Influence of starvation and clofibrate administration on oxidative phosphorylation by rat liver mitochondria

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Whole cells, homogenates and mitochondria obtained from the livers of albino rats which were starved for 6 days or more showed a 50% decrease in oxidative activity. The decrease could be corrected by the addition of cytochrome *c* *in vitro*. The phosphorylative activity of mitochondria remained unaffected. The decrease in oxidative rate was not observed when starving animals were given the anti-hypercholesterolaemic drug clofibrate. The total cellular concentration of cytochrome *c* was not affected by starvation. However, the concentration of the pigment in hepatic mitochondria isolated from starving animals was less than half that in normal mitochondria. Clofibrate-treated animals did not show a decreased concentration of cytochrome *c* in hepatic mitochondria. Mitochondria isolated from starving animals, though deficient in cytochrome *c*, did not show any decrease in succinate dehydrogenase activity or in the rate of substrate-dependent reduction of potassium ferricyanide or attendant phosphorylation. In coupled mitochondria, ferricyanide may not accept electrons from the cytochrome *c* in the respiratory chain. Starvation decreases the concentration of high-affinity binding sites for cytochrome *c* on the mitochondrial membrane. The dissociation constant increases in magnitude.

Starvation causes a drastic decrease in the content of mitochondria in liver (Allard *et al.*, 1957; Scarpelli *et al.*, 1971). It was shown in our laboratory that the decrease can be effectively prevented by the administration of the anti-hypercholesterolaemic drug clofibrate [ethyl α -(*p*-chlorophenoxy)isobutyrate] during starvation (Chhabra *et al.*, 1979). The mitochondria-proliferating property of the drug, first reported from our laboratory (Kurup *et al.*, 1970), has been confirmed in many laboratories (Gear *et al.*, 1974; Subramanian *et al.*, 1975). Since the condition of starvation marks the ascendancy of energy-yielding catabolic processes over energy-consuming anabolic reactions, a general derangement of membranes may be expected. However, subcellular organelles other than mitochondria do not show such a drastic decrease. The specific degradation of mitochondrial membranes may reflect an effort on the part of the starving animal to minimize the production of ATP. Investigation of the influence of starvation on mitochondrial oxidative phosphorylation therefore assumes importance. The results presented in this paper give clear indication that during starvation mitochondrial oxidation decreases, presumably ow-

ing to a specific deficiency of one of the components of the respiratory chain, namely cytochrome *c*.

Materials and methods

Animals and administration of the drug

Male albino rats, 3 months old and weighing 135–150 g, from the Central Animal Facility of this Institute were fed with the stock diet described by Kurup *et al.* (1970). When the animals weighed 140–160 g, diet was withdrawn from the experimental animals (starved) for the time period indicated. Water was allowed *ad lib.*, and vitamins were given twice a week, as for control animals, which received the diet *ad lib.* Wherever indicated, clofibrate (50 mg/rat per day) was given orally as water emulsion in 0.2 ml. With a view to minimizing variations, an equal number of control and experimental animals were killed at any one time. All killings were done at about 09:00 h.

In 8 days of starvation, the animals lost 35% of their initial weight. Except for the direct effects of starvation, the animals were healthy, normal and devoid of any symptoms of infection. Under our experimental conditions, the starved animals sur-

vived for more than 10 days. Resumption of feeding on the tenth day of starvation resulted in speedy reversal of the effects of starvation. The animals regained their initial weight in 1 week. All the metabolic changes were reversed in 4 days (Chhabra *et al.*, 1979).

Chemicals

All chemicals used were of the purest grade available. Cytochrome *c* (type II or type III) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All solutions were prepared in water triple-distilled in an all-quartz apparatus.

Preparation of liver suspension

Suspensions of liver cells were prepared essentially by the 'micro-slice' technique developed by Reddy & Sastry (1979) for brain tissue. Fresh liver (1 g) was made into a fine mince (by using a razor blade) in 2.5 ml of 0.25 M-sucrose and passed, under suction, through a nylon bolting cloth [149 μ m (100 mesh); Tobler and Traber, New York, NY, U.S.A.] stretched over a Buchner funnel. The tissue remaining on the mesh was gently disturbed with a blunt glass rod until most of the tissue passed through. The cells were counted with a haemocytometer.

