Changes in the Liver Mitochondrial Oxidation of Succinate during Cold-Exposure

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1. Exposure of rats to low environmental temperature resulted in increased activities of several hepatic oxidative-enzyme systems. 2. Simultaneous with increase in liver ubiquinone in cold-exposed rats, the ubiquinone-dependent succinate-neotetrazolium chloride reductase activity also increased. Such an increase could also be obtained by enriching liver with ubiquinone by feeding with an exogenous source. 3. Succinate-neotetrazolium chloride reductase activity could be increased by preincubation of mitochondria with succinate and the mechanism of this activation appears to be different from that obtained on addition of ubiquinone. 4. Succinate-neotetrazolium chloride reductase activity was found to be more labile than succinate dehydrogenase on freezing and thawing and storage, and the presence of succinate gave protection against this loss in hepatic mitochondria obtained from both normal and cold-exposed animals.

The biochemical basis of production of extra heat during chronic cold-exposure of animals has been a subject of extensive investigation (summarized by Masaro, 1966). To explain the increased heat production, Potter (1958) postulated that cold-exposure may activate electron-transport pathways of low phosphorylating efficiency. Uncoupling of oxidative phosphorylation in liver mitochondria of cold-exposed animals was reported by some investigators (Smith & Fairhurst, 1958; Laindes & Beyer, 1960), but this could not be reproduced by other workers (Frehn & Anthony, 1963; Patkin & Masaro, 1960; Aldridge & Stoner, 1963). However, several reports are available on increased activities of electron-transport systems in mitochondria from livers of cold-exposed animals which are not coupled to energy conservation and therefore can be considered as calorogenic shunt pathways (Hannon, 1963; Beyer, 1963; Reynafarje & Chaffe, 1960). Ubiquinone, an electron-transport component, increased in concentration several-fold in livers of cold-exposed rats (Beyer, Noble & Herschfeld, 1962) owing to a combination of increased synthesis and decreased degradation (Aithal, Joshi & Ramasarma, 1968). Beyer *et al.* (1962) put forward an interesting hypothesis that the increased ubiquinone might play a significant role in heat production. Succinate-neotetrazolium reductase (EC 1.3.99.1) is known to be an ubiquinone-dependent enzyme system (Lester & Smith, 1961), and this system may therefore act as a bypass of electrons. Further, the stimulation of the

activity in oxidative metabolism can be brought about by modulation of activity of the preformed enzyme or by increasing its concentration by *de novo* synthesis. With these observations in view, the oxidation of succinate by liver mitochondria in cold-exposed rats was studied with special reference to succinate dehydrogenase (EC 1.3.99.1) and succinate-neotetrazolium reductase.

EXPERIMENTAL

Animals. Male albino rats (weighing 150-170 g) from the stock colony were used. The diet consisted of vitamin-free casein (20%), peanut oil (5%), sugar (10%), salt mixture (U.S. Pharmacopeia, 1947) (5%) and starch (60%). Vitamins were provided according to U.S. Pharmacopeia (1947). Food and water were given *ad lib*.

Exposure to cold. The rats were exposed to low environmental temperature of 0-5°C in a refrigerated chamber which was provided with a slow stream of fresh air. During this period these animals received vitamin B-complex daily and fat-soluble vitamins three times a week instead of on alternate days and weekly respectively, given for control animals at room temperature. The quantity of food consumed also progressively increased up to twofold during cold exposure.

Subcellular fractions. The livers were perfused with cold 0.9% NaCl before removal from the animals, which were killed by stunning and decapitation. They were homogenized in 10 vol. of cold 0.25 M-sucrose medium [containing 0.01 M-tris (pH 7.4) and 5 mM-EDTA] in a Potter-Elvehjem homogenizer. The subcellular fractions were obtained by differential centrifugation by the procedure of Schneider & Hogeboom (1950), and were

washed once with the homogenizing medium and resuspended in it (1 ml/g of liver homogenized).

