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CYTOCHROME *c* STIMULATED OXIDATION OF ETHANOL BY LIVER MITOCHONDRIA

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Abstract—The addition of ethanol, NAD, alcohol dehydrogenase and cytochrome *c* to liver mitochondria gives rise to an oxygen consumption which is proportional to the amount of ethanol oxidized. The system is insensitive to rotenone and antimycin and not coupled to phosphorylation and is in these respects identical with the well-known NADH-cytochrome *c* reductase present in the outer membrane of liver mitochondria. The respiration is directly proportional to the added cytochrome *c* in the range of 1·6–16·3 μmoles/l. and follows the Michaelis–Menten equation for different ethanol concentrations (K_m = 6·2 mmoles/l). As cytochrome *c* is easily washed out of mitochondria during fractionation, the externally added cytochrome *c* probably replaces it *in vitro*. It is therefore suggested that cytochrome *c* may alternate between the outer and inner membrane and may be available *in vivo* for the oxidation of extramitochondrial NADH by means of the NADH-cytochrome *c* reductase in the outer membrane.

MOST OF the alcohol dehydrogenase (ADH) in the liver has been shown to be present in the soluble fraction of the cell (Dianzani,[1] Nyberg *et al.*,[2] Büttner[3]). Thus ethanol is oxidized to acetaldehyde mainly outside the mitochondria. Various mechanisms for the transport of reducing equivalents from the extramitochondrial NADH into mitochondria have been suggested (Bücher and Klingenberg,[4] Sacktor,[5] Borst,[6] Whereat *et al.*[7]).

So far none has been proved to function *in vivo*, although several observations argue in favour of the oxaloacetate–malate shuttle as a possible mechanism (Hassinen,[8] Williamson *et al.*,[9] Hommes and Richters[10]). The difficulty is, however, that the concentration of NADH inside the mitochondria is much larger than in the cytoplasm. A transfer will therefore not be possible unless energy is supplied in some way.

In the present paper, another mechanism will be discussed, in which NADH, formed by the oxidation of ethanol outside the mitochondria, is re-oxidized by a cytochrome *c* stimulated mitochondrial system. The system is insensitive to rotenone and antimycin and not coupled to phosphorylation and is in these respects identical with the NADH-cytochrome *c* reductase shown to be present in liver mitochondria (Raw *et al.*,[11] Raw and Mahler,[12] Lehninger *et al.*,[13] Lehninger,[14] Ernster *et al.*,[15] Ernster *et al.*[16]).

EXPERIMENTAL

Male Wistar rats weighing 200–300 g from the stock of this laboratory were used in the experiments. The animals were killed by a blow on the neck and exsanguinated.

The liver was rapidly removed, chilled in ice-cold, 0·25 M sucrose with 3·4 mM Tris–HCl (pH 7·4) and 1·0 mM EGTA. After scissoring and rinsing, the liver was homogenized in a loosely fitted, Potter–Elvehjem all-glass homogenizer. A suspension of liver mitochondria was made by a method described by Chappel and Hansford.[17] The mitochondrial pellet was washed twice and the fluffy layer was removed after each centrifugation. This procedure was carried out with 3·4 mM Tris–HCl (pH 7·4) and 1·0 mM EGTA in the 0·25 M sucrose. The pellet was finally suspended in 0·25 M sucrose–Tris–HCl without EGTA.

The incubation medium in the Warburg vessels contained 3 μmoles of ATP, 10 μmoles of Mg^{2+}, 45 μmoles of glucose, 50 μmoles of phosphate buffer (pH 7·4) and 0·5 mg of hexokinase (Sigma, Grade III). Each vessel contained mitochondria from 300 mg of liver, giving a final volume of 2·0 ml. Either one of the following substances or combinations of them were added; 3·4–34 μmoles ethanol, 2 μmoles NAD, 3·2–80·0 nmoles cytochrome *c* and 0·8 μg rotenone. The incubation was performed at 30° for 30 min, including 5 min for temperature equilibration. It was found that the hexokinase used (Sigma, Grade III) was contaminated with alcohol dehydrogenase which was able to oxidize 0·14 μmole of ethanol per min/mg hexokinase at pH 7·4.