Isolation of mitochondria

Liver mitochondria were isolated as described by Kurup *et al.* (1970). Mitochondria were depleted of cytochrome *c* by salt treatment (Jacobs & Sanadi, 1960).

Assay of oxidative phosphorylation

The rate of oxygen uptake was measured polarographically by using a Gilson model K-ICT-C Oxygraph fitted with a Clark oxygen electrode. The reaction medium contained 100 mM-Tris/HCl buffer, pH 7.4, 20 mM-potassium phosphate buffer, pH 7.4, 7 mM-MgCl₂, 2 mM-EDTA, 50 mM-sucrose and up to 1.5 mg of freshly prepared liver mitochondria in a total reaction volume of 1.5 ml. After the addition of the substrate (500 mM-succinate or 100 mM-glutamate + 200 mM-malate; 50 μ l), the rate of State-3 oxidation (Chance & Williams, 1955) was measured after the addition of 300 nmol of ADP in 30 μ l. Respiratory control index was calculated as the ratio of oxidation rates in State 3 and State 4 (ADP exhausted).

Oxidative phosphorylation was also measured manometrically by using a Gilson differential respirometer. Phosphorylation coupled to the first and second sites was measured with ferricyanide as the electron acceptor. Phosphorylation coupled specifically to the third site was measured with ascorbate + NNN'N' - tetramethyl-*p*-phenylenediamine. The details were essentially as described by

Panini & Kurup (1974). When succinate or ascorbate was used as electron donor, rotenone (10 μ g) was added to the reaction system. Protein was measured by the biuret method (Gornall *et al.*, 1949), deoxycholate being used for solubilization. The assay of oxidative phosphorylation was carried out soon after isolation of mitochondria and was completed within 1–2 h after isolation.

Determination of cytochrome *c*

Perfused liver (0.5 g fresh wt.) or mitochondria (20 mg of protein) isolated from such livers was washed repeatedly with acetone, chloroform/methanol (2:1, v/v) and acid/acetone as described by Rieske (1967). The haem content was calculated from the difference spectrum (reduced—oxidized) of the pyridine haemochrome. The spectra were taken in a Cary 14 recording spectrophotometer.

Binding sites for cytochrome *c*

High-affinity binding sites for cytochrome *c* on the mitochondrial membrane were determined essentially as described by Hanstein & Hatefi (1974). The experimental conditions were essentially the same as used for the determination of oxidative phosphorylation. Equilibrium binding was carried out in a medium containing 50 mM-Tris/HCl buffer, pH 7.4, 10 mM-potassium phosphate buffer, pH 7.4, 1 mM-EDTA, 3 mM-MgCl₂ and 5 mg of cytochrome *c*-depleted mitochondria (protein) and various concentrations of cytochrome *c* in 30 ml. After incubation for 10 min at 30°C, mitochondria were sedimented by centrifugation at 15000 *g* for 10 min. The tubes were kept inverted over a filter paper for 15 min. The sides and mouth of the tubes were wiped free of the adhering fluid and the pigment bound to the membrane was determined spectrophotometrically, after conversion into the pyridine haemochrome, in a Beckman-26 recording spectrophotometer.

Enzyme assays

The activity of ATPase (ATP phosphohydrolase, EC 3.6.1.3) was measured essentially as described by Panini & Kurup (1974). Mitochondrial concentration (300 μ g of protein) and time of incubation (1 min or 4 min) were so adjusted as to ensure linearity in both the latent and the uncoupler-stimulated activity. Succinate dehydrogenase [succinate-(acceptor) oxidoreductase, EC 1.3.99.1] activity was measured as described by Bernath & Singer (1962). Succinate oxidase activity was measured as described by Potter (1972).

In all determinations of enzyme activities and rates of oxidative phosphorylation, an equal number of control and experimental samples were used at any one time, to minimize errors owing to variations. Also, unless otherwise stated, all determina-

tions were made without undue lapse of time after isolation of mitochondria.