Enzyme assays. Succinate dehydrogenase activity was determined at 25°C spectrophotometrically with either dichlorophenol-indophenol or phenazine methosulphate-dichlorophenol-indophenol as electron acceptor, by the methods of Green, Mii & Kohout (1955) and Arrigoni & Singer (1958) respectively. Succinate oxidase and cytochrome oxidase (EC 1.9.3.1) were determined at 30°C manometrically by measuring oxygen uptake (Potter, 1957). The details of these methods were described by Aithal & Ramasarma (1969). Oxidation of succinate coupled to phosphorylation was determined at 30°C by the method of Green, Smith & Zeigler (1957) by using a hexokinase trap. Succinate-neotetrazolium reductase activity in mitochondria was determined by measuring the formazan produced on enzymic reduction of neotetrazolium (Slater, 1963). The reaction mixture consisted of 70 µmol of potassium phosphate buffer, pH 7.4, 50 µmol of succinate, 1.5 µmol of neotetrazolium, 0.6 µmol of KCN (freshly neutralized) and about 1 mg of mitochondrial protein in a total volume of 1.1 ml. The reaction mixture was incubated for 10 min at 37°C and then 1.0 ml of 10% (w/v) trichloroacetic acid was added. The formazan produced was extracted with 5 ml of ethyl acetate and measured by its E_{510} . NADH-neotetrazolium reductase (EC 1.6.99.3) activity was similarly determined except that 1.5 µmol of NADH was used in place of succinate and the incubation time decreased to 5 min. NADH-cytochrome *c* reductase (EC 1.6.99.3) was measured at 25°C by the method of Mackler & Green (1956). Only about 0.1 mg of protein was used when microsomal fractions were tested. The enzyme activities are expressed as units/mg of protein, 1 unit being 1 µmol of dye reduced/min or 1 µg-atom of oxygen utilized/min.

Preincubation of mitochondria with succinate and washing. Mitochondria (about 1 mg of protein) were preincubated in potassium phosphate buffer (70 µmol), pH 7.4, with 50 µmol of succinate in a volume of 1.3 ml at 37°C for 7 min (Kimura, Hauber & Singer, 1967). The enzyme activities were then determined without further addition of succinate. The activated preparation was diluted with 2 vol. of the sucrose medium and sedimented at 8800g for 10 min in a refrigerated Sorvall RC-2 centrifuge. The sediment obtained was again suspended in 2 vol. of the sucrose medium and re-centrifuged. The process was repeated another time. In these experiments generally about 50 mg of mitochondrial protein was used.

Determinations. Protein was determined by the biuret method (Gornall, Bardawill & David, 1949) by using deoxycholate for solubilization. Inorganic phosphate was measured by the method of Fiske & SubbaRow (1925). Ubiquinone was determined spectrophotometrically from the decrease in E_{275} obtained on addition of NaBH₄ to an ethanolic solution of the sample obtained after column chromatography of non-saponifiable lipids of the livers (Joshi, Jayaraman & Ramasarma, 1963).

RESULTS

Changes in some oxidative enzyme systems. Exposure of rats to low environmental temperature resulted in increased activities of several hepatic oxidative-enzyme systems (Table 1). These results

are in general agreement with previous reports (Hannon, 1963; Beyer, 1963; Reynafarje & Chaffe, 1960). Activities of both succinate oxidase and succinate dehydrogenase in mitochondria increased progressively with the period of exposure to cold. However, succinate dehydrogenase activity tended to decrease on prolonged exposure, though the activity was still above that in unexposed rats, an observation similar to that reported for malate dehydrogenase (Hannon, 1960). The results on oxidative phosphorylation again confirm the lack of uncoupling. Although there was no appreciable increase in NADH-cytochrome *c* reductase activity, NADH-neotetrazolium reductase activity showed a twofold increase. This is surprising, since the two activities are supposed to be a measure of the same enzyme protein. Therefore it seems possible that this enzyme system using the unspecific acceptors may form an alternative calorigenic electron-transport route.

