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INFLUENCE OF ETHANOL ON ISOLATED MITOCHONDRIA

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Abstract—Effects of ethanol on the oxidative phosphorylation with glutamate as substrate and on the swelling capacity of isolated rat liver mitochondria have been studied.

Ethanol in concentrations from 1 to 10% (v/v) was found to depress the oxygen consumption and the phosphate uptake of freshly prepared mitochondria. Mitochondria aged for 24 hr at 2° *in vitro*, showed a much reduced respiration and phosphorylation. Increasing ethanol concentrations caused increasing oxidative rates in the aged mitochondria, whereas their phosphate uptake remained nearly unchanged.

Swelling of the freshly isolated mitochondria was prevented completely by ethanol in relatively high concentrations. The above-cited findings show that ethanol is not a suitable vehicle for water-insoluble agents in functional studies of mitochondria.

BEER and QUASTEL[1] investigating the effects of alcohol administration on the respiration of rat brain mitochondria found no inhibition of mitochondrial oxidative processes by aliphatic alcohols given in pharmacologically active concentrations. These findings are in agreement with results obtained by Kiessling,[2] who did not observe any impairment of the respiration in isolated rat brain mitochondria with succinate, glutamate, pyruvate or α-ketoglutarate as substrates, following the injection of ethanol. Later on Kiessling and Tilander[3] reported that liver mitochondria isolated from rats given a prolonged alcohol feeding have a reduced capacity to oxidize pyruvate and succinate. In a communication by Christophersen[4] the effect of ethanol on mitochondrial oxidations had been studied *in vitro*, the oxidation of β-hydroxybutyrate was found to be stimulated by relatively low concentrations of ethanol, while with pyruvate, α-ketoglutarate and succinate as substrates ethanol did depress the O_2 uptake. The P/O ratios were not lowered by ethanol concentrations which increased the β-hydroxybutyrate oxidation.

These findings of Christophersen[4] are of special interest for the biochemist, because ethanol is not so rarely used as a vehicle for water-insoluble compounds whose influence on the oxidative phosphorylation is to be studied *in vitro*, and because under determinate conditions ethanol *per se* may affect the oxidative phosphorylation. In the present communication the effects of various concentrations of ethanol on the respiration and formation of high-energy phosphate with glutamate as substrate were studied in isolated mitochondria.

MATERIALS AND METHODS

Liver mitochondria from adult male Sprague-Dawley rats were prepared according to the method described by Hogeboom *et al.*[5] After liver homogenization in a glass tube with Teflon pestle in ice cold 0·25 M sucrose containing 0·001 M ethylene-diamine-tetraacetic acid (EDTA) the nuclei and cell debris were removed by centrifugation at 800 *g* for 15 min. The mitochondria from the supernatant were sedimented at 10,000 *g* for 15 min, washed twice in the homogenizing medium and resuspended in the same medium. One ml of the final stock suspension contained the mitochondria isolated from 1 g of wet liver. All steps of the preparation were performed at 0–4° and pH 7·4.

The O_2 uptake was measured according to the Warburg technique at 30° with air as gas phase and 20% potassium hydroxide in the center well. The incubation mixture contained 0·02 M Trisbuffer pH 7·4, 0·02 M orthophosphate pH 7·4, 0·02 M potassium chloride, 0·008 M magnesium chloride, 0·025 M glucose, 0·01 M glutamate, 0·001 M ATP, 0·01 M sodium fluoride, 0·25 M sucrose. 0·2 ml of mitochondrial suspension was added last. The reaction medium containing all components except the hexokinase, was preincubated for 6 min to allow time for gas equilibration and adjustment of the manometers. The reaction was started by tipping in the hexokinase (lyophil. 200 M.K. units). Duration of experiment was 15 min, the final volume was 1 ml. The phosphate consumption was calculated from the disappearance rate of orthophosphate in incubation medium, measured by the method of Fiske and SubbaRow.[6]

Ethanol (absolute grade) was added to the reaction mixture prior to the addition of the mitochondria.