Results

Oxidative phosphorylation

Under our experimental conditions the animals could survive starvation for more than 10 days (Chhabra *et al.*, 1979). Mitochondria isolated from the livers of rats that were starved for up to 4 days did not show any decrease in the rate of oxidative phosphorylation. However, when animals were starved further, mitochondrial oxidation showed a measurable and progressive decrease. The oxidative activity of hepatic mitochondria isolated from rats that were starved for 8 days was decreased by half when compared with normal mitochondria (Table 1). The fact that this observation held good for all the three commonly used substrates which activate different segments of the respiratory chain indicated that the decreased oxidation could be due to a defect in the terminal part (cytochrome oxidase). In agreement with this, addition of cytochrome *c* to the reaction system almost doubled the rate of oxidation by mitochondria from starved animals. Addition of the pigment to freshly prepared normal mitochondria did not stimulate oxidation (Table 1). It is noteworthy that starvation affects only the oxidative activity of mitochondria. The ability of mitochondria to phosphorylate (P/O ratio) remained unaffected by starvation. Interestingly enough, hepatic mitochondria isolated from starving animals that were being fed with clofibrate did not show any decrease in oxidation. Addition of cytochrome *c* to these preparations did not stimulate oxidation.

Respiratory control and ADP/O ratio

In agreement with the results presented in Table 1, on starvation of the animal for 8 days, the rate of active respiration (State 3) of hepatic mitochondria decreased by 40% ($P < 0.05$) with both glutamate + malate and succinate. The ADP/O ratio was not affected (Table 2). As shown in Table 1, the decrease in oxidation could be corrected by the addition of cytochrome *c* to the reaction system. In the presence of cytochrome *c*, however, the ADP/O ratio tended to decrease. Oxidation, however, remained sensitive to antimycin A as well as to cyanide. Addition of cytochrome *c* to mitochondria from control animals did not appreciably change the State-3 oxidation rate, although the ADP/O ratio showed a significant decrease.

In agreement with the results in Table 1, when the starved animals were being fed with clofibrate, mitochondrial oxidative activity was not decreased (Table 2). In all respects, these mitochondria behaved like the organelles isolated from normal animals.

It is noteworthy that mitochondria isolated from starved animals which showed considerable decrease in State-3 oxidation did not show a similar decrease in State-4 oxidation. This was particularly true when glutamate + malate was used as the substrate. This resulted in a lowering of respiratory-control index (Table 2).

ATPase activity

State-4 oxidative activity remained high, suggesting partial uncoupling. This was tested by measuring the latent and uncoupler-stimulated ATPase activity of these preparations. The results

Table 1. *Effect of starvation and clofibrate administration on oxidative phosphorylation by rat liver mitochondria*
Rats were starved for 8 days where indicated. Clofibrate (50 mg/rat per day) was administered orally as indicated. Oxygen uptake determined manometrically is expressed as ng-atoms of O/min per mg of mitochondrial protein. The concentration of cytochrome *c* (Cyt. *c*), if added, was 16 μ M. The values are the means $\pm$ S.D. for six independent determinations (animals). **Significance $P < 0.01$ (fed versus starved).

