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Studies on the Intracellular Localization and Incorporation of ^{59}Fe into Catalase in Rat Liver

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Nyberg, Schuberth & Änggård (1953) found that about 61 % of the catalase activity was present in the mitochondrial fraction of rat liver and only about 3·7 % in the mitochondrial fractions of horse and guinea-pig liver. Greenfield & Price (1956) claimed that catalase is present mainly in the mitochondrial fraction of rat liver but that it is eluted rapidly from mitochondria by 0·25M-sucrose solution, which they used as the homogenizing medium. They also reported the isolation of catalase from rat-liver mitochondria and showed that elution of catalase from mitochondria could be prevented by adding either polyvinylpyrrolidone or albumin to the homogenizing medium. Ludewig & Chanutin (1950), however, noted that about 45 % of the catalase activity of rat liver is present in mitochondria. Thomson & Klipfel (1957) reported that, during density-gradient centrifugation, rat-liver catalase and uricase sediment closely together in a particulate fraction that is lighter than the particles containing succinate dehydrogenase and cytochrome oxidase, the enzyme markers for mitochondria. de Duve, Beaufay, Jacques, Rahman-Li & Sellinger (1960) observed a close relationship in the sedimentation properties of particles containing uricase, catalase and D-amino acid oxidase, and suggested that these enzymes are present in subcellular particles distinct from both mitochondria and lysosomes.

de Duve, Wattiaux & Baudhuin (1962) point out that the prevention of elution of catalase from rat-liver mitochondria by polyvinylpyrrolidone could be an absorption artifact, since the addition of polyvinylpyrrolidone to 0·25M-sucrose homogenates of rat liver containing a high proportion of soluble catalase also decreased the amount of enzyme present in the supernatant fraction. Intracellular redistribution of catalase during the incubation of mouse-liver slices was found by Adams & Elizabeth (1956), Adams & Burgess (1958, 1959*a*, *b*) and Adams (1960). Roodyn, Suttie & Work (1962) observed negligible rates of incorporation of ^{14}C-labelled amino acids into catalase, malate dehydrogenase and cytochrome *c* under optimum conditions for the incorporation of radioactive amino acids into mitochondria. These authors, however, suggested that their failure to incorporate radioactive amino acids into the soluble enzymes isolated from mitochondria could be explained if these enzymes are not truly mitochondrial, and they emphasized the necessity for a re-examination of the intracellular localization of these enzymes in liver. Peters & Higashi (1963) suggested that the mitochondrial catalase of rat liver has its origin in vesicles that sediment with a granular microsome fraction and that it is then transferred gradually to mitochondria. They also considered the possibility that enzymically inactive microsomal catalase is a precursor of active catalase.

The present work was undertaken with a view to relating the intracellular localization of catalase to its biosynthesis in rat liver. The incorporation of ^{59}Fe into catalase has been studied mainly to assess whether there is any direct participation of mitochondria in the biogenesis of catalase. A preliminary account of some of this work has been published (Radhakrishnan & Sarma, 1963).

MATERIALS AND METHODS

Animals. The experimental animals were male albino rats of this Institute strain weighing about 200–250 g.

Chemicals. ATP (sodium salt) and NAD were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The sodium salts of creatine phosphate, creatine phosphokinase, GSH, α-oxoglutaric acid and protoporphyrin were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. GTP was a sample from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., obtained as a gift from Dr T. A. Sundararajan.

[2-^{14}C]Glycine and $^{59}FeCl_3$ were obtained from the Atomic Energy Establishment, Trombay, India.

Preparation of cell fractions. Rats were starved for 15 hr. and killed by a blow on the head followed by decapitation. The livers were perfused with cold 0·25M-, 0·33M- or 0·88M-sucrose solution, excised, chilled immediately, washed and homogenized in the medium of same sucrose concentration with a Potter–Elvehjem-type glass homogenizer. The homogenates were filtered through surgical gauze to remove fibres and clumps of unbroken cells. The cell fractions were isolated at 0–4° by the conventional procedure of Schneider & Hogeboom (1950) with a PR–2 centrifuge (International Equipment Co., U.S.A.) and a Spinco model L ultracentrifuge (Beckman/Spinco, U.S.A.).

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as the standard.

Catalase activity was measured by the titrimetric method of Euler & Josephson (1927) as described by Radhakrishnan, Raghupathy & Sarma (1963). Catalase activity was calculated in terms of ml. of 0·1 N-potassium permanganate equivalent to hydrogen peroxide consumed/min./mg. of protein.