The energy conserved during microsomal electron transport is negligible and presumably is dissipated as heat. The results for microsomal oxidation of NADH by cytochrome *c*, also shown by Beyer (1963), or by neotetrazolium increased nearly twofold, implicating such a possibility.

Changes in succinate-neotetrazolium reductase activity. Simultaneously with the increase in concentration of ubiquinone, mitochondrial succinate-neotetrazolium reductase activity increased progressively with the period of cold-exposure (Fig. 1). Similar increase in the enzyme activity could also be obtained in livers enriched in ubiquinone by orally dosing rats at room temperature with exogenous ubiquinone (Table 2). This is the first demonstration of increased activity of this ubiquinone-dependent enzyme system by artificially increasing ubiquinone concentration in intact animals. But it should be pointed out that a large proportion of the increased ubiquinone does not participate *in toto* in this enzyme system, since the specific activity of the enzyme on the basis of ubiquinone content progressively decreased.

Effect of preincubation of mitochondria with succinate and subsequent washing. The activity of succinate dehydrogenase increased severalfold when soluble or particulate enzyme preparations were preincubated with succinate for a short time (Kimura *et al.* 1967; Kearney, 1957). It was suggested that the increased activity resulted from conformational changes in the enzyme protein molecule. We have found that the rat liver mitochondrial succinate dehydrogenase could be activated about twofold by such treatment both in normal and cold-exposed animals (Table 3).

Succinate-neotetrazolium reductase activity in rat liver mitochondria could also be activated by preincubation with succinate (Table 3). As with

Table 1. *Changes in the mitochondrial and microsomal enzyme activities in livers of rats exposed to cold*

Rats were exposed to low environmental temperature (0–5°C) for the specified number of days. Mitochondrial and microsomal preparations from the livers of such animals were tested for the enzyme activities as described in the Experimental section: succinate dehydrogenase was determined by the dichlorophenol-indophenol method. The values given are means ± s.d. of the samples independently processed from six rats in each group.

Activity	Enzyme activity (munits/mg. of protein)				
	0	5	10	20	40
Mitochondrial enzymes					
Oxidation of succinate					
(a) Succinate oxidase	76 ± 11	88 ± 9	—	102 ± 13	108 ± 11
(b) Succinate dehydrogenase	7.3 ± 0.8	9.2 ± 0.6	11.3 ± 1.6	10.6 ± 1.3	10.1 ± 0.7
(c) Coupled phosphorylation					
Oxygen uptake	43 ± 5	49 ± 4	55 ± 6	62 ± 3	66 ± 5
P _i esterified	65 ± 8	84 ± 5	78 ± 8	81 ± 9	95 ± 7
P/O ratio	1.5 ± 0.2	1.7 ± 0.2	1.4 ± 0.4	1.3 ± 0.2	1.4 ± 0.3
(d) Succinate-neotetrazolium reductase	5.1 ± 0.4	6.7 ± 0.6	7.5 ± 0.8	7.0 ± 0.7	8.1 ± 0.6
Cytochrome oxidase	420 ± 57	470 ± 62	472 ± 39	510 ± 45	484 ± 52
NADH–cytochrome c reductase	48 ± 8	50 ± 8	53 ± 9	53 ± 9	52 ± 7
NADH–neotetrazolium reductase	39 ± 5	76 ± 4	85 ± 10	79 ± 9	90 ± 6
Microsomal enzymes					
NADH–cytochrome c reductase	345 ± 28	420 ± 44	404 ± 36	460 ± 38	560 ± 33
NADH–neotetrazolium reductase	82 ± 7	130 ± 11	154 ± 17	140 ± 16	152 ± 22

Fig. 1. Changes in mitochondrial ubiquinone concentration (○) and succinate–neotetrazolium reductase activity (●) in livers of rats exposed to cold. Each point represents the mean value obtained from independent analyses of six rats.

succinate dehydrogenase activity, the extent of activation on preincubation was higher in liver mitochondria from cold-exposed rats, indicating increased enzyme concentration.