Substrate	Measurement	Addition	Fed	Starved	Fed + clofibrate	Starved + clofibrate
Glutamate + malate	O ₂ uptake	—	111 $\pm$ 17	58 $\pm$ 6**	98 $\pm$ 10	103 $\pm$ 21
	O ₂ uptake	Cyt. <i>c</i>	114 $\pm$ 21	97 $\pm$ 13	87 $\pm$ 6	104 $\pm$ 20
	P/O	—	2.9 $\pm$ 0.2	2.9 $\pm$ 0.2	2.6 $\pm$ 0.1	2.5 $\pm$ 0.3
	P/O	Cyt. <i>c</i>	2.9 $\pm$ 0.2	2.8 $\pm$ 0.1	2.5 $\pm$ 0.3	2.5 $\pm$ 0.2
Succinate	O ₂ uptake	—	182 $\pm$ 17	96 $\pm$ 13**	193 $\pm$ 20	176 $\pm$ 16
	O ₂ uptake	Cyt. <i>c</i>	177 $\pm$ 22	183 $\pm$ 15	178 $\pm$ 11	193 $\pm$ 20
	P/O	—	1.7 $\pm$ 0.1	1.6 $\pm$ 0.1	1.8 $\pm$ 0.1	1.8 $\pm$ 0.3
	P/O	Cyt. <i>c</i>	1.9 $\pm$ 0.3	1.4 $\pm$ 0.1	1.5 $\pm$ 0.1	1.7 $\pm$ 0.3
Ascorbate + NNN' <i>N'</i> - tetramethyl- <i>p</i> - phenylenediamine	O ₂ uptake	—	213 $\pm$ 29	137 $\pm$ 16**	238 $\pm$ 21	259 $\pm$ 59
	O ₂ uptake	Cyt. <i>c</i>	221 $\pm$ 17	281 $\pm$ 28	256 $\pm$ 26	297 $\pm$ 59
	P/O	—	0.9 $\pm$ 0.1	0.9 $\pm$ 0.2	0.81 $\pm$ 0	0.8 $\pm$ 0.2
	P/O	Cyt. <i>c</i>	0.8 $\pm$ 0.1	0.7 $\pm$ 0.1	0.7 $\pm$ 0	0.8 $\pm$ 0.3

Table 2. *Effect of starvation and clofibrate administration on respiratory control in rat liver mitochondria*

Oxidative phosphorylation was assayed polarographically with and without the addition of cytochrome *c* (Cyt. *c*; 16 μ M), in a Gilson model K-ICT-C Oxygraph fitted with a Clark electrode. Starvation was for 8 days. Clofibrate (50 mg/rat per day) was given orally. Respiratory-control index (RCI) is calculated as the ratio of the rate of oxygen uptake in the presence of ADP (State 3) to that after the added ADP was exhausted. The values are means $\pm$ S.D. for five or six independent determinations (animals). Oxygen uptake is expressed as ng-atoms of O/min per mg of mitochondrial protein. Significance (fed versus starved): * $P < 0.05$; ** $P < 0.01$.

Substrate	Measurement	Addition	Fed	Starved	Fed + clofibrate	Starved + clofibrate
Glutamate + malate	State 3	—	141 $\pm$ 14	85 $\pm$ 29*	117 $\pm$ 7	106 $\pm$ 13
	State 3	Cyt. <i>c</i>	156 $\pm$ 24	128 $\pm$ 2	127 $\pm$ 14	118 $\pm$ 15
	RCI	—	6.0 $\pm$ 0.3	4.1 $\pm$ 1.1	8.0 $\pm$ 0.7**	5.8 $\pm$ 0.7
	RCI	Cyt. <i>c</i>	6.0 $\pm$ 0.7	4.0 $\pm$ 1.0	6.9 $\pm$ 0.3	5.2 $\pm$ 0.6
	ADP/O	—	2.4 $\pm$ 0.2	2.1 $\pm$ 0.4	2.3 $\pm$ 0	2.5 $\pm$ 0.2
	ADP/O	Cyt. <i>c</i>	2.0 $\pm$ 0.2	1.7 $\pm$ 0.1	2.2 $\pm$ 0	2.2 $\pm$ 0.1
Succinate	State 3	—	228 $\pm$ 21	150 $\pm$ 12**	222 $\pm$ 10	208 $\pm$ 13
	State 3	Cyt. <i>c</i>	247 $\pm$ 28	235 $\pm$ 11	247 $\pm$ 2	211 $\pm$ 24
	RCI	—	4.8 $\pm$ 0.6	4.5 $\pm$ 1.0	4.6 $\pm$ 0.3	4.8 $\pm$ 0.7
	RCI	Cyt. <i>c</i>	4.4 $\pm$ 1.0	3.2 $\pm$ 0.7	3.6 $\pm$ 0.4	3.7 $\pm$ 0.5
	ADP/O	—	1.5 $\pm$ 0.3	1.3 $\pm$ 0.1	1.4 $\pm$ 0.1	1.5 $\pm$ 0.1
	ADP/O	Cyt. <i>c</i>	1.3 $\pm$ 0.1	1.0 $\pm$ 0.1	1.3 $\pm$ 0	1.4 $\pm$ 0.1

Table 3. *Effect of starvation and clofibrate administration on ATPase activity of rat liver mitochondria*

The activity of ATPase in freshly prepared hepatic mitochondria was determined as described by Panini & Kurup (1974). Wherever indicated animals were starved for 8 days. Other details are given in the Materials and methods section. Uncoupler-stimulated activity was determined after the addition of 0.5 mM-dinitrophenol. The values are means $\pm$ S.D. for four independent determinations.