Succinate-oxidase activity was measured manometrically according to the method of Schneider & Potter (1943). The succinate-oxidase activity was calculated as μl. of oxygen consumed/min./mg. of protein.

Washing of mitochondria. The mitochondrial pellets obtained by the differential centrifugation of 20 ml. portions of 10% (w/v) homogenates of rat liver in 0·25M-, 0·33M- or 0·88M-sucrose solution were uniformly suspended twice with 10 and 5 ml. and later four times with 2·5 ml. portions of sucrose solution of the same concentration as that with which the original homogenate was prepared. The mitochondria from the suspension were isolated each time by resedimentation and the catalase activity in the washings was determined.

Measurement of radioactivity. The ^{14}C-labelled samples were plated as infinitely thin films on stainless-steel planchets and counted with a Geiger tube (TGC 2; window thickness, 1·9 mg./cm.^{2}) attached to a 151–A-type decade scaler (Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.). The counts were corrected for self-absorption and background.

The ^{59}Fe radioactivity of the samples was measured in a well-type scintillation counter (model DS5-5; Nuclear–Chicago Corp.).

Incorporation of ^{59}Fe *into catalase in the mitochondrial and* 100000*g supernatant fractions* in vitro. A homogenate of perfused rat liver (0·3 g./ml.) was made up in 0·25M-sucrose containing sodium–potassium phosphate buffer, pH 7·2 (0·154M), nicotinamide (100 mM), NAD (300μM), ATP (200μM), GSH (1 mM), α-oxoglutaric acid (2 mM) and $MgCl_2$ (3 mM). A portion (100 ml.) of this suspension was mixed with $^{59}FeCl_3$ ($9{\cdot}9 \times 10^4$ counts/min.). Portions (10ml.) of the mixture in ten 50 ml. Pyrex Erlenmeyer flasks were incubated in air at 37° in a Gallenkamp metabolic shaker for 60 min. The flasks were then rapidly chilled and the contents were pooled and diluted with 2 vol. of 0·25M-sucrose solution and cell fractions were isolated according to the method of Schneider & Hogeboom (1950). The particulate fractions were homogenized and all the fractions were dialysed thoroughly with stirring against ice-cold water till the diffusate was free of ^{59}Fe. They were then made up to a definite volume and samples were taken for measuring ^{59}Fe radioactivity. Haem from the subcellular fractions was extracted with butan-2-one at pH 2·0 by the method of Teale (1959) and its ^{59}Fe radioactivity was measured.

For studying the effect of 2,4-dinitrophenol on the incorporation of ^{59}Fe in rat-liver cell fractions, the incubation medium was essentially as above except that the homogenate was divided into two equal portions, of which one received 2,4-dinitrophenol (0·1 mM). The two portions were preincubated separately for 10 min. before incubation with $^{59}FeCl_3$.

Isolation of ^{59}Fe*-labelled catalase from the* 100000*g supernatant fractions of rat-liver homogenate.* The procedure was based on the method of Loftfield & Bonnichsen (1956). Ferritin was removed from the dialysed supernatant by the addition of 0·5 vol. of saturated ammonium sulphate solution. To the supernatant solution more ammonium sulphate was added to give 60% saturation. The precipitate formed was recovered and dialysed against 0·1M-phosphate buffer, pH 7·0. Ice-cold dialysed solution (4 vol.) was shaken with 1 vol. of ice-cold chloroform–ethanol (1:30, v/v) to denature the haemoglobin. After centrifugation in the cold, the supernatant solution was dialysed against several changes of cold water and freeze-dried. The powder was dissolved in 2 ml. of 0·1M-phosphate buffer, pH 7·2, and loaded on a column (2 cm. × 65 cm.) of Sephadex G-75 (medium grade; Pharmacia, Uppsala, Sweden) that had been previously equilibrated with the same buffer. The column was developed at 5° and 3 ml. fractions were collected. The ^{59}Fe radioactivity in the fractions was measured as above and E_{408} of the fractions was measured in a Beckman DU spectrophotometer. Fractions containing catalase were pooled, dialysed thoroughly against cold water and freeze-dried. The ^{59}Fe radioactivity in the freeze-dried powder was measured and a portion of it was dissolved in 3 ml. of 0·1M-phosphate buffer, pH 7·2, for measurement of ^{59}Fe radioactivity, catalase activity and E_{408}.