These results suggest that the increased activity in the cold-exposed rats was due to an increase in the concentration of the enzyme and not to alteration of the activity of the existing protein, in the event of which the maximum 'succinate-activated' rates

would be expected to be the same as the unexposed control values.

Aithal & Ramasarma (1969) showed that on washing with sucrose medium the 'succinate-activated' preparations of mitochondria revert to basal activity. When this experiment was repeated with mitochondrial samples from cold-exposed rats the washing procedure caused the activity to revert to the initial value for the mitochondrial preparation (Table 3). Therefore the activation by succinate was an easily reversible process in both mitochondrial preparations. The same results were obtained with succinate-neotetrazolium reductase (Table 3).

Addition of ubiquinone *in vitro* is known to activate succinate-neotetrazolium reductase (Lester & Smith, 1961), but not succinate dehydrogenase. The combined effect of ubiquinone and preincubation with succinate was more than the sum of the individual effects (Table 4). The two types of activation appear to be achieved by different mechanisms.

Effect of storage and freezing and thawing of mitochondria. It was previously shown that the presence of succinate during storage of mitochondria stabilized the succinate dehydrogenase activity particularly against the lability of the partially

Table 2. *Effect of supplementing ubiquinone in the diet on the mitochondrial succinate-neotetrazolium reductase in livers of rats at room temperature*

Ubiquinone-9 dissolved in peanut oil was administered orally (1 mg/day per rat). The enzyme activity was determined as described in the Experimental section. The values are means of independent determinations of three rats in each group.

Experimental period (days)	Ubiquinone (nmol/g of liver)	Activity of succinate-neotetrazolium reductase (munits/mg of protein)
0	150	4.1
10	220	4.9
40	530	7.0

Table 3. *Effect of preincubation with succinate and subsequent washing of mitochondria on the activities of succinate dehydrogenase and succinate-neotetrazolium reductase in livers of rats exposed to cold*

Mitochondria were preincubated once with succinate and washed with the homogenizing medium by diluting with it and washing as described in the Experimental section. Each sample was again preincubated with succinate and the enzyme activities thus obtained are referred as 'succinate-activated in assay'. Succinate dehydrogenase was assayed by the phenazine methosulphate-dichlorophenol-indophenol method. The values are the means of two independently analysed samples from three pooled livers. The rats were exposed to 0–5°C for 15 days as described in the Experimental section.

Activity	Treatment of mitochondria	Enzyme activity (munits/mg of protein)			
		Room temperature (about 25°C)		Cold exposed (0–5°C)	
		Unactivated	'Succinate-activated in assay'	Unactivated	'Succinate-activated in assay'
Succinate dehydrogenase	None	17.1	41.4	23.5	51.6
	Preincubated once with succinate and washed once	18.6	42.0	27.2	50.8
	Preincubated once with succinate and washed twice	17.8	39.7	21.8	51.3
Succinate-neotetrazolium reductase	None	4.8	7.8	6.4	9.5
	Preincubated once with succinate and washed once	5.4	8.2	6.8	9.8
	Preincubated once with succinate and washed twice	5.2	7.6	6.5	9.7

Table 4. *Effect of addition of ubiquinone in vitro on the mitochondrial succinate-neotetrazolium reductase activity*

Ubiquinone-9 (10 μ g) in 0.05 ml of ethanol was dispersed in the phosphate buffer used in the assay to which mitochondria (1 mg of protein) were added. Preincubation with succinate was then carried out. The values are means of independent analysis of two mitochondrial samples.