ATPase activity	Activity (nmol of P _i liberated/min per mg of protein)			
	Fed	Starved	Fed + clofibrate	Starved + clofibrate
Latent	21 $\pm$ 1	45 $\pm$ 6	23 $\pm$ 4	20 $\pm$ 4
Dinitrophenol-stimulated	208 $\pm$ 28	203 $\pm$ 26	185 $\pm$ 19	179 $\pm$ 15

presented in Table 3 show that the latent ATPase activity increased 2-fold on starvation. This effect of starvation was not observed in drug-fed animals. Also, addition of cytochrome *c* to the reaction system did not have any effect on latent ATPase activity.

The deleterious effects of starvation on mitochondrial oxidation and latent ATPase activity were completely reversed when animals starved for 8 days were re-fed for 4 days (results not shown). We have shown (Chhabra *et al.*, 1979) that all the metabolic changes produced by starvation are reversed on resumption of feeding.

Succinate oxidase activity

Succinate oxidase activity of freshly isolated mitochondria and liver homogenate decreased on starvation (Table 4). The decrease could be corrected by the addition of cytochrome *c*. Starvation of clofibrate-fed animals did not lead to any decrease in succinate oxidase activity. Addition of cytochrome *c*

to preparations from normal or clofibrate-fed animals did not result in any significant stimulation of activity.

Succinate oxidation by liver cells also decreased by 50% on starvation. Addition of cytochrome *c* to such cells stimulated the rate of oxidation and brought it to the same value as obtained with cells obtained from the livers of control animals (Table 4). In contrast, cell preparations from starved animals that were fed with the drug did not show any decrease in succinate oxidase activity. Addition of cytochrome *c* to such cells did not cause any significant increase in oxidase activity.

Ferricyanide reduction

With a view to localizing the deleterious effect of starvation on respiratory function, the activity of different segments of the electron-transport chain was determined. The succinate dehydrogenase activity of mitochondria (356 ng-atoms of O/min per mg of protein) was not affected by starvation

Table 4. *Effect of starvation and clofibrate administration on succinate oxidase activity of cells, homogenate and mitochondria of rat liver*

Experimental details were the same as given in the legend for Table 1. Starvation was for 8 days; clofibrate (50 mg/rat per day) was administered orally. Oxygen uptake was determined manometrically with fresh preparations as described by Potter (1972). Cytochrome *c* (16 μ M) was added to the reaction system as indicated. The values are the means $\pm$ S.D. for four independent determinations. Where no S.D. is given, the values are means of two determinations. **Significance $P < 0.01$ (fed versus starved).

Liver preparation	Addition	Succinate oxidase activity (ng-atoms of O/min per mg of protein)			
		Fed	Starved	Fed + clofibrate	Starved + clofibrate
Cells	None	30 $\pm$ 4	14 $\pm$ 6**	27 $\pm$ 3	38
	Cytochrome <i>c</i>	45 $\pm$ 6	56 $\pm$ 9	36 $\pm$ 3	38 $\pm$ 9
Homogenate	None	62 $\pm$ 14	39 $\pm$ 4**	82	79 $\pm$ 9
	Cytochrome <i>c</i>	69 $\pm$ 9	73 $\pm$ 8	86	77
Mitochondria	None	194	115**	202	202
	Cytochrome <i>c</i>	212	220	218	218

Table 5. *Effect of starvation and clofibrate administration on ferricyanide reduction by rat liver mitochondria*

The reaction system (1 ml) contained 3 mM-KCN and 10 mM-K₃Fe(CN)₆ in addition to the reagents used for the manometric determination of oxidative phosphorylation. Rotenone (1.3 μ M) was added when succinate was used as substrate. Mitochondria (1 mg of protein) were added last. After incubation for 10 min at 30°C, the reaction was stopped by the addition of 1 ml of HClO₄ (10%, w/v). Ferricyanide concentration was measured by the A_{420} by using a Unicam SP.100 spectrophotometer. The values are the means $\pm$ S.D. for four determinations. The animals were starved for 8 days where indicated. The rate of ferricyanide reduction is expressed in nmol/min per mg of protein.