Paper electrophoresis of catalase isolated from the Sephadex G-75 column. Catalase solution (0·2 ml.) was spotted at the cathode end on three Whatman no. 3MM paper strips (2 cm. × 45 cm.) and electrophoresis was conducted at 0–5° for 5 hr. at 400v and 15 mA in a horizontal Durrum-type electrophoresis apparatus with 0·05M-veronal buffer, pH 8·6. After being dried, the first strip was cut transversely into pieces 1 cm. wide, each piece was suspended in 3 ml. of 0·05M-veronal buffer, pH 8·6, and the ^{59}Fe radioactivity was measured in all eluents. The haemin was detected on the second strip by the benzidine-spray method of Connelly, Morrison & Stotz (1958), and catalase activity was located on the third strip by the method of Jermyn (1953).

Isolation of catalase from mitochondria. The procedure was the same as described above for the isolation of catalase from the 100000*g* supernatant fraction, except that the mitochondria were initially subjected to ultrasonic treatment in a Raytheon sonic oscillator (10 kcyc./sec.) for 3 min.

Incorporation of ^{59}Fe *into subcellular fractions of rat liver and into catalase of the mitochondrial and* 100000*g supernatant fractions* in vivo. Five rats were each given intraperitoneal injections of $^{59}FeCl_3$ ($8{\cdot}9 \times 10^5$ counts/min.) in 0·5 ml. of 0·9% sodium chloride. After exactly 15 hr. the animals were killed and the livers were immediately perfused with cold 0·88M-sucrose. The pooled livers were immediately homogenized with ice-cold 0·88M-sucrose. The isolation of cell fractions, the estimation of total ^{59}Fe radioactivity and the ^{59}Fe radioactivity in the haem fraction of the subcellular fractions, and the isolation of ^{59}Fe-labelled catalase from the mitochondrial and the 100000*g* supernatant fractions were carried out as above.

Synthesis of ^{59}Fe*-labelled haemin.* The mitochondrial pellet obtained by the differential centrifugation of 150 ml. of a 30% (w/v) homogenate of rat liver in 0·25M-sucrose was homogenized in a medium containing sodium–potassium phosphate buffer, pH 7·4 (0·154M), protoporphyrin (900μM), $NaHCO_3$ (38·5 mM), GSH (1 mM) and β-mercapto-

ethanol (50 μl.). Then 100 ml. of this suspension was mixed thoroughly with $^{59}FeCl_3$ ($3{\cdot}3 \times 10^6$ counts/min.), and 10 ml. portions were placed in 50 ml. Pyrex Erlenmeyer flasks. The flasks were flushed with nitrogen, stoppered and incubated at 37° in the metabolic shaker for 60 min. The contents of the flasks were then pooled and the ^{59}Fe-labelled haem was extracted by the method of Teale (1959). It was converted into pyridine haemochromogen as described by Richmond, Altman & Salomon (1954) and transferred to a column (2 cm. × 30 cm.) of Celite. The column was developed with 8% (v/v) acetic acid in chloroform, and two distinct bands were obtained. The first band was reddish violet and the second band was brownish red. The second band was eluted with 10% (v/v) acetic acid in chloroform, the eluate was evaporated *in vacuo* to dryness and the residue dissolved in a small quantity of 8% (v/v) acetic acid in chloroform. The solution was filtered and treated with ether and light petroleum. The crystals that formed were dried *in vacuo* and further identified spectrophotometrically as haemin by means of its pyridine haemochromogen (Drabkin, 1942). The pyridine haemochromogen exhibited absorption maxima at 558 and 525 mμ. The molar extinction coefficient of the pyridine haemochromogen was 31000 at 558 mμ, which agrees with the value reported by Drabkin (1942). No attempt was made to characterize the second pigment. It was devoid of ^{59}Fe radioactivity.

Incorporation of ^{59}Fe *into protoporphyrin by the subcellular fractions of rat liver.* A homogenate of rat liver (0·1 g./ml.) in 0·25M-sucrose was prepared and a portion of it was subjected to differential centrifugation. The particulate fractions obtained were separately homogenized with 0·25M-sucrose. The boiled whole homogenate, whole homogenate, nuclear, mitochondrial, microsomal homogenates and 100000*g* supernatant fractions (8 ml. in each case) were taken for incubation in six separate 50 ml. Pyrex Erlenmeyer flasks and each fraction was diluted with 12 ml. of 0·25M-sucrose in 0·154M-sodium–potassium phosphate buffer, pH 7·4. In addition, the final incubation medium in each flask contained protoporphyrin (210 μM), ATP (100 μM), GSH (0·5 mM), β-mercaptoethanol (5 μl.) and $^{59}FeCl_3$ ($1{\cdot}8 \times 10^5$ counts/min.), with 200 μg. of carrier iron as $FeCl_3$, in a total volume of 20 ml. The flasks were flushed with nitrogen, stoppered and incubated at 37° in the metabolic shaker for 60 min. The ^{59}Fe-labelled haem was isolated and its radioactivity was determined as described above.