As a check on the mitochondrial function, the respiratory control was estimated as the ratio between the respiration obtained with 30 μmoles of pyruvate, 3 μmoles of malate and with ATP, Mg^{2+}, phosphate buffer, glucose and hexokinase as stated above and the respiration obtained when glucose and hexokinase were omitted. No respiratory control ratio less than 4 was accepted (the mean value of all the experiments included was $4·3 \pm 0·1$). The P/O ratio was estimated by the isotope method of Lindberg and Ernster[18] and the protein content of mitochondria according to Lowry *et al.*[19]

The oxygen consumption was recorded polarographically at 28° in an Oxygraph, Model KM (Gilson Medical Electronics). The incubation vessel contained 0·50 mmole sucrose, 0·20 mmole Tris–HCl buffer, pH 7·3, 25 μmoles phosphate, 5 μmoles magnesium and the mitochondria from 100 mg of liver tissue in a volume of 2·0 ml. The additions made were either 7 μmoles glutamate and 0·5 μmole ADP or 70 μmoles ethanol, 2·0 μmoles NAD, 0·5 mg of hexokinase, Grade III and 40 nmoles cytochrome *c*. The inhibition of respiration was studied in the presence of 3·3 μg of antimycin A and/or 2 μmoles of KCN. Most of the results presented were obtained by the Warburg manometric technique but the fundamental effects were checked with oxygraph recordings.

Ethanol was determined at pH 9·5 by the yeast alcohol dehydrogenase method, according to Dickinson and Dalziel,[20] after removal of protein with trichloroacetic acid. Values for the endogenous respiration were subtracted from the respiration obtained in the presence of a substrate. The statistical evaluation was made using Student's *t*-test. Hexokinase Grade III, NAD, ATP and cytochrome *c* Type III were purchased from the Sigma Chemical Co.

RESULTS

The addition of ethanol, NAD and ADH to mitochondria gave rise to oxygen consumption, which is partly caused by acetaldehyde oxidation in the mitochondria (Hedlund and Kiessling,[21] Kiessling,[22] Kiessling *et al.*[23]). This respiration is completely

inhibited by rotenone and antimycin (Table 1, Fig. 1). The addition of cytochrome *c* increases the respiration further and this increase is insensitive to rotenone and antimycin but is completely blocked by KCN (Table 1, Fig. 1).

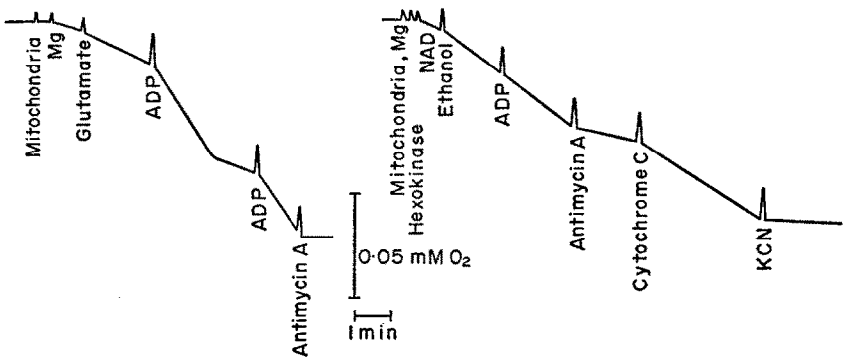

FIG. 1. Polarographic recording of oxygen consumption by rat liver mitochondria after additions as indicated in the figure. The respiratory control with glutamate is 4·71, the ADP:O ratio 2·84 and the coupled respiration rate 43·0 nmoles O_2/min/mg protein. The respiration after ethanol and ADP is 21·1 nmoles O_2/min/mg protein and after cytochrome *c* 18·2 nmoles O_2/min/mg protein. For details of the incubation procedure, see Experimental.

TABLE 1. EFFECT OF CYTOCHROME *c* ON MITOCHONDRIAL RESPIRATION AND P/O WITH ETHANOL, NAD AND HEXOKINASE

Additions	Respiration (μatom O_2/hr/10 mg protein)	P/O
Ethanol + NAD + hexokinase	16·4 ± 0·6	1·3 ± 0·2
Ethanol + NAD + hexokinase + rotenone	4·6 ± 0·4	
Ethanol + NAD + hexokinase + cytochrome *c*	25·4 ± 1·9	0·9 ± 0·1
Ethanol + NAD + hexokinase + cytochrome *c* + rotenone	12·9 ± 1·8	0·5 ± 0·1
NAD + cytochrome *c*	3·8	

The hexokinase used contains alcohol dehydrogenase as a contamination in sufficient amounts to reduce NAD at such a rate that NADH is always present outside the mitochondria (see Experimental).