Substrate	Measurement	Fed	Starved	Fed + clofibrate	Starved + clofibrate
Pyruvate + malate	K ₃ Fe(CN) ₆ reduction	147 $\pm$ 9	149 $\pm$ 20	142 $\pm$ 15	126 $\pm$ 6
	P/2e ⁻	1.7 $\pm$ 0.1	1.4 $\pm$ 0.2	1.4 $\pm$ 0.2	1.5 $\pm$ 0
Succinate	K ₃ Fe(CN) ₆ reduction	407 $\pm$ 28	398 $\pm$ 52	355 $\pm$ 10	327 $\pm$ 35
	P/2e ⁻	0.7 $\pm$ 0.1	0.6 $\pm$ 0.1	0.7 $\pm$ 0	0.8 $\pm$ 0.1

(375 ng-atoms of O/min per mg after 8 days). Also, ferricyanide reductase activity of freshly prepared mitochondria, which eliminates the third site of phosphorylation, did not decrease on starvation (Table 5). When ferricyanide was used as the electron sink, the rates of electron transport and phosphorylation were similar to those obtained with oxygen, but were unaffected by starvation. Addition of cytochrome *c* had no stimulatory effect on the rate of ferricyanide reduction. Also, removal of cytochrome *c* from the mitochondria by extraction with salt solution, which decreased the respiratory activity to zero, did not have any influence on the rate of ferricyanide reduction and attendant phosphorylation (results not shown). Ferricyanide reduction was sensitive to antimycin A in all cases and to rotenone when pyruvate + malate was used as the substrate.

Influence of freezing and thawing

It is known that freezing and thawing, which

evidently causes membrane damage, decreases mitochondrial respiratory rate (Krishnakantha *et al.*, 1975). Drastic decreases of respiratory control and ADP/O ratios were also observed. Addition of cytochrome *c* to frozen and thawed preparations caused an increase in respiratory rate (Table 6). However, full activity was not regained. Also, the loss of phosphorylation was not corrected.

Freezing and thawing also decreased ferricyanide reductase activity and completely abolished attendant phosphorylation. Addition of cytochrome *c* to the frozen preparation did not enhance ferricyanide reduction. Also, the frozen and thawed preparation showed considerable antimycin A-insensitive activity, in agreement with earlier observations (Lee *et al.*, 1967).

K_m of cytochrome oxidase

Within limits, the increase in oxidative activity on addition of cytochrome *c* to mitochondria isolated from starved animals was dependent on the con-

Table 6. *Effect of freezing and thawing on respiratory activity of hepatic mitochondria isolated from normal and starved rats*

Mitochondria isolated from livers of control and starved (8 days) rats were kept frozen overnight at -15°C . Cytochrome *c* (Cyt. *c*; $17\mu\text{M}$) and antimycin A ($1\mu\text{g}/\text{mg}$ of protein) were added to the reaction system as indicated. Activity is expressed as ng-atoms of O taken up or nmol of $\text{K}_3\text{Fe}(\text{CN})_6$ reduced/min per mg of mitochondrial protein. Other conditions were the same as described in the legends for Tables 1 and 5. There was no phosphorylation with frozen preparations.