Additions	Enzyme activity (munits/mg of protein)	
	Unactivated	'Succinate- activated in assay'*
Nil	5.1	8.6
Ubiquinone	7.2	9.8

* Explained in Table 3.

activated state obtained on brief exposure of rats to hypobaric conditions (Aithal & Ramasarma, 1969). Table 5 shows that there was a small decrease during storage in the activity of succinate dehydrogenase in cold-exposed samples. However, addition of small quantities of succinate gave protection to the enzyme from the deleterious effect of repeated freezing and thawing after storage at -20°C . If the mitochondrial preparations were activated by preincubation with succinate and then stored frozen along with the added succinate, the increased activity was retained even after 20 days at -20°C and three freezings and thawings, without further preincubation procedure.

Similar results were obtained with succinate-neotetrazolium reductase except that the decrease in activity during storage was more rapid than with

Table 5. *Protection by succinate of the activated succinate dehydrogenase during freezing and thawing and storage*

Mitochondrial samples were stored in the sucrose medium. Succinate (50 μ mol/ml of medium) was added to two samples, one of which was incubated for 7 min at 37°C . All the samples were frozen at -20°C . The tubes were thawed at 5, 10 and 20 days and portions assayed for succinate dehydrogenase (by the phenazine methosulphate-dichlorophenol-indophenol method) and succinate-neotetrazolium reductase. The values are the means of two independently analysed samples from three pooled livers. The rats were exposed to $0-5^{\circ}\text{C}$ for 15 days as described in the Experimental section.

Activity	Storage at -20°C		Treatment of mitochondria before storage	Preincuba- tion at 37°C for 7 min	Enzyme activity (munits/mg of protein)			
					Room temperature (about 25°C)		Cold exposed ($0-5^{\circ}\text{C}$)	
	Days	Thawings	Addition		Un- activated	'Succinate activated in assay'*	Un- activated	'Succinate activated in assay'*
Succinate dehydrogenase		Initial	—	—	17.3	41.4	23.6	52.0
	5	1	—	—	17.8	41.7	23.2	50.8
			Succinate	—	17.5	40.8	23.5	50.6
			Succinate	+	41.7	—	52.4	—
	10	2	—	—	16.6	40.2	22.7	50.3
			Succinate	—	17.0	40.7	22.8	51.4
			Succinate	+	41.0	—	51.2	—
	20	3	—	—	16.2	40.1	22.1	50.2
			Succinate	—	16.9	41.2	23.4	51.6
			Succinate	+	41.3	—	51.8	—
Succinate-neotetra- zolium reductase		Initial	—	—	4.9	8.1	6.3	9.3
	5	1	—	—	4.4	—	6.0	—
			Succinate	—	4.6	—	6.2	—
			Succinate	+	7.8	—	9.1	—
	10	2	—	—	3.8	6.2	5.4	8.2
			Succinate	—	4.3	7.5	5.8	9.0
			Succinate	+	7.8	—	8.8	—
	20	3	—	—	3.2	5.7	5.1	7.6
			Succinate	—	4.1	7.6	5.6	9.1
			Succinate	+	7.9	—	9.0	—

* Explained in Table 3.

succinate dehydrogenase (Table 5). Preincubation with succinate gave full protection against this loss in mitochondria from both normal and cold-exposed animals.

DISCUSSION

Activation of succinate-neotetrazolium reductase activity on preincubation with succinate, shown for the first time, is of special interest because although the same flavoprotein is involved in all cases only the assays involving reductions of dichlorophenol-indophenol, phenazine methosulphate or neotetrazolium are activated by preincubation with succinate, but not the reduction of FMNH₂ of fumarate (Kimura *et al.* 1967). Further, previous experiments from this laboratory showed activation only of succinate dehydrogenase and not of succinate-neotetrazolium reductase in the liver mitochondria of rats exposed to hypobaric conditions (Aithal & Ramasarma, 1969). It is therefore possible that the active sites at which the dyes interact with the protein in these assays are distinct and may be differentially altered.

