The Localization of Amine Oxidase in the Liver Cell

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Of all the mammalian tissues studied, the liver is the most active source of amine oxidase. This enzyme is known to be associated with the insoluble fraction of the tissue, but no study of its localization in the liver cell has so far been made. Recent work on other enzymes has shown that a number of oxidases are associated with the mitochondria. The enzymes which have been studied include cytochrome oxidase, the succinoxidase system (Hogeboom, Claude & Hotchkiss, 1946), cytochrome reductase (Hogeboom & Schneider, 1950), octanoic acid oxidase (Schneider, 1948), the oxidases of the citric acid cycle (Kennedy & Lehninger, 1949), and the enzymes involved in the synthesis of citrulline (Leuthardt & Müller, 1948). It was therefore of interest to find out whether the mitochondria were also the principal site of amine oxidase activity.

In the present investigation, use has been made of the observation of Hogeboom, Schneider & Pallade (1948), that well defined fractions of liver-cell constituents could be obtained by the differential centrifugation of homogenates prepared in sucrose. In hypertonic sucrose (0·88 M), Hogeboom *et al.* (1948) obtained sedimentation of the bulk of the mitochondria by centrifugation at 24,000 *g* for 20 min., and of the microsomes by centrifugation at 41,000 *g* for 2 hr. Using isotonic sucrose (0·25 M), Schneider (1948) sedimented the mitochondria by centrifugation at 8500 *g* for 10 min., and the microsomes by centrifugation at 18,000 *g* for 60 min.

Using a similar procedure to that described by these authors, various fractions of mammalian liver have been prepared and examined for amine oxidase activity.

METHODS

The preparation of homogenates. Experiments were carried out on rat liver. The liver was minced with scissors, and forced through a wire mesh on to a cooled watch glass. The resulting pulp was weighed and homogenized in a small volume of sucrose solution in an all-glass homogenizer for about 5 min.; more sucrose solution was then added so that 1 g. of tissue was present in 10 ml. of the homogenate. In different experiments the sucrose concentration was either 0·88 M (hypertonic) or 0·25 M (isotonic).

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Centrifugation procedure. About 10 ml. of the homogenate were retained for determination of amine oxidase activity; the rest was centrifuged at 600 *g* for 5 min. to sediment the unbroken cells, incompletely homogenized cell fragments, nuclei, and red blood cells. The sediments obtained were resuspended in sucrose solution up to the original volume. When hypertonic sucrose was used, this preliminary centrifugation of the supernatant was repeated twice more because of the high viscosity of the medium. This will be more fully described in the experimental part.

The supernatant fraction contained the soluble cell constituents and formed elements, mainly mitochondria and microsomes, but a few unsedimented red blood cells and nuclei were also present. This fraction is called the first supernatant, of which about 10 ml. were retained. The remainder was subjected to high-speed centrifugation on an International refrigerated centrifuge, as described in the experimental part. Each of the fractions obtained was made up with sucrose solution so that 1·0 ml. of the fraction corresponded with 1·0 ml. of the homogenate. The temperature was kept as near as possible to 0° throughout.

Manometric procedure. The amine oxidase activity of each fraction was determined in conical Warburg flasks. The main compartment of each flask contained: 1·0 ml. of fraction +0·4 ml. of 0·067 M-sodium phosphate buffer, pH 7·4, +0·2 ml. of 0·1 M-semicarbazide. The side bulb of each flask contained either the substrate, 0·4 ml. of an 0·05 M-solution of tyramine hydrochloride, or 0·4 ml. of water in the blank. The inner compartments contained a filter paper and 0·3 ml. of N-KOH. The determinations were carried out in oxygen at 37°. Readings were taken at 5 min. intervals and the rate of tyramine oxidation was calculated from the oxygen uptake during the first 15 min. of the reaction. Amine oxidase activity is expressed in terms of q_{O_2}, that is, the μl./hr. of O_2 consumed by 1·0 ml. of each fraction (derived from 100 mg. fresh weight of tissue).

Preliminary experiments had shown that the amine oxidase activity of liver homogenates prepared in sucrose was increased by the addition of 0·4 ml. of 0·067 M-sodium phosphate buffer, pH 7·4, per flask. No further increase in activity was observed when more buffer was added.

Nitrogen determinations. It was not convenient to estimate the nitrogen content of the various fractions in the presence of large amounts of sucrose. Nitrogen determinations were therefore carried out on the protein precipitates of each fraction. The protein precipitant used was trichloroacetic acid; 5 ml. of a 2·5% (w/v) solution were added to 1·0 ml. of the fraction. The precipitated protein was transferred quantitatively to micro-Kjeldahl flasks and digested with the sulphuric acid-phosphoric acid mixture described by Campbell & Hanna (1937). Steam distillation was carried out in the Markham apparatus (Markham, 1942). The protein nitrogen content of each fraction is given as mg./ml.