Reaction	Mitochondria	Measurement	Addition	Fed	Starved
Succinate oxidation	Fresh	Oxygen uptake	—	239	151
			Cyt. <i>c</i>	—	218
		P/O	—	1.4	1.2
	Frozen	Oxygen uptake	Cyt. <i>c</i>	—	1.2
			—	58	37
		P/O	Cyt. <i>c</i>	177	138
Succinate- $\text{K}_3\text{Fe}(\text{CN})_6$ reduction	Fresh	$\text{K}_3\text{Fe}(\text{CN})_6$ reduction	—	253	241
			—	1.0	0.6
		P/ $2e^-$	—	158	180
	Frozen	$\text{K}_3\text{Fe}(\text{CN})_6$ reduction	—	111	225
			Cyt. <i>c</i>	95	30
			Antimycin A		

Table 7. *Effect of starvation and clofibrate administration on the cytochrome *c* content of hepatic tissue and isolated mitochondria*

The procedure for the removal of contaminating haem and the determination of cytochrome *c* are given in the Materials and methods section. From the reduced-oxidized spectra of the pyridine haemochrome, cytochrome *c* content was estimated by using $A_{550} = 18.5\text{mm}^{-1}\cdot\text{cm}^{-1}$. Starvation was for 8 days. Clofibrate ($50\text{mg}/\text{rat}$ per day) was administered as indicated. The values are the means $\pm$ S.D. for six or eight independent estimations. **Significance $P < 0.01$ (fed versus starved).

Hepatic preparation	Fed	Starved	Fed + clofibrate	Starved + clofibrate
Tissue (nmol/g fresh wt. of liver)	12.8 ± 3.3	13.0 ± 3.1	12.0 ± 2.3	14.2 ± 1.4
Mitochondria (pmol/mg of protein)	218 ± 20	$99 \pm 20^{**}$	203 ± 9	189 ± 10

centration of cytochrome *c*. This raised the possibility that the increased requirement for the pigment was due to a change in the affinity of cytochrome oxidase for cytochrome *c*. The K_m of cytochrome oxidase for ferrocytochrome *c* was determined by depleting intact mitochondria of the pigment by extraction with salt (Jacobs & Sanadi, 1960). In all experiments, depleted mitochondria showed zero oxidation. The K_m for mitochondria from control animals was $6.1 \pm 0.3\mu\text{M}$ and that for 8-day starved animals was $5.6 \pm 1.8\mu\text{M}$ (mean $\pm$ S.D. for six determinations). This indicated that the affinity of cytochrome oxidase for cytochrome *c* did not change during starvation. The requirement for cytochrome *c* shown by mitochondria isolated from starved animals is not due to an increase in K_m for the pigment. Administration of clofibrate appeared to enhance the affinity of cytochrome oxidase for cytochrome *c*, the K_m values being $3.0 \pm 0.5\mu\text{M}$ and

$2.9 \pm 0.2\mu\text{M}$ for mitochondria from clofibrate-fed control and clofibrate-fed starved (8 days) animals respectively.

*Concentration of cytochrome *c* in mitochondria*

The above observations raised the possibility that starvation might decrease the concentrations of cytochrome *c* in the tissue. The concentrations of the pigment in whole liver and isolated mitochondria were determined. The results are presented in Table 7. Strangely enough, the total quantity of cytochrome *c* + *c*₁ present in the liver tissue was unaffected by starvation. However, mitochondria isolated from starved animals contained less than half the cytochrome *c* present in mitochondria isolated from control animals. Administration of clofibrate during starvation prevented the decrease in mitochondrial cytochrome *c* (Table 7). Our observation that the concentration of cytochrome *c* in

Fig. 1. *Binding of cytochrome c to rat liver mitochondria*

Cytochrome *c* (concentration indicated) was mixed with 5 mg of freshly prepared rat liver mitochondria, which had been previously depleted of cytochrome *c* by salt treatment. The details are given in the Materials and Methods section. The pattern of binding of the pigment to mitochondria from control (●) and 8-day-starved (○) animals is shown. The saturation of high-affinity binding sites is indicated in inset (a) and the Scatchard plot is shown in inset (b) (abbreviation: cyt. *c*, cytochrome *c*). The Scatchard plots were fitted by the method of least squares.

rat liver does not change during starvation is in agreement with earlier observations (Lemberg & Barrett, 1973).

