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ACTIVATION OF THE EXTERNAL PATHWAY OF NADH OXIDATION IN LIVER MITOCHONDRIA OF COLD-ADAPTED RATS

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Summary

15 min cold exposure of rats adapted to cold results in switching on a pathway of the fast oxidation of extramitochondrial NADH in the isolated liver mitochondria. This pathway is sensitive to mersalyl and cyanide, resistant to amytal and antimycin A, and can be stimulated by dinitrophenol. A portion of the endogenous cytochrome *c* pool can easily be removed by washing mitochondria of the cold-exposed rats.

A scheme is discussed, postulating desorption of the inner membrane-bound cytochrome *c* into intermembrane space of mitochondria, resulting in formation of a link between the non-phosphorylating NADH-cytochrome *c* reductase in the outer mitochondrial membrane and cytochrome *c* oxidase in the inner membrane. It is suggested that such an oxidative pathway is involved in the urgent heat production in liver in response to the cold treatment.

Introduction

A cold-induced increase in the heat production by warm-blooded animals is always accompanied by a stimulation of oxygen consumption in a number of tissues. Liver was found to be involved in responses of this type [1]. It is in liver that the P/O ratio decrease was first described in the cold-adapted animals [2,3]. Later Beyer [4] showed the decrease in the amytal sensitivity of respiration of the liver pieces of cold-adapted rats, suggesting the activation of the external pathway of NADH oxidation in mitochondria and/or microsomes. It was proposed in this group [5] that the mechanism of the cold adaptation-induced activation of the external oxidation pathway consists in the partial desorption of cytochrome *c* pool from the inner mitochondrial membrane into the intermembrane space. In this case, a link may be formed between (i) the extremely active system of free (non-coupled) electron transfer

via NADH-cytochrome b_5 reductase (fp_5) and cytochrome b_5, localized in the outer mitochondrial membrane, and (ii) cytochrome oxidase, another potent electron transfer system localized in the inner membrane. As a result, energy coupling sites I and II of the respiratory chain can be bypassed.

In the present paper the data supporting this hypothesis will be presented.

Methods

Female Wistar rats (150 g) were studied. The animals were kept in 0.05 m^3 cages containing 6 to 10 rats. The experiments were carried out mainly with two groups of animals. One group (control) was kept at 24—26°C up to the moment of decapitation. The other group was exposed for 6—8 h to 2—4°C daily and to about 24—26°C for the rest of the time for about 1.5 months; 3—14 h before decapitation, the animal was kept at room temperature and then placed into a cold room (2—4°C) for 15 min.

Twice distilled water was used to dissolve all water-soluble reactants. Mitochondria were isolated and suspended in the medium containing 0.3 M sucrose, 10 mM Tris · HCl, pH 7.4. Cooled for 1 min, liver was homogenized manually with a Teflon pestle for 40 s (the medium-to-tissue ratio was 8 : 1). Then the mixture was centrifuged for 10 min at 600 × *g*. The resulting supernatant (up to the very sediment) should be poured off and used for second centrifugation (for 10 min at 12 000 × *g*). The mitochondrial sediment was suspended in isolation medium (about 40 mg protein per ml of the medium). Protein was determined by the Biuret method. About 2 mg of mitochondrial protein per ml were added into the polarographic cell. To measure mitochondrial respiration a slightly hypo-osmotic incubation medium was used, containing 70 mM KCl, 10 mM Tris · HCl and 5 mM potassium phosphate, pH 7.5.

Oxygen concentration was measured by a Clark type electrode and LP-60 polarograph. The temperature of the incubation medium was 27—28°C.

Differential spectra of mitochondrial cytochromes (reduced minus oxidized) were measured in a low temperature differential double-beam spectrophotometer. For reduction or oxidation of cytochromes, cyanide or amytal, respectively, the following solution was added: 0.3 M sucrose, 10 mM Tris · HCl, pH 7.4, 40 μM 2,4-dinitrophenol and 0.5 mM NADH. Absorption (ΔD^{λ}) in the maximum of the α-band of cytochrome c_1 and c was measured against the baseline drawn through 590 nm and 572 nm points of the spectrum.

The cytochrome c-to-c_1 ratio was calculated as

$$\frac{[\text{cyt} \cdot c]}{[\text{cyt} \cdot c_1]} = \frac{\Delta A_{550}}{\Delta A_{554}} \times \frac{K_{c_1}}{K_c},$$

where ΔA_{550} and ΔA_{554} are the absorptions at corresponding wavelengths; K_c and K_{c_1}, cytochrome c and c_1 extinction coefficients equal to 22 and 19 absorbance units · cm^{-1} · mM^{-1}, respectively.