Incorporation of [2-^{14}C]*glycine into the supernatant fraction from rat-liver homogenate.* A homogenate of perfused rat liver (0·3 g./ml.) was prepared with 0·25M-sucrose solution in 0·154M-sodium–potassium phosphate buffer, pH 7·2. Of the supernatant fraction obtained from it, 5 ml. portions were taken in 50 ml. Pyrex Erlenmeyer flasks for incubation. The incubation medium in each flask contained rat-liver supernatant (5 ml.), creatine phosphate (3·1 mM), creatine phosphokinase (50 μg.), GTP (61·55 μM), $MgCl_2$ (184·6 μM), ATP (0·461 mM) and [2-^{14}C]glycine ($2{\cdot}9 \times 10^5$ counts/min.) in a total volume of 6·5 ml. The final volume in each flask was made up to 6·5 ml. by the addition of 1·5 ml. of 0·25M-sucrose in 0·154M-sodium–potassium phosphate buffer, pH 7·2. The flasks were flushed with $N_2 + CO_2$ (95:5), stoppered and incubated at 37° in a metabolic shaker. Four separate sets of flasks contained the complete system as above minus creatine phosphate, $MgCl_2$, ATP and GTP respectively, whereas in two different sets of flasks ribonuclease or chloramphenicol at the levels of 500 μg./flask was included. At the end of 60 min. the protein was precipitated by the addition of trichloroacetic acid to give a final concentration of 5% (w/v). The protein precipitates were washed thrice with 5% trichloroacetic acid, once with ethanol, three times with hot ethanol–ether (3:1, v/v) and finally with ether. They were plated on stainless-steel planchets and the radioactivity was measured.

Incorporation of ^{59}Fe-*labelled haemin into catalase.* The experiment was carried out essentially as described in the preceding paragraph, but on a semi-preparative scale. An incubation mixture was prepared with 50 ml. of the supernatant fraction obtained by the differential centrifugation of perfused rat-liver homogenate (0·3 g./ml.) in 0·25M-sucrose containing sodium–potassium phosphate buffer, pH 7·2 (0·154M), creatine phosphate (3·33 mM), creatine phosphokinase (500 μg.), ATP (0·5 mM), GTP (66·6 μM), $MgCl_2$ (0·2 mM) and ^{59}Fe-labelled haemin ($3{\cdot}5 \times 10^3$ counts/min.) in a total volume of 60 ml. The final volume of the mixture was made up to 60 ml. by the addition of 10 ml. of 0·25M-sucrose solution in 0·154M-sodium–potassium phosphate buffer, pH 7·2. Portions (10 ml.) of this mixture were taken in 50 ml. Pyrex Erlenmeyer flasks and flushed with $N_2 + CO_2$ (95:5), stoppered and incubated at 37° for 60 min. At the end of the incubation period, 50 ml. of a cold rat-liver homogenate (0·3 g./ml.) was added as a source of carrier catalase, and labelled catalase was isolated as described above and the ^{59}Fe radioactivity was measured. Subsequently, the ^{59}Fe-labelled haem was quantitatively cleaved from the isolated catalase with butan-2-one (Teale, 1959), cold haemin being used as the carrier, and the radioactivity was measured.

RESULTS

Intracellular localization of catalase in rat liver. Schneider & Hogeboom (1950) showed that the morphological and cytological properties of liver mitochondria are best preserved in 0·88M-sucrose solution even though the biochemical activity is affected in some respects. The data presented in Table 1 reveal the intracellular distribution of catalase, and the percentage distribution of protein in rat liver when 0·25M-, 0·33M- and 0·88M-sucrose solutions respectively were used as homogenizing media. The subcellular distribution of succinate-oxidase activity was taken as a marker for mitochondria when iso-osmotic and hyperosmotic sucrose solutions were used as the homogenizing media. The distribution of catalase is essentially independent of sucrose concentration. In all cases at least half of the catalase is present in the 100000*g* supernatant fraction and only about 25% is in mitochondria.

