainder of these enzymes was recovered in soluble form and presumably occurs in the cell sap.

4. D-Amino acid oxidase and catalase showed similar distributions, indicating that the bulk of these enzymes is associated with non-mitochondrial particles with sedimentation properties similar to those of the lysosomes and of the urate-oxidase-containing 'microbodies'.

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18. RESOLUTION OF MITOCHONDRIAL FRACTIONS FROM RAT LIVER INTO THREE DISTINCT POPULATIONS OF CYTOPLASMIC PARTICLES BY MEANS OF DENSITY EQUILIBRATION IN VARIOUS GRADIENTS

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Beaufay, Bendall, Baudhuin, Wattiaux & de Duve (1959) described experiments in which mitochondrial fractions from rat liver were partly resolved into their components by density equilibration in sucrose gradients, with either H_2O or D_2O as solvent. The results obtained in these investigations provided additional confirmation of the existence of lysosomes as a separate group of particles, distinct from mitochondria, and suggested further that urate oxidase, though almost

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