The cytochrome concentration was measured in the mitochondrial suspension before washing and after the first and the second washings of mitochondria. The procedure of washing was the following. Mitochondria were incubated for 1—2 min at 27°C in the incubation mixture: 70 mM KCl, 10 mM Tris · HCl, 5 mM potassium phosphate, 4 mM glutamate and 1 mM malate (pH 7.5). Then

40 μM dinitrophenol was added. 1 min later the mitochondrial suspension was diluted with a five-fold volume of the cold isolation mixture (0.3 M sucrose, 10 mM Tris · HCl; pH 7.4) and then the suspension was centrifuged for 10 min at 12000 × *g*. The mitochondrial sediment was resuspended in the isolation mixture and used for measuring the difference spectrum.

Tris (from Sigma), cytochrome *c* (from Biomed-Krakov), NADH, NADPH and ascorbate (from Reanal) were used. Dinitrophenol was twice crystallized in alcolhol.

Results

In Table I the rates of oxidation of several hydrogen donors are given. The following changes in the respiratory activity parameters are demonstrated in mitochondria from the cold-adapted rats exposed to cold before decapitation:

1. The stimulation of glutamate + malate oxidation in the absence of dinitrophenol.
2. The lowering of the maximal rate of glutamate + malate oxidation observed in the presence of dinitrophenol.
3. The decrease in the sensitivity of glutamate + malate oxidation to amytal and antimycin A.
4. The great stimulation of oxidation of added NADH and some increase in the ascorbate oxidations in the presence of antimycin A and amytal. This

TABLE I

EFFECT OF COLD TREATMENT OF RAT ON THE RESPIRATION OF LIVER MITOCHONDRIA

In parentheses is given the number of animals. Incubation mixture: 70 mM KCl, 10 mM Tris · HCl, 5 mM potassium phosphate, 4 mM glutamate, 1 mM malate, pH 7.5. Additions: $4 \cdot 10^{-5}$ M dinitrophenol, 1.6 mM amytal, $8 \cdot 10^{-7}$ M antimycin A, 0.6 mM NADH, $2 \cdot 10^{-5}$ M cytochrome *c*, 2 mM ascorbate, 1.5 mM NaCN. Control group: animals were kept at 24—26°C. Cold-treated group: animals were exposed for 6—8 h to 2—4°C daily and to 24—26°C for the rest of the time for about 1.5 months; 3 h before decapitation, they were kept at room temperature and then placed in the cold (2—4°C) for 15 min.

Additions	Oxygen consumption (nmol $O_2 \cdot mg^{-1}$ protein $\cdot min^{-1}$)	
	Control group	Cold-treated group
No additions	7.2 ± 0.3 (9)	9.2 ± 0.2 (8)
Dinitrophenol	25.2 ± 1.0 (9)	11.0 ± 0.3 (8)
Dinitrophenol + amytal	4.8 ± 0.1 (9)	8.0 ± 0.1 (8)
Dinitrophenol + amytal + antimycin A	3.3 ± 0.2 (9)	5.8 ± 0.2 (8)
Dinitrophenol + amytal + antimycin A + NADH	3.8 ± 0.3 (9)	18.7 ± 1.1 (8)
Dinitrophenol + amytal + antimycin A + NADH + cytochrome *c*	25.8 ± 0.5 (9)	26.6 ± 1.0 (8)
Dinitrophenol + amytal + antimycin A + ascorbate	2.1 ± 0.2 (8)	10.9 ± 0.6 (8)
Dinitrophenol + amytal + antimycin A + NADH + NaCN	—	1.1 ± 0.1 (5)

TABLE II

THE EFFECT OF THE WASHING OF MITOCHONDRIA ON THE CYTOCHROME *c*-TO-CYTOCHROME c_1 RATIO

Incubation mixture: 0.3 M sucrose, 10 mM Tris · HCl, pH 7.5; 40 μM dinitrophenol, 0.5 mM NADH. 2 mM NaCN was added to one spectrophotometric cell and 3.2 mM amytal to the other. For conditions of washing of mitochondria see Methods. Cold treatment as in Table I.