In several experiments the O_2 uptake of aged mitochondria was measured. For that purpose a portion of freshly prepared mitochondrial suspension was allowed to age by standing for 24 hr at 2°. Swelling studies were performed with mitochondria isolated in 0·25 M sucrose without EDTA (pH 7·4). For measuring the swelling rate usually 0·02 M Tris-buffer was added to the mentioned mitochondria suspension in sucrose in order to adjust a nearly neutral environment of pH 7·4. Moreover in separate experiments the mitochondrial swelling was determined in the incubation medium (without hexokinase) used for the estimation of the oxidative phosphorylation. The rate of swelling was determined at the wavelength of 520 nm.

RESULTS AND DISCUSSION

The inhibitory effect of various concentrations of ethanol on the O_2 consumption and phosphate uptake of freshly prepared mitochondria is shown in Table 1. With

TABLE 1. EFFECT OF INCREASED ETHANOL CONCENTRATIONS ON THE O_2 CONSUMPTION AND PHOSPHATE UPTAKE OF FRESH ISOLATED MITOCHONDRIA

Ethanol (final concentration, %)	O_2 consumption (μA_0)	Phosphate uptake (%)	No. of rats
0	8·3 ± 0·14	100	4
1	7·8 ± 0·18	81	4
5	5·7 ± 0·19	75	4
10	4·6 ± 0·25	66	4

increasing ethanol concentrations from 0 to 10% (v/v), the O_2 consumption gradually decreases by 45 per cent approximately, the phosphate uptake is reduced by about 33 per cent. Without ethanol addition, the liver mitochondria show normal rates of oxidative phosphorylation in our experiments similar to those described in the pertinent literature.

The aged mitochondria show a markedly decreased initial O_2 consumption in comparison to the freshly prepared ones. In sharp contrast to the foregoing experiments, ethanol addition provokes an increase of the O_2 consumption by more than 100 per cent, whereas the phosphate uptake remains unchanged (Table 2).

TABLE 2. EFFECT OF INCREASED ETHANOL CONCENTRATIONS ON THE O_2 CONSUMPTION AND PHOSPHATE UPTAKE OF MITOCHONDRIA AGED FOR 24 HR AT 2°

Ethanol (final concentration, %)	O_2 consumption (μA_0)	Phosphate uptake (%)	No. of rats
0	2·0 ± 0·25	24	4
5	3·3 ± 0·12	25	4
10	4·3 ± 0·28	25	4

Glycerol (0·5%, v/v) added to the reaction medium, does not influence the oxidative phosphorylation of isolated liver mitochondria in any way.

The effects of ethanol on the mitochondrial function observed by us with glutamate as substrate are in accordance to those of former experiments performed with substrates others than glutamate, e.g. pyruvate, succinate and α-ketoglutarate.[4] The inhibitory influence of ethanol on glutamate oxidation can hardly be explained by a genuine uncoupling effect, for at lower ethanol concentrations the P/O ratio remained nearly unchanged. Only at higher ethanol concentrations a gradual decrease of the P/O ratio was found, and a simultaneous decrease of the oxidation rate, that pointed rather to a general toxicity for the enzyme systems related to oxidative phosphorylation. Aging of mitochondria prior to incubation with ethanol might support the glutamate transport into the mitochondria to the respiratory enzyme sites, thus increasing the substrate supply and consecutively the oxidation rate. This assumption could provide an explanation for the strange phenomenon of an increased O_2 uptake by aged mitochondria in contact with ethanol. Simultaneously, the ethanol added seems to impair the phosphorylating factors.

Swelling studies indicate that ethanol (final concentration 10%, v/v) inhibits swelling of liver mitochondria suspended in a sucrose-Tris buffer solution, by about 60 per cent. With mitochondria suspended in the reaction medium (without hexokinase), the ethanol does prevent the swelling process at all (Fig. 1). All swelling studies had to be performed on freshly prepared mitochondria, because, after 3 hr of aging, isolated mitochondria have usually reached a maximum of spontaneous swelling.