EXPERIMENTAL

Experiments in isotonic sucrose

Four experiments were carried out in which homogenates were prepared in 0·25 M-sucrose. During the first stages the same procedure was followed in all experiments. The enzymic activities and protein nitrogen contents of the different fractions are given in Table 1. In these experiments, the amine oxidase activity of the homogenates varied from a q_{O_2} of 126 to 260, and the protein nitrogen content from 2·38 to 3·35 mg./100 mg. fresh weight of tissue. After low-speed centrifugation at 600 *g*, 71–75 % of the activity of the homogenate was found in the supernatant fraction. The change in the enzymic activity per mg. protein nitrogen was slight.

In each experiment the first supernatant was centrifuged at 22,000 *g* for 45 min. on the refrigerated centrifuge. This was done in order to separate the soluble protein from the formed elements, the mitochondria and the microsomes, which were sedimented. The resulting supernatant had no enzymic activity in two experiments, and 3 and 12 % of the activity of the homogenate in the other two. The protein nitrogen content of this fraction varied from 0·96 to 1·20 mg./ml. Thus centrifugation at 22,000 *g* removed a considerable amount of enzymically inactive protein.

Practically all the enzymic activity of the first supernatant was present in the sediment obtained after high-speed centrifugation. This sediment contained the mitochondria and probably the bulk of the microsomes. The enzymic activity of this fraction per mg. protein nitrogen was higher than that of the starting material. This can be seen from the data given in Table 1.

The further treatment of the sediments obtained in the first high-speed centrifugation differed in the three experiments in which a further centrifugation was carried out. The results are given in Table 2. In Exp. 2 the sediment containing mitochondria and microsomes was suspended in isotonic sucrose, and again centrifuged at 22,000 *g* for 45 min., in order to wash the formed elements and to remove remaining traces of soluble protein. It could be expected that by this treatment the mitochondria and microsomes were again sedimented together. From Tables 1 and 2 it can be seen that after the second high-speed centrifugation the enzymic activity per mg. protein nitrogen of the sediment was slightly increased.

In Exps. 3 and 4 the sedimented mitochondria and microsomes were suspended in isotonic sucrose

Table 1. *Amine oxidase activity and protein nitrogen content of centrifuged homogenates of rat liver in* 0·25 M-*sucrose*

(q_{O_2}: μl. O_2/hr./ml. of fraction. P.N.: mg. protein nitrogen/ml. of fraction. Figures in parentheses give the q_{O_2} and P.N. content as % of that of the homogenates.)

Centrifugation *g*	Centrifugation Time (min.)	Fraction	Exp. 1 q_{O_2}	Exp. 1 P.N.	Exp. 2 q_{O_2}	Exp. 2 P.N.	Exp. 3 q_{O_2}	Exp. 3 P.N.	Exp. 4 q_{O_2}	Exp. 4 P.N.
		Homogenate*	214 (100)	2·38 (100)	260 (100)	2·93 (100)	164 (100)	3·35 (100)	126 (100)	3·04 (100)
600	5	Supernatant*	156 (73)	1·81 (74)	184 (71)	2·28 (78)	120 (73)	2·48 (74)	94 (75)	2·26 (74)
		Sediment	54 (25)	0·75 (31)	90 (34)	0·88 (30)	56 (34)	1·05 (31)	42 (33)	1·19 (29)
22,000	45	Supernatant	0 (0)	0·96 (40)	8 (3)	1·17 (40)	0 (0)	1·15 (35)	16 (12)	1·20 (39)
		Sediment*	151 (70)	0·95 (40)	198 (76)	1·14 (39)	124 (75)	1·34 (40)	100 (79)	1·11 (36)

* Fraction which is subjected to further centrifugation.

Table 2. *Further treatment of the last sediment from experiments shown in Table* 1. *Medium:* 0·25 M-*sucrose*

Exp.	2		3		4	
Centrifugation: *g*	22,000		1,800		8,500	
Time (min.)	45		45		10	
	q_{O_2}	P.N.	q_{O_2}	P.N.	q_{O_2}	P.N.
Supernatant	20 (7)	0·27 (9)	20 (12)	0·58 (17)	20 (16)	0·48 (16)
Sediment	188 (72)	0·88 (30)	92 (56)	0·91 (27)	62 (49)	0·60 (20)

Table 3. *Enzymic activity and protein nitrogen content of centrifuged homogenates of rat liver. Medium:* 0·88 M-*sucrose*

(Data are given as in Table 1.)