In Table 2 are given the catalase activities of the washings of mitochondria. It is clear that catalase is easily eluted from mitochondria when 0·25M- and 0·33M-sucrose solutions are used for washing, but

Table 1. *Intracellular distribution of catalase in rat liver with different concentrations of sucrose solutions for homogenization and differential centrifugation*

Cell fractions from 10 ml. of the 10% homogenates of rat liver in 0·25 M-, 0·33 M-, or 0·88 M-sucrose solution were isolated by differential centrifugation. Experimental details are given in the text. The results are given as means ± S.D. (four determinations).

Medium	0·25 M-Sucrose			0·33 M-Sucrose		0·88 M-Sucrose		
Fraction	Protein (%)	Catalase activity (%)	Succinate-oxidase activity (%)	Protein (%)	Catalase activity (%)	Protein (%)	Catalase activity (%)	Succinate-oxidase activity (%)
Whole homogenate	100	100	100	100	100	100	100	100
Nuclei	12 ± 1·58	10·5 ± 1·11	19 ± 1·60	16 ± 1·20	11 ± 2·23	13 ± 2·54	10 ± 1·58	7 ± 1·60
Mitochondria	24 ± 3·10	26·25 ± 2·38	70·75 ± 2·88	26 ± 1·58	26 ± 2·91	25 ± 2·91	23 ± 2·23	85 ± 3·16
Microsomes	16 ± 1·60	6 ± 1·58	3 ± 1·50	18·5 ± 1·10	6 ± 1·58	17·75 ± 1·92	8·25 ± 1·92	3 ± 1·22
Supernatant	45 ± 1·87	53·25 ± 1·08	Nil	38 ± 1·58	55·5 ± 1·65	43 ± 2·23	58·75 ± 0·83	Nil

not when 0·88 M-sucrose is used. Our results with 0·25 M-sucrose solution are in agreement with the report of Greenfield & Price (1956).

Incorporation of ^{59}Fe *into haem in subcellular fractions of rat liver* in vivo *and* in vitro. The findings of Loftfield & Bonnichsen (1956) were confirmed in the experiments on the distribution of ^{59}Fe radioactivity *in vivo* in the subcellular fractions of rat liver. The incorporation of ^{59}Fe *in vitro* into the subcellular fractions of liver tissue and the effect of 2,4-dinitrophenol on this incorporation are shown in Table 3. The total ^{59}Fe radioactivities in the microsomal and the 100000g supernatant fractions were higher than the ^{59}Fe radioactivities in nuclear and mitochondrial fractions, as in the experiments *in vivo*. However, the labelled haem was present mainly in the mitochondrial fraction. This is presumably due to the presence of haem-synthetase or ferrochelatase activity in mitochondria (Wintrobe, 1957; Minakami, Yoneyama & Yoshikawa, 1958; Minakami, 1958; Rimington, 1958; Nishida & Labbe, 1959; Porra & Jones, 1963*a*). 2,4-Dinitrophenol (0·1 mM) had an inhibitory effect on the incorporation of ^{59}Fe into the subcellular fractions of liver tissue. The most marked inhibition was observed with the incorporation into the mitochondrial fraction. Thus incorporation of ^{59}Fe into the subcellular fractions is an energy-dependent process.

Incorporation of ^{59}Fe *into catalase in mitochondria and cell sap* in vivo *and* in vitro. The results summarized in Table 4 illustrate the incorporation of ^{59}Fe into catalase isolated from the mitochondrial and the 100000g supernatant fractions of rat liver both *in vivo* and *in vitro*. Loftfield & Bonnichsen (1956) observed that, during the short periods after the intraperitoneal injection of $^{59}FeCl_3$ into rats, the iron-containing proteins attained the maximum specific radioactivity. In the present experiments the catalase was isolated from the mitochondrial and the 100000g supernatant fractions of rat livers after an intraperitoneal injection of $^{59}FeCl_3$. In the elution patterns of ^{59}Fe-labelled catalase, isolated from the mitochondrial and the 100000g supernatant fractions, on Sephadex G-75 columns, ^{59}Fe radioactivity, E_{408} and catalase activity were all superimposable on a single peak. However, some inactive material without any ^{59}Fe radioactivity but with absorption at 280 mμ was eluted from the columns after the elution of catalase. The paper-electrophoretic pattern of ^{59}Fe-labelled catalase isolated after passing through Sephadex G-75 column also indicated that the ^{59}Fe radioactivity was concentrated on a single band that was positive to the haemin and catalase tests on the paper, thereby establishing that the catalase sample isolated in the investigation was uncontaminated with other iron-containing proteins. An almost

identical pattern of results was obtained with catalase labelled with ^{59}Fe *in vitro*.