Animals	Cytochrome *c*-to-cytochrome c_1 ratio		
	Before washing	After the 1st washing	After the 2nd washing
Control	1.76 · 0.05 (7)	1.71 · 0.01 (7)	1.63 : 0.01 (7)
Cold-treated	1.65 ± 0.07 (6)	1.22 ± 0.02 (6)	0.82 · 0.01 (6)

difference disappeared when the reaction mixture was supplemented with cytochrome *c* which stimulated respiration much stronger in the control group than in the cold-treated one.

Respiration in the presence of NADH was sensitive to cyanide.

One can mention that both the rate of glutamate + malate oxidation in the presence of dinitrophenol and the degree of dinitrophenol-induced stimulation of oxidation of these substrates are rather low. However, adding EDTA or changing the procedure of isolation of mitochondria to enhance these parameters diminished the above-listed differences between the control and the cold-treated groups. There are some indications that trace amounts of Ca^{2+} may be critical for demonstration of the cold-induced changes in mitochondrial functions.

In the next series of experiments, the effect of washing rat liver mitochondria on the cytochrome *c* content was studied. As can be seen in Table II, washing decreases cytochrome *c*-to-cytochrome c_1 ratio in mitochondria of the cold-treated but not of the control group of rats. After the 2nd washing this ratio was found to be two-fold lower in the cold-treated group than in the control one.

Apparently, some loss of the mitochondrial cytochrome *c* pool and its dilution in the incubation medium takes place under conditions of polarographic measurement of respiration of the mitochondria from the cold-treated group. This may be responsible for the cold-induced decrease in the rate of the uncoupled oxidation of glutamate + malate (see above Table I) and *N,N,N',N'*-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) + ascorbate (Table III). In Table III one can see the effect of added cytochrome *c* which was found to increase the TMPD + ascorbate oxidation rate in the cold-adapted rats exposed to cold before decapitation, this rate in the control group remaining unaffected.

It is noteworthy that the cold-induced effect on the ascorbate + TMPD oxidation is due to the influence of 15 min cold exposure of the cold-acclimatized animal before decapitation. At the same time, the cold acclimatization per se proves insufficient to induce the observed effect (Table III).

TABLE III

EFFECT OF COLD TREATMENT ON THE RATE OF ASCORBATE OXIDATION BY RAT LIVER MITOCHONDRIA IN THE PRESENCE OF TMPD

Incubation mixture: 70 mM Tris · HCl, 5 mM potassium phosphate, pH 7.5; additions: 2 mM ascorbate, 400 μM TMPD, 5 μM cytochrome *c*.

Animals	Oxygen comsumption (nmol O_2 · mg^{-1} protein · min^{-1})	
	Ascorbate, TMPD	TMPD, ascorbate, cytochrome *c*
Control	30.7 ± 1.0 (5)	33.8 ± 1.6 (5)
Cold-adapted + 3 h at 24°C before decapitation	31.0 ± 2.7 (5)	39.3 ± 3.1 (50
Cold-adapted + 3 h at 24°C + 15 min at 2—4°C before decapitation	15.9 ± 1.0 (8)	36.6 ± 2.6 (8)

TABLE IV

THE ROLE OF COLD ADAPTATION AND SHORT-TERM COLD EXPOSURE IN THE RESPIRATORY ACTIVITY CHANGES OF RAT LIVER MITOCHONDRIA

Incubation mixure: 70 mM KCl, 10 mM Tris · HCl, 5 mM potassium phosphate, 1 mM malate, 4 mM glutamate, pH 7.5. Consecutive additions: $4 \cdot 10^{-5}$ M dinitrophenol, 1.6 mM amytal, $8 \cdot 10^{-7}$M antimycin A, 0.6 mM NADH.

Animals	Oxygen consumption (nmol O_2 · mg^{-1} protein · min^{-1})			
	—	Dinitrophenol	Amytal + antimycin A	NADH
Control	7.1 ± 0.3 (8)	16.9 ± 0.6 (8)	3.5 ± 0.4 (8)	3.9 ± 0.5 (8)
Cold-adapted + 14 h at 14°C	7.7 ± 0.3 (8)	11.7 ± 0.5 (8)	3.7 ± 0.3 (8)	4.2 ± 0.3 (8)
Cold-adapted + 14 h at 24°C + 15 min at 3°C	10.5 ± 0.2 (15)	11.0 ± 0.2 (15)	6.1 ± 0.7 (15)	18.6 ± 0.6 (15)

TABLE V

THE ROLE OF ION COMPOSITION OF INCUBATION MIXTURE IN DEMONSTRATING THE COLD TREATMENT-INDUCED INITIATION OF EXTERNAL NADH OXIDATION

In all the samples there are 4 mM glutamate, 1 mM malate, $4 \cdot 10^{-5}$ M dinitrophenol, 1.6 mM amytal, $8 \cdot 10^{-7}$ M antimycin A, 0.6 mM NADH.