Excess of ubiquinone found in cold-exposed rat livers may be responsible in part for the increased succinate-neotetrazolium reductase activity, although the results on succinate dehydrogenase, particularly those obtained on activation by preincubation with succinate, indicate the presence of an increased quantity of the enzyme protein also. It should be pointed out that this system may contribute only a small fraction of the total extra heat generated.

The above studies concern the changes in oxidative metabolism in the liver tissue which contribute only part of the heat production during non-shivering thermogenesis. Other tissues such as the skeletal muscle, the brain and the gut also have an important role. This study of the enzyme system in the liver suggests that a combination of several of the electron-transport systems would probably account for the extra heat. These experiments also show that uncoupling of mitochondrial oxidative phosphorylation is not the mechanism of extra heat production in the liver although such a process may occur in brown-fat mitochondria due to the presence of endogenous free fatty acids (Aldridge & Street, 1968; Guillory & Racker, 1968).

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REFERENCES

- Aithal, H. N., Joshi, V. C. & Ramasarma, T. (1968). *Biochim. biophys. Acta*, **162**, 66.
 Aithal, H. N. & Ramasarma, T. (1969). *Biochem. J.* **115**, 77.
 Aldridge, W. N. & Stoner, H. B. (1963). *Biochim. biophys. Acta*, **78**, 736.
 Aldridge, W. N. & Street, B. W. (1968). *Biochem. J.* **107**, 315.
 Arrigoni, O. & Singer, T. P. (1958). *J. biol. Chem.* **234**, 666.
 Beyer, R. E. (1963). *Fedn Proc. Fedn Am. Socs exp. Biol.* **22** (Suppl. 3), 874.
 Beyer, R. E., Noble, W. M. & Herschfeld, T. J. (1962). *Can. J. Biochem. Physiol.* **40**, 511.
 Fiske, C. H. & SubbaRow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Frehn, J. L. & Anthony, A. (1963). *Am. J. Physiol.* **203**, 821.
 Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
 Green, D. E., Mii, S. & Kohout, D. M. (1955). *J. biol. Chem.* **217**, 651.
 Green, D. E., Smith, A. L. & Zeigler, D. M. (1957). *Biochim. biophys. Acta*, **23**, 516.
 Guillory, R. J. & Racker, E. (1968). *Biochim. biophys. Acta*, **153**, 490.
 Hannon, J. P. (1960). *Am. J. Physiol.* **198**, 740.
 Hannon, J. P. (1963). *Fedn Proc. Fedn Am. Socs exp. Biol.* **22** (Suppl. 3), 856.
 Joshi, V. C., Jayaraman, J. & Ramasarma, T. (1963). *Indian J. exp. Biol.* **1**, 113.
 Kearney, E. B. (1957). *J. biol. Chem.* **229**, 363.
 Kimura, T., Hauber, J. & Singer, T. P. (1967). *J. biol. Chem.* **242**, 4987.
 Laines, S. P. & Beyer, R. E. (1960). *Nature, Lond.*, **188**, 1196.
 Lester, R. L. & Smith, A. L. (1961). *Biochim. biophys. Acta*, **47**, 475.
 Mackler, B. & Green, D. E. (1956). *Biochim. biophys. Acta*, **21**, 1.
 Masaro, E. J. (1966). *Physiol. Rev.* **46**, 67.
 Patkin, J. & Masaro, E. J. (1960). *Am. J. Physiol.* **199**, 201.
 Potter, V. R. (1957). In *Manometric Techniques*, p. 170. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
 Potter, V. R. (1958). *Fedn Proc. Fedn Am. Socs exp. Biol.* **17**, 1060.
 Reynafarje, B. & Chaffe, R. R. J. (1960). *Proc. Soc. exp. Biol. Med.* **103**, 225.
 Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
 Slater, T. F. (1963). *Biochim. biophys. Acta*, **77**, 365.
 Smith, R. E. & Fairhurst, A. S. (1958). *Fedn Proc. Fedn Am. Socs exp. Biol.* **17**, 151.