It is obvious from Table 4 that the ^{59}Fe radioactivities of catalase isolated from 100000*g* supernatant fractions in experiments both *in vivo* and *in vitro* were higher than the ^{59}Fe radioactivities in catalase isolated from mitochondria. The specific radioactivity of catalase, expressed as the ratio of ^{59}Fe radioactivity to E_{408}, was also higher for catalase isolated from the 100000*g* supernatant fraction than from the sample isolated from mitochondria in the experiments *in vivo*. However, the experiments *in vitro* demonstrate that the specific radioactivity of catalase isolated from mitochondria is greater than the specific radioactivity of catalase obtained from 100000*g* supernatant fraction. This can, however, be attributed to the rapid haem-synthesizing capacity of liver mito-

Table 2. *Catalase activity in the washings of rat-liver mitochondria*

Experimental details are given in the text. Catalase activity is expressed in terms of ml. of 0·1 N-potassium permanganate equivalent to hydrogen peroxide consumed/min./mg. of protein. The results are given as means ± S.D. (four determinations).

	Catalase activity		
Medium ... No. of washings	0·25 M-Sucrose	0·33 M-Sucrose	0·88 M-Sucrose
1	35·5 ± 3·20	25·25 ± 2·16	21 ± 1·58
2	73·5 ± 2·29	69·75 ± 2·16	18 ± 1·11
3	56·25 ± 1·29	32·25 ± 1·47	11·25 ± 0·82
4	56·75 ± 1·47	33 ± 1·12	9·5 ± 1·11
5	59 ± 0·70	29 ± 0·70	7·75 ± 0·82
6	49·5 ± 0·5	34·75 ± 0·82	5·75 ± 0·82
7	50·5 ± 1·12	20·75 ± 0·83	4·75 ± 0·82
8	32 ± 1·11	29·75 ± 0·82	4 ± 0·70

Table 3. *Influence of 2,4-dinitrophenol on the incorporation of* ^{59}Fe *in the subcellular fractions of rat-liver tissue* in vitro

Experimental details are given in the text. The concentration of 2,4-dinitrophenol used was 0·1 mM. The incubation was carried out at 37° for 60 min. with $^{59}FeCl_3$ ($3·84 \times 10^6$ counts/min.). The results are given as means ± S.D. (four determinations).

	Total ^{59}Fe radioactivity (counts/min.)*			^{59}Fe radioactivity in haem (counts/min.)*		
Fraction	Control	DNP-treated	Percentage inhibition	Control	DNP-treated	Percentage inhibition
Whole homogenate	584500 ± 5408	435750 ± 5959	25·44 ± 1·41	8241 ± 180	5576 ± 89	32·28 ± 2·56
Nuclei	77612 ± 757	69087 ± 908	10·97 ± 1·55	567 ± 10	526 ± 15	7·23 ± 0·81
Mitochondria	26422 ± 580	13325 ± 410	49·57 ± 0·25	4420 ± 72	1935 ± 46	56·19 ± 1·71
Microsomes	216650 ± 4522	174450 ± 3235	19·42 ± 2·87	1216 ± 99	1040 ± 93	14·55 ± 0·89
Supernatant	220875 ± 2880	172575 ± 2039	21·86 ± 0·42	2257 ± 55	1965 ± 51	12·96 ± 0·60

* Values are corrected for background.

Table 4. *Incorporation of* ^{59}Fe *into catalase isolated from the mitochondrial and* 100000*g supernatant fractions of rat liver*

Experimental details are given in the text. The results are given as means ± S.D. (three determinations).

	in vivo			*in vitro*		
Fraction	Total ^{59}Fe radioactivity (counts/min.)*	Percentage incorporation	Sp. radioactivity (counts/min./E_{408})	Total ^{59}Fe radioactivity (counts/min.)*	Percentage incorporation	Sp. radioactivity (counts/min./E_{408})
Mitochondria	516 ± 52	0·011 ± 0·001	350 ± 36	16350 ± 735	1·644 ± 0·751	8023 ± 120
Supernatant	7463 ± 463	0·168 ± 0·010	958 ± 61	26066 ± 839	2·626 ± 0·103	4231 ± 93

* Values are corrected for background.

chondria or to a dilution effect attributable to the high concentration of catalase in the 100000*g* supernatant fraction.