Figure 2 shows that this rotenone insensitive respiration is directly proportional to cytochrome *c* in the range of 1·6–16·3 μmoles/l. No oxygen consumption occurs in the absence of mitochondria which excludes auto-oxidation of cytochrome *c*. An increase in the cytochrome *c* stimulated respiration corresponds to an increase in the ethanol disappearance (Fig. 2). The ratios between oxygen consumption and ethanol disappearance at various concentrations of cytochrome *c* are given in Table 2.

Ethanol in final concentrations ranging from 1·7 to 17·0 μmoles/l. gave rise to an oxygen consumption which follows the Michaelis–Menten equation, when the cytochrome *c* level is kept at a level giving optimal stimulation of the respiration. Plotting the data for ethanol concentrations and the corresponding oxygen uptake in the

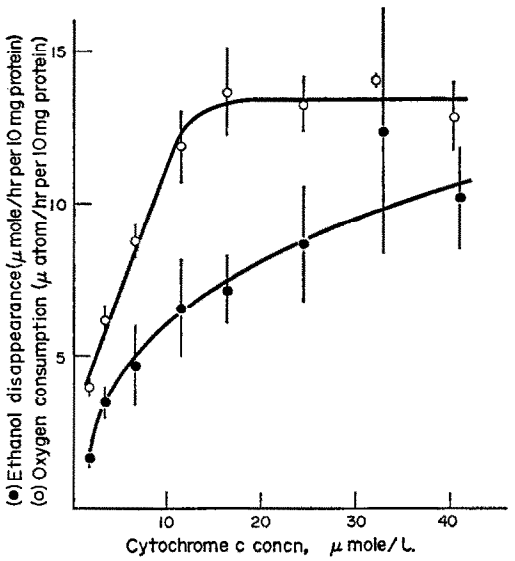

FIG. 2. The effect of different concentrations of cytochrome *c* on the respiratory rate and of ethanol disappearance when rat liver mitochondria were incubated with 34 μmoles ethanol, 2 μmoles NAD, 0·5 mg hexokinase and 0·8 μg rotenone in a final volume of 2 ml. The hexokinase was contaminated with alcohol dehydrogenase. For further details, see Experimental. Each point represents the mean value of seven different experiments, except the two points where cytochrome *c* is over 30 μmoles which are based on three different experiments. The bars indicate the standard error.

double reciprocal manner gives a line from which K_m can be estimated (Fig. 3). K_m was 6·2 mmoles of ethanol/l. The P/O ratio is 1·9 without cytochrome *c* and 0·9 with cytochrome *c*. In the presence of rotenone, the P/O ratio is only 0·2–0·5.

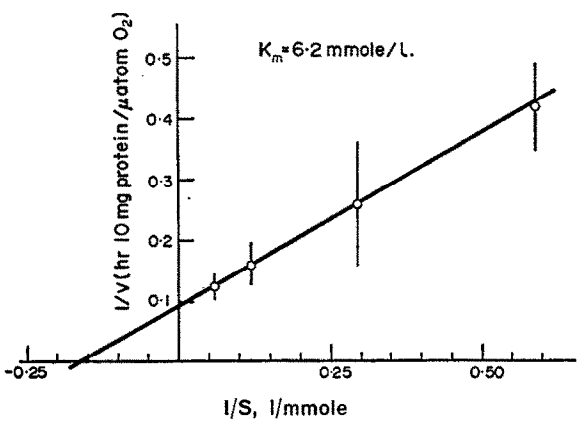

FIG. 3. Double reciprocal plot of the effect of different ethanol concentrations (*S*) on the respiratory rate (*v*) in rat liver mitochondria incubated with 1 mM NAD, 0·25 mg/ml hexokinase, 0·4 μg/ml rotenone and 16·3 μM cytochrome *c*. The hexokinase used contained alcohol dehydrogenase as a contamination, in sufficient amounts for the formation of NADH throughout the experiement. For details of the experimental procedure, see Experimental. Each point represents the mean value of seven experiments and the bars indicate the standard error.