Centrifugation			Exp. 5		Exp. 6	
g	Time (min.)	Fraction	q_{O_2}	P.N.	q_{O_2}	P.N.
		Homogenate*	222 (100)	2·65 (100)	180 (100)	2·78 (100)
600	3 × 5	Supernatant*	160 (72)	2·08 (78)	144 (80)	2·17 (78)
		Sediment	49 (22)	1·13 (42)	40 (22)	0·68 (24)
22,000	45	Supernatant	40 (18)	1·39 (52)	24 (13)	1·47 (53)
		Sediment*	108 (48)	0·65 (24)	122 (68)	0·75 (27)
22,000	45	Supernatant	24 (11)	0·22 (8)	20 (11)	0·24 (8)
		Sediment	110 (49)	0·48 (18)	80 (44)	0·41 (14)

* Fraction subjected to further centrifugation.

and centrifuged at a lower speed, at room temperature, in order to separate the mitochondria from the microsomes; the latter can be assumed to have remained suspended in the supernatant, whereas the mitochondria were sedimented. The speed and time of centrifugation were different in the two experiments, as shown on Table 2, but the results were similar. In Exp. 3, the supernatant contained 12 %, and the sediment 56 % of the activity of the homogenate.

Experiments with hypertonic sucrose

Two experiments were carried out in which the homogenate was prepared in 0·88 M-sucrose. The results are summarized in Table 3. As in the experiments described above, the homogenate was centrifuged at 600 *g* for 5 min., but in order to ensure that all the larger cell fragments had sedimented in the more viscous medium, the centrifugation of the supernatant fluid was repeated twice. The three sediments were pooled and made up to the original volume of the homogenate.

In these experiments the supernatant had 72 and 80 % of the enzymic activity of the homogenate, and the activities per mg. protein nitrogen were unchanged. When this fraction was centrifuged at 22,000 *g* for 45 min., a supernatant was obtained which contained the soluble cell constituents and most of the microsomes, and a sediment which contained mainly mitochondria but probably also some microsomes. The sediment was resuspended in 0·88 M-sucrose and again centrifuged at 22,000 *g* for 45 min.

From Table 3 it can be seen that the supernatant fraction containing the soluble material and microsomes had some enzymic activity and that, on resuspension and resedimentation of the mitochondrial fractions, some more enzymic activity appeared in the supernatant. The activity/mg. protein nitrogen of the twice sedimented mitochondria was three times that of the original homogenate.

Separation of mitochondria and microsomes

In the preceding section two different methods have been employed to obtain a preparation of mitochondria free from microsomes. In the first, in isotonic sucrose, the mitochondria and microsomes were first brought down together, and then separated by resuspension and sedimentation of the mitochondria by centrifugation at lower speed (Exps. 3 and 4). In the second method, a fraction of mitochondria was obtained by repeated centrifugation at 22,000 *g* in hypertonic sucrose (Exps. 5 and 6). All the fractions which contained mitochondria stained characteristically with Janus green B at a concentration of 1 in 10,000.

Tables 4 and 5 give a summary of the results obtained. In these two tables the enzymic activities and protein nitrogen contents of the fractions are expressed as a percentage of the values for the first supernatant. Table 4 shows clearly that the removal of soluble cell protein did not noticeably affect enzymic activity. The data given in Table 5 are explained by the removal of some enzymic activity with the microsome fraction. In the two experiments carried out in hypertonic sucrose (Exps. 5 and 6), the enzymic activity per mg. protein nitrogen

was slightly higher than in the two preceding experiments in isotonic sucrose. Whether this was due to any difference in the composition of the mitochondrial fraction, cannot be decided.

Table 4. *Enzymic activity and protein nitrogen content of once sedimented mitochondria plus microsomes*

(Data are given as percentage of the q_{O_2} and P.N. of the supernatant from low-speed centrifugation of the original homogenate.)

Exp.	q_{O_2} (%)	P.N. (%)
1	96	52
2	108	50
3	103	54
4	106	49
Mean	**103**	**51**

Table 5. *Enzymic activity and protein nitrogen content of fractions containing twice sedimented mitochondria*

(Data are given as in Table 4.)