Haem synthesis by chelation of ^{59}Fe *into protoporphyrin.* The incorporation of Fe^{2+} ions into protoporphyrin to form haem is an enzymic process (Krueger, Melnick & Klein, 1956; Schwartz, Cartwright, Smith & Wintrobe, 1959; Nevé, 1961). From the results in Table 5 it is evident that mitochondria have a higher haem-synthesizing activity than the 100000*g* supernatant fraction. The present findings are in agreement with the reports of Nishida & Labbe (1959) and Minakami *et al.* (1958).

Incorporation of ^{59}*Fe-labelled haemin into catalase* in vitro *under conditions favourable for the incorporation of* [2-^{14}C]*glycine into proteins in the absence of mitochondria.* In Table 6 are shown data on the capacity of rat-liver homogenate free of nuclear and mitochondrial fractions to incorporate [2-^{14}C]-glycine into proteins. The conditions were chosen after preliminary experiments to find the optimum conditions for the incorporation of [^{14}C]glycine.

Table 5. *Incorporation of* ^{59}Fe *into protoporphyrin by rat-liver fractions*

Experimental details are given in the text. The results are given as means ± S.D. (four determinations).

Fraction	Total ^{59}Fe radioactivity (counts/min.)*	Percentage incorporation
Whole homogenate	107342 ± 5094	100
Boiled whole homogenate	726 ± 32	0·67 ± 0·02
Nuclei	10311 ± 334	9·61 ± 0·69
Mitochondria	60515 ± 648	56·53 ± 3·11
Microsomes	19537 ± 501	18·24 ± 0·82
Supernatant	17907 ± 623	16·71 ± 0·70

* Values are corrected for background.

The results suggest that all the ingredients, ATP-generating system, Mg^{2+} ions, GTP and ATP, are required in the incubation medium for maximum incorporation. Ribonuclease had an inhibitory effect, as reported by Zamecnik & Keller (1954); however, chloramphenicol was not inhibitory (Franklin, 1963), but had a slight stimulatory effect. The incorporation of ^{59}Fe-labelled haemin into catalase in rat-liver homogenate free of nuclear and mitochondrial fractions under the same conditions is summarized in Table 7. As with the incorporation of [2-^{14}C]glycine into proteins, the complete system minus any one of the components was less active than the complete system in the incorporation of ^{59}Fe-labelled haemin into catalase. The ^{59}Fe-labelled haemin that is incorporated into catalase was recovered quantitatively after cleaving the haem from the protein.

DISCUSSION

The biosynthesis of tissue haemoproteins that play an important role in the respiration of aerobic cells has been studied extensively by Theorell, Beznak, Bonnichsen, Paul & Akeson (1951), Loftfield & Bonnichsen (1956) and Marsh & Drabkin (1957). Sano & Granick (1961) have proposed a scheme for the synthesis of protoporphyrin IX in liver cells that involves the participation of mitochondria in the initial synthesis of δ-aminolaevulate and in the final oxidation of coproporphyrinogen III to protoporphyrin IX. The subsequent incorporation of iron into protoporphyrin to form haem has also been shown to be essentially a mitochondrial function (Sano & Granick, 1961). There is no doubt that protoporphyrin is the direct precursor of haem and that the insertion of iron is enzymically catalysed. Porra & Jones (1963*b*) observed ferrochelatase activity in extracts of

Table 6. *Incorporation of* [2-^{14}C]*glycine in rat-liver homogenates free of nuclear and mitochondrial fractions* in vitro

The complete system contained: creatine phosphate (3·1 mM), creatine phosphokinase (50 μg.), GTP (61·55 μM), $MgCl_2$ (184·6 μM), ATP (0·461 mM), [2-^{14}C]glycine (2·9 × 10^5 counts/min.) and rat-liver supernatant fraction (5·0 ml.) in a total volume of 6·5 ml. in 0·25M-sucrose containing sodium–potassium phosphate buffer, pH 7·2 (0·154M). The incubation was carried out at 37° for 60 min. as described in the text. The results are given as means ± S.D. (three determinations).