Binding sites for cytochrome c

The results presented in Table 7 raised the possibility that in the starving animal the ability of mitochondria to bind cytochrome *c* is impaired. The binding of cytochrome *c* to depleted mitochondria from control and starved animals was therefore studied. Equilibrium binding showed a biphasic pattern similar to that observed by Hanstein & Hatefi (1974) for uncoupler binding. From the part of Fig. 1 at high concentrations of cytochrome *c*, which represents low-affinity binding, the contribution by non-specific binding was calculated as 4.8 and 4.3 pmol/mg of protein per μM -cytochrome *c* respectively for normal and defective mitochondria. By subtracting the contribution of non-specific binding from total binding, a saturation curve characteristic of specific binding was obtained in both cases (Fig. 1, inset a). The concentration of binding sites and dissociation constants were calculated from Scatchard plots (Fig. 1, inset b).

The concentration of high-affinity binding sites for cytochrome *c* was 269 pmol/mg of mitochondrial protein, for mitochondria freshly isolated from the

livers of control animals. Mitochondria isolated from 8-day-starved animals showed a 35% decrease in the concentration of the binding sites for the pigment (177 pmol/mg of protein). The dissociation constant (K_d) showed a concomitant increase ($K_d = 60.5 \mu\text{M}$ for mitochondria from control animals; $K_d = 83.5 \mu\text{M}$ for mitochondria from 8-day-starved animals). The results show that in normal mitochondria more than 80% of the binding sites are saturated, whereas in mitochondria from starved animals only 55% of the sites are saturated.

Discussion

The results presented in this paper give clear indications that on starvation hepatic mitochondria show a decreased rate of substrate oxidation, owing to a specific decrease of one of the electron-transport components, namely cytochrome *c*. Strangely enough, mitochondria isolated from starved animals, which show a 50% decrease in cytochrome *c* content, also show about the same decrease in oxidative activity. This would indicate that the amount of cytochrome *c* present in the mitochondrion is enough to saturate the binding sites on the organelle, a surmise amply supported by the results of our binding studies. The ability of exogenously added

cytochrome *c* to be incorporated into the mitochondrial structure in deficient organelles (Wainio, 1970) is consistent with the location of the pigment on the mitochondrial inner membrane as an extrinsic protein (Racker, 1970). Our results would be inconsistent with the view that the mitochondrial outer membrane is impenetrable to externally added cytochrome *c* (Wojtczak & Zaluska, 1969). Our results also do not offer any direct support to the view which ascribes a coupling role for the pigment (Avi-dor & Streichman, 1969).

The decrease in cytochrome *c* concentration in mitochondria isolated from starved animals does not appear to be an artifact of isolation or a reflection of the general degradative environment prevailing in the cell during starvation. Homogenates and whole cells also show decreased oxidation. The concentration of cytochrome *c* in liver tissue does not decrease on starvation. This would mean that under the conditions prevailing in the cell the pigment does not attach itself to the mitochondrion. Again, this does not seem to be due to general membrane damage, at least of the type that occurs during freezing and thawing. The possibility of starvation-induced modifications in the molecule of the pigment requires investigation.

Storage of isolated mitochondria for long periods is known adversely to affect oxidative phosphorylation and respiratory control (Inamdar *et al.*, 1974, 1975). In our experiments this could not have contributed to the difference, because we have not allowed the mitochondria to 'age' after isolation. Mitochondrial respiratory control (succinate as substrate) did not show any significant change between control and starved groups (Table 2).

Another point that merits discussion is the observation that phosphorylation and electron-transport activity associated with ferricyanide reduction are not affected by depletion of mitochondrial cytochrome *c*. This would make it unlikely that ferricyanide taps electrons from cytochrome *c* in the respiratory chain, as is generally believed. The possibility that the iron-sulphur centre in the ubiquinone-cytochrome *c* reductase (Complex III) segment of the chain (Silman *et al.*, 1967) is the species actually involved in this interaction appears attractive.

The disassociation of cytochrome *c* from the mitochondrial membrane, together with the decrease in the population of the organelles (Scarpelli *et al.*, 1971; Chhabra *et al.*, 1979) themselves, offers a plausible explanation for the well-known lowering of basal metabolic rate in starvation. The intriguing point is how the mitochondrial membrane or the pigment becomes modified in starvation so that the association between them is hampered. The lowering of basal metabolic rate in thyroidectomy

involves, in contrast, a decrease in the concentration of cytochrome *c* in the tissue itself.

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