Karler, *et al.*[7] observed that ethanol inhibited both spontaneous and drug-induced mitochondrial swelling. Furthermore they reported that on the one hand ethanol *per se* did not uncouple the oxidative phosphorylation (with pyruvate and succinate as substrates) and on the other it did block the characteristic metabolic action of

various uncoupling agents. These observations were interpreted to indicate that ethanol affected oxidative phosphorylation only by inhibiting mitochondrial swelling. The mechanism of the antiswelling activity of ethanol is not clear. Swelling appears to be dependent upon intact respiration, and substances such as cyanide that inhibit respiration can also inhibit mitochondrial swelling.[8, 9] Perhaps the addition of ethanol

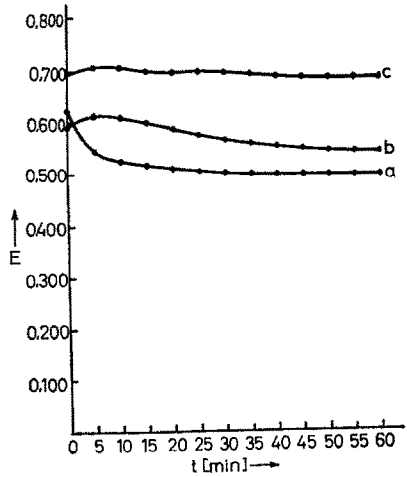

FIG. 1. Effect of ethanol on rat liver mitochondria swelling (mean values of 4 separate experiments)
a. spontaneous swelling in 0·25 M sucrose–0·02 M Tris buffer;
b. swelling in 0·25 M sucrose–0·02 M Tris buffer and added ethanol (final concentration 10%, v/v);
c. swelling in a medium identical with respiratory medium containing ethanol (final concentration 10%, v/v).

also provides an environment of osmolarity for the mitochondria, which prevents swelling. In view of the fact that the mitochondrial membranes are rich in lipids, it is perhaps not surprising that ethanol may alter their properties. Remarkable is that Johnson and Lardy have observed substrate-selective effects on mitochondrial oxidations by varying the tonicity of the medium, to change the permeability of mitochondria.[10]

From our results can be drawn the practical conclusion that ethanol is only restrictedly suitable as vehicle of water-insoluble agents (e.g. potential "uncouplers") and additive to the reaction medium for functional estimations in isolated mitochondria, because in concentrations more than 1% (v/v) it diminishes the oxidative and phosphorylative processes in a considerable degree and impairs mitochondrial swelling.

REFERENCES

1. C. T. BEER and J. H. QUASTEL, *Canad. J. Biochem.* **36**, 543 (1958).
2. K.-H. KIESSLING, *Expl Cell Res.* **26**, 432 (1962).
3. K.-H. KIESSLING and K. TILANDER, *Expl Cell Res.* **30**, 476 (1963).
4. B. O. CHRISTOPHERSEN, *Biochim. biophys. Acta* **86**, 14 (1964).
5. G. H. HOGEBOOM, W. C. SCHNEIDER and G. E. PALLADE, *J. biol. Chem.* **172**, 619 (1948).

6. C. H. FISKE and Y. SUBBAROW, in (Eds. S. P. COLOWICK and N. O. KAPLAN), *Methods in Enzymology*, Vol. **3**, p. 843, Academic Press, New York (1957).
7. R. KARLER, TH. S. SULKOWSKI and J. T. MIYAHARA, *Biochem. Pharmac.* **14**, 1025 (1965).
8. F. E. HUNTER, JR., J. DAVIS and L. CARLAT, *Biochim. biophys. Acta* **20**, 237 (1956).
9. A. L. LEHNINGER and B. L. RAY, *Biochim. biophys. Acta*, **26**, 643 (1957).
10. D. JOHNSON and H. LARDY, *Nature, Lond.* **181**, 701 (1958).