Flask no.	Incubation medium	Sp. radioactivity (counts/min./mg. of protein)*	Percentage incorporation
1	Complete system	285 ± 7	0·098 ± 0·002
2	Complete system minus $MgCl_2$	12 ± 2	0·004 ± 0·002
3	Complete system minus creatine phosphate	190 ± 8	0·065 ± 0·003
4	Complete system minus ATP	25 ± 4	0·008 ± 0·002
5	Complete system minus GTP	185 ± 14	0·064 ± 0·048
6	Complete system plus ribonuclease	186 ± 9	0·064 ± 0·033
7	Complete system plus chloramphenicol	327 ± 10	0·112 ± 0·032

* Values are corrected for background and self-absorption.

Table 7. *Incorporation of* ^{59}*Fe-labelled haemin into catalase in rat-liver homogenate free of nuclear and mitochondrial fractions*

The complete incubation medium contained 50 ml. of the supernatant fraction obtained by the differential centrifugation of rat-liver homogenate (0·3 g./ml.) in 0·25M-sucrose containing sodium–potassium phosphate buffer, pH 7·2 (0·154M), creatine phosphate (3·33 mM), creatine phosphokinase (500 μg.), ATP (0·5 mM), GTP (66·6 μM), $MgCl_2$ (0·2 mM) and ^{59}Fe-labelled haemin ($3·5 \times 10^3$ counts/min.) in a total volume of 60 ml. in 0·25M-sucrose containing sodium–potassium phosphate buffer, pH 7·2 (0·154M). Then 10 ml. portions of this mixture were taken in six 50 ml. Pyrex Erlenmeyer flasks and the incubation was carried out at 37° for 60 min. as described in the text. The results are given as means ± S.D. (three determinations).

Omission from complete incubation medium	^{59}Fe radioactivity (counts/min.)*	Percentage incorporation of ^{59}Fe haem in catalase	^{59}Fe radioactivity in haem recovered from catalase (counts/min.)*
None	280±13	7·81±0·96	266±12
$MgCl_2$	16±3	0·46±0·09	10±3
Creatine phosphate	174±16	4·96±0·33	153±21
ATP	34±5	0·98±0·14	27±4
GTP	154±12	4·41±0·35	142±9

* Values are corrected for background.

several micro-organisms and have considered its significance in the biosynthesis of haem prosthetic groups. We have found maximum 'haem-synthetase' activity in the mitochondrial fraction of rat liver. Radhakrishnan & Sarma (1963) found that the catalase activity is greater in 100000*g* supernatant fraction than in mitochondria in the liver tissue of other animals as well. A similar observation was made in rat liver by Higashi & Peters (1963).

Results reported in the present paper indicate that preformed haem prosthetic group can combine with apocatalase that is synthesized in the cell sap. Beljanski (1955) reported that apocatalase synthesized by a porphyrin-less mutant of *Escherichia coli* can combine *in vitro* with haemin to give a product with the properties of normal catalase. Jensen (1957) also found that a haem-requiring mutant of *Staphylococcus aureus* behaves similarly. The isolation of crystalline catalase and a crystalline apocatalase from a haem-requiring mutant of *S. aureus* grown in the presence and in the absence of haem respectively was reported by Jensen & Hyde (1963). Since apocatalase can be biosynthesized in the absence of prosthetic group it follows that proper folding of the polypeptides of apocatalase does not depend on the prosthetic group (Chantrenne, 1962).

We conclude that mitochondria play a role in the synthesis of haem and that it combines with apocatalase which is formed in the cell sap.

SUMMARY

1. The intracellular distribution of catalase in rat liver has been examined. Catalase activity was greater in the 100000*g* supernatant than in mitochondria and was independent of the concentration of sucrose in the homogenizing media.

2. Catalase was eluted from mitochondria by washing with 0·25M- or 0·33M-sucrose solution, but not with 0·88M-sucrose.

3. In experiments both *in vivo* and *in vitro*, the ^{59}Fe radioactivity in catalase isolated from the 100000*g* supernatant fraction of rat liver was higher than in that from mitochondria.

4. The incorporation of ^{59}Fe into haem *in vitro* occurred more actively with mitochondria than with other subcellular fractions.

5. Under conditions favourable for the incorporation of [2-^{14}C]glycine into proteins *in vitro* in the absence of mitochondria, preformed ^{59}Fe-labelled haemin was incorporated into catalase.

6. It is suggested that mitochondria have a role in the biosynthesis of haem prosthetic groups of catalase.

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