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# Effect of Alcohols on the Functional Organization of the Inner Mitochondrial Membrane

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## *Abstract*

Monohydric alcohols extract phospholipids from beef heart mitochondria with an efficacy which depends on the chain length of the alcohol. Succinoxidase and ATPase activities are affected by alcohols in a similar way; alcohols make ATPase oligomycin-insensitive at concentrations decreasing with the chain length of the alcohol. Oxidative phosphorylation is inhibited at much lower concentrations of alcohols. Hydrophobic bonds must play a role in the organization of all of the activities considered.

The forces which are responsible for the organization of the inner mitochondrial membrane are still incompletely understood, but several lines of indirect evidence are available for the demonstration that hydrophobic interactions play a main role in the lipid–protein associations in mitochondria.[1–3]

Monohydric alcohols have been found in our laboratory to inhibit the interaction of phospholipid micelles with lipid-depleted mitochondria with an efficacy depending on the chain length of the alcohol[4] and this has been taken as evidence for the hydrophobic nature of this interaction. It has been considered therefore of importance to investigate whether the converse is also true; in other words do alcohols break lipid–protein bonds in the native mitochondrial membrane with increased efficacy proportional to the hydrophobicity of their molecules? Such a demonstration would provide a direct indication of the presence of hydrophobic interactions between proteins and phospholipids in mitochondria. Other examples found by similar means to depend upon the weakening of hydrophobic bonds, include the dissociation of the subunits of $\beta$-galactosidase[6] and the conversion of $P_{430}$ to $P_{420}$[7,8]. In erythrocyte membranes the results of solvent extraction studies have permitted the postulation of several possible types of interactions between lipids and proteins.[9] It is furthermore of interest to