Animals	Oxygen consumption (nmol O_2 · mg^{-1} protein · min^{-1})		
	70 mM KCl, 5 mM potassium phosphate	70 mM KCl, 5 mM potassium phosphate, 20 μM $CaCl_2$	30 mM KCl, 5 mM potassium phosphate
Control	3.0 ± 0.3 (6)	6.7 ± 0.7 (8)	6.5 ± 0.6 (5)
Control + 15 min at 4°C	2.9 ± 0.2 (5)	4.2 ± 0.2 (6)	5.2 ± 1.0 (5)
Cold-adapted + 14 h at 24°C	3.6 ± 0.4 (7)	17.7 ± 1.7 (7)	18.3 ± 2.2 (5)
Cold-adapted + 14 h at 24°C + 15 min at 3°C	19.8 + 1.2 (11)	—	13.5 ± 0.8 (7)

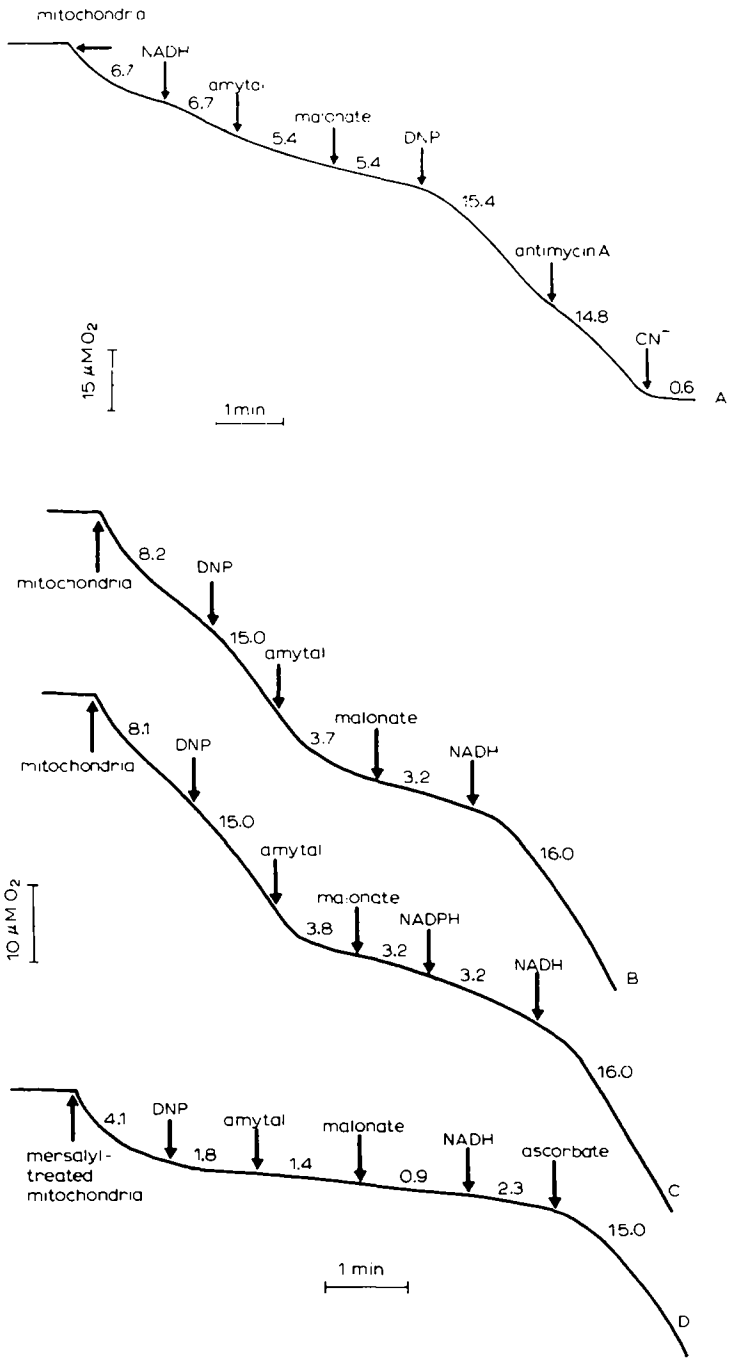

Fig. 1. Kinetics of oxygen consumption by liver mitochondria from the cold-treated rat. Conditions of the cold treatment as in Table I. Incubation mixture: 70 mM KCl, 10 mM Tris · HCl, 5 mM potassium phosphate, pH 7.5, and in Expts. B—D. 4 mM glutamate and 1 mM malate. The numbers near the curves give the rates of oxygen consumption (nmol $O_2 \cdot mg^{-1}$ protein $\cdot min^{-1}$). Additions: mitochondria (2 mg of protein/ml), $4 \cdot 10^{-5}$ M dinitrophenol (DNP), 1.6 mM amytal, $8 \cdot 10^{-7}$ M antimycin A, 2 mM malonate, 0.6 mM NADH or NADPH, 2 mM ascorbate and 1.5 mM NaCN.

The kinetics of the oxygen consumption by mitochondria of a cold-treated rat are given in Fig. 1. It is demonstrated that added NADH (but not NADPH) induces fast oxygen consumption by mitochondria in a mersalyl-sensitive, amytal- and antimycin A-insensitive fashion. Ascorbate oxidation proved insensitive to mersalyl. Dinitrophenol was found to stimulate NADH oxidation in the presence of amytal and malonate. Amytal could be replaced by rotenone (not shown). Antimycin A addition after dinitrophenol did not change the NADH oxidation rate.

The role of some conditions in vivo and in vitro in demonstrating the effects described, are analyzed in Tables IV and V. It was revealed that in slightly hypotonic incubation mixture which was used in all the above experiments, one can observe the cold-induced increase in the rates of glutamate + malate and added NADH oxidation only if the cold-adapted animals were exposed to the cold for 15 min before decapitation. Without this short-term cold treatment both these parameters were at the same level in cold-adapted and control groups. A certain difference between these two groups was observed when the respiration was measured under more hypotonic conditions (the KCl concentration was lowered from 70 mM down to 30 mM), or when the incubation mixture with 70 mM KCl was supplemented with $2 \cdot 10^{-5}$ M $CaCl_2$ (Table V). One can see that in both cases, the rate of the added NADH oxidation was much higher in cold-adapted rats regardless of whether they were or were not exposed to the cold immediately before decapitation.

Discussion

Mechanism of the added NADH oxidation in liver mitochondria from cold-treated rats

The above data indicate that NADH, when added to the liver mitochondria isolated from cold-treated rats, is oxidized via the following pathway:

added NADH $\rightarrow$ fp_5 $\rightarrow$ b_5 $\rightarrow$ $c_{i.m.}$ $\rightarrow$ c_m $\rightarrow$ $a + a_3$ $\rightarrow$ O_2,

where fp_5 denotes NADH-cytochrome b_5 reductase flavoprotein, b_5 cytochrome b_5, $c_{i.m.}$ cytochrome c in the intermembrane space of the mitochondrion, c_m the inner membrane-bound cytochrome c, and $a + a_3$ cytochrome c oxidase.

Involvement of the fp_5-b_5 system in this process is demonstrated by the inhibitor analysis. it was found that the added NADH oxidation is a mersalyl-sensitive, amytal- and antimycinresistant reaction whose rate is almost as high as the rate of uncoupled glutamate + malate oxidation. NADPH could not substituted for NADH in this system. In liver mitochondria, there is no pathway other than fp_5-b_5 to maintain fast oxidation of added NADH which would be characterized by such the inhibitor sensitivity.

As for the participation of cytochrome c oxidase in the above pathway, this is favoured by the fact of the cyanide sensitivity of the added NADH oxidation. It should be noted that the added NADH oxidation is stimulated by dinitrophenol.

In all probability, the role of the link between cytochrome b_5 and cytochrome c oxidase is performed by the intermembrane cytochrome c which is desorbed from the inner membrane surface during cold treatment of the

animal. Indeed, washing of mitochondria from the cold-treated group results in the loss of a portion of the cytochrome *c* pool. Another fact suggesting the presence of cytochrome *c* in the water phase outside the inner mitochondrial membrane is oxidation of ascorbate without added cytochrome *c* or TMPD in mitochondria from the cold-treated rats. It is well known that ascorbate, like extramitochondrial NADH, cannot rapidly reduce membrane-bound cytochrome *c*.

The role of short-term cold exposure and cold adaptation in the external pathway activation

The data of Tables III—V clearly show that both cold adaptation and 15 min cold exposure before decapitation are necessary to induce external NADH oxidation.

The cold adaptation per se affects the external NADH oxidation by the liver mitochondria rather slightly. So, differences between control (warmth-adapted) and cold-adapted groups could be revealed only in certain special conditions, namely in hypotonic KCl solution or in the presence of a small amount of Ca^{2+} (to show Ca^{2+} requirement of large scale changes in energy coupling in liver mitochondria from cold-acclimatized rats, see ref. 6). Short-term cold exposure of the warmth-adapted rats was without effect in all the conditions tested (Table V).

Generally, the ion composition of the incubation mixture was found to be important for demonstration of the cold exposure-induced external NADH oxidation.

Some swelling of mitochondria seems to be favourable for the external NADH oxidase activity to be revealed. EDTA inhibition of several types of mitochondrial swelling, was shown to decrease (in the 0.5 mM concentration) the cold-induced oxidation of the added NADH. On the other hand, the cobra venom phospholipase treatment, lowering of pH, pre-incubation of mitochondria with oleate and some other swelling-inducing influences brought about appearance of some amytal- and antimycin-resistant NADH oxidation in mitochondria of the control group.

Thus, cold treatment of the animal seems to unmask the ability of liver mitochondria to oxidize the added NADH, the effect being mediated by desorption of the membrane-bound cytochrome *c*.

Activation of the external NADH oxidation may play a role in the necessary heat production by the liver tissue in response to a fast decrease in the ambient temperature, since two of the three energy-conserving sites of the mitochondrial NADH oxidase system prove to be shunted by the very active fp_5—b_5 pathway of free (non-coupled) electron transfer.

If one suggests that the observed activation of external NADH oxidation is of a physiological significance, the question should be answered where would this NADH come from in vivo. First of all, it may be produced by glycolysis. Oxidation of NADH via the external pathway demonstrates much lower respiratory control than that of intramitochondrial NADH or succinate. Activation of the external pathway may stimulate the glycolytic NADH oxidation, and hence stimulate glycolysis, at the respiratory control state. In this connection, the cold stress-induced glycolysis activation observed in our group several

years ago can be mentioned [7]. So, the described phenomenon may contribute to the substrate mobilization under cold conditions.

The external pathway activation may be not only of a regulatory importance. It may also ensue a substantial decrease in the P/O ratio of liver mitochondria in the cold-acclimatized rats. To achieve this effect it would be necessary to equilibrate NADH/NAD^+ across the inner mitochondrial membrane. Such process might be carried out by a shuttle organized by systems responsible for transport of NAD-linked substrates through the inner membrane.

Apparently the above heat-regulatory mechanism is specific for the cold stress conditions rather than for prolonged acclimatization of rats to the constant decrease of the temperature. Besides the above-mentioned fact that maximal NADH oxidation increase requires both cold adaptation and 15 min cold exposure directly before the experiment, we would like to note one more observation which is critical for demonstrating the described phenomenon. It was found that rats continuously kept at 2—4°C in solitary cages, did not demonstrate such pronounced increase of the added NADH oxidation under the in vitro conditions used in the majority of the above experiments. This means that the observed response is specific for a peculiar type of cold adaptation. Several rats kept together in a cage, tend to cling to one another to get warm when the ambient temperature lowers. Such "collective heat regulation" is inevitably accompanied by adaptation to the cold stress taking place every time when an animal finds itself outside the group or on its periphery.

The observed response of liver mitochondria can be compared with those in some other tissues. A change in mitochondrial energetics, induced by a short-term cold exposure of a coldadapted warm-blooded animals was first discovered in a pigeon skeletal muscle studied in this laboratory [8,9]. The change in question is uncoupling of the phosphorylating respiratory chain due to the free fatty acid accumulation in mitochondria [5]. A similar effect was later described in mitochondria from brown adipose tissue (for review, see ref. 10), in which a special mechanism of regulation of the membrane H^+ conductance was recently described [11]. Perhaps a cold exposure-induced increase in the glutamate + malate oxidation in the absence of uncoupler and ADP is indicative of a partial uncoupling in liver mitochondria (see Table I). However, the most pronounced change in properties of mitochondria from liver of cold-treated rats is initiation of the external NADH oxidation via the amytal- and antimycin-resistant pathway.

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