TABLE 2. OXYGEN CONSUMPTION IN RELATION TO THE ETHANOL DISAPPEARANCE AT DIFFERENT CONCENTRATIONS OF CYTOCHROME *c*

Cytochrome *c* (μmoles/l.)	μatom O_2/μmole ethanol
1·6	2·35
3·3	1·77
6·5	1·87
11·4	1·80
16·3	1·90
24·4	1·53
32.6	1·25
40·7	1·26

DISCUSSION

Evidence has been provided for the occurrence of a NADH-cytochrome *c* reductase within the liver mitochondria, which may be responsible for the rotenone- and antimycin-insensitive oxidation of exogenous NADH by added cytochrome *c* (Raw *et al.*,[11] Raw and Mahler,[12] Lehninger *et al.*,[13] Lehninger,[14] Ernster *et al.*,[15] Ernster *et al.*[16]). Recently it has been shown by Sottocasa *et al.*[24] that the enzyme system is associated with the outer membrane. This location facilitates the mitochondrial oxidation of NADH formed in the cytoplasm, for example by ethanol oxidation. One condition is that cytochrome *c* must be present in the outer membrane or in the soluble fraction. In the experiments with isolated mitochondria shown in Fig. 2, various concentrations of cytochrome *c* were added. With concentrations of cytochrome *c* ranging from 1·6 to 40·7 μmoles/l., a stimulation of the respiration is obtained. Ethanol, NAD and ADH act as a NADH-generating system.

It is, of course, impossible to draw any conclusions from these *in vitro* experiments about the physiological significance of the system in connection with ethanol oxidation. It only shows that, in the presence of mitochondria, cytochrome *c*, ethanol, NAD and ADH, the NADH formed may be re-oxidized and that the respiration is roughly proportional to the amount of ethanol which has disappeared.

Several investigations suggest that the biosynthesis of cytochrome *c* occurs in the endoplasmic reticulum (González-Cadavid and Campbell,[25] Davidian *et al.*,[26] Kadenbach[27]). Recently Davidian and Penniall[28] and Kadenbach[29] presented results indicating that the product of the synthetic activity of the endoplasmic reticulum must be a nonfunctional form of cytochrome *c* and that it is this entity which is transferred to the mitochondria.

From 50 to 90 per cent of the cytochrome *c* of the liver cell has been reported to occur inside the mitochondria (González-Cadavid and Campbell,[25] Davidian *et al.*[26]). The rest is present in the soluble fraction, the microsomes and the nuclei (Davidian *et al.*[26]). On the basis of figures reported by Loud[30] showing that the mitochondria occupy some 20 per cent of the cytoplasmic volume, a calculation reveals a concentration of cytochrome *c* in the cytoplasm ranging from 1·7 to 8·4 μmole/l. This is within the range that causes a stimulation of the respiration with ethanol, NAD and ADH added to isolated mitochondria (Fig. 1).

According to Muscatello and Carafoli[31], a difference exists in the permeability

properties to cytochrome *c* of the two faces of the inner mitochondrial membrane. It has been suggested that a permeability barrier for cytochrome *c* exists at the inner but not at the outer face. If this is true, cytochrome *c* may alternate between the two membranes and also be available for NADH-cytochrome *c* reductase in the outer membrane. Racker[32] has outlined a topographical model of the inner membrane. In the model cytochrome *c* is located at the outer side of the membrane. This location facilitates an alternation of cytochrome *c* between the two membranes.

The complete abolition of the cytochrome *c* stimulated respiration by KCN shows that the final part of the respiratory chain is involved in this system (Fig. 1). That cytochrome *c* has to be added to mitochondria in order to make the NADH-cytochrome *c* reductase work may be explained by cytochrome *c* being washed out of the mitochondria. This may be expected, as the bulk of the extramitochondrial cytochrome *c* results from a redistribution of mitochondrial cytochrome *c* during cell fractionation (Kadenbach[33]). This cytochrome *c* is easily replaced in an *in vitro* system by adding cytochrome *c* to the medium.

Thus, not only the minor amounts of cytochrome *c* located in the cytoplasm but also the easily washed-out part of the mitochondrial cytochrome *c* may be available *in vivo* for the oxidation of extramitochondrial NADH by means of the NADH-cytochrome *c* reductase in the outer membrane.

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