Exp.	q_{O_2} (%)	P.N. (%)
3	77	37
4	66	27
5	69	23
6	56	19
Mean	**67**	**26·5**

DISCUSSION

It has been shown that of the total amine oxidase activity of a liver homogenate, about 50% is associated with those granules which are of the size and staining properties characteristic of mitochondria. It seems likely, however, that the percentage activity resident in the mitochondria is greater than this figure. In the preliminary low-speed centrifugation of the homogenate, about 25% of the total enzymic activity was brought down in the sediment. It can be safely assumed that most of this fraction consisted of unbroken cells and incompletely homogenized cell fragments, which retained enzymic activity. The activity of the mitochondrial fraction expressed as a percentage of the activity of the first supernatant, gives probably a better measure of the contribution of the mitochondria to the amine oxidase activity of the liver as a whole. Table 5 shows that 56–77% of the enzymic activity of the first supernatant was present in this fraction. The finding that amine oxidase is present in the mitochondria is in agreement with a statement by Cotzias & Dole (1951); no full description of this observation has been published.

In isotonic sucrose, the mitochondria and microsomes were brought down together by high-speed centrifugation. This fraction contained practically all the enzymic activity of the first supernatant. It remained constant on resuspension and resedimentation at 22,000 *g*, but was reduced by low-speed centrifugation at 1800 *g*. This indicates that the microsomes also contain amine oxidase, and that together with the mitochondria they account for all the amine oxidase activity of the liver tissue.

It is still under discussion to what extent the microsomes are pre-formed elements of the liver cell. The recent observations of Harman (1950) and of Still & Kaplan (1950), that prolonged homogenization in a Waring blender results in the transfer of succinoxidase from the fraction containing mitochondria to that containing microsomes, raises the possibility that in homogenates prepared by the milder methods used in this paper, there may be some disintegration of the mitochondria. It can therefore not be decided if the amine oxidase activity of the smaller particles is due to the presence of fragmented mitochondria.

The sedimentation of the cytoplasmic granules results in a purification of amine oxidase; this is explained by the removal of a considerable amount of soluble protein which is without activity. From the data given it is apparent that the isolated mitochondria are two to three times more active than the original homogenate.

The particulate matter of the liver cell is known to contain the respiratory enzymes, which have been studied by methods similar to those employed in this work. It is of interest that amine oxidase has a similar distribution, although it is an oxidase for which any connexion with the cytochrome-cytochrome oxidase system has never been demonstrated.

SUMMARY

1. The distribution of amine oxidase in homogenates of rat liver, prepared in either isotonic or hypertonic sucrose, has been studied.

2. Evidence has been obtained that about two-thirds of the enzymic activity is present in the mitochondria; the remainder is present in the microsome fraction.

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The Branched-Chain Fatty Acids of Ox Fat

1. THE ISOLATION FROM OX SUET OF A C_{17} BRANCHED-CHAIN SATURATED FATTY ACID

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The occurrence of high molecular weight branched-chain saturated fatty acids has been reported in certain bacterial lipids (Anderson, 1927, 1941; Velick, 1944; Hofmann & Lucas, 1950) and in wool wax (Weitkamp, 1945). Recent investigations in this laboratory have revealed the presence in butterfat of (*a*) two isomeric C_{17} saturated fatty acids, each with a methyl side chain (Hansen & Shorland, 1951), (*b*) a multi-branched C_{20} fatty acid (Hansen & Shorland, 1952), (*c*) a C_{18} methyl branched-chain fatty acid (Hansen, Shorland & Cooke, 1951).

In this paper is described a C_{17} branched-chain acid isolated from ox suet by the processes of hydrogenation, fractional distillation *in vacuo*, and fractional crystallization.

EXPERIMENTAL

The fat used in this work was extracted from the suet (15·5 lb.) of a 4-year-old 'prime' heifer (dressed weight approximately 600 lb.). Following the determination of the fatty acid composition of a sample of the fat, 162 g. of the fractionated methyl esters of the C_{18} unsaturated acids were hydrogenated at 150° and at atmospheric pressure, using a nickel catalyst supported on kieselguhr.

Repeated crystallization from 15 vol. acetone at −30° removed the solid products of hydrogenation and yielded

Table 1. *Fractionation of ox-fat liquid methyl esters in micro column*

(Pressure approx. 0·05 mm. Hg.)

Fraction	Wt. (g.)	b.p. (°)	Saponification equiv.	Iodine value (Wijs)	
FL1	0·20	117–122	290·7	3·4	Liquid
FL2	0·60	122–128	290·5	1·6	Liquid
FL3	0·93	128–130	300·7	1·2	Liquid
FL4	0·46	130–131	319·3	2·2	Liquid
FLR	0·44	—	—	—	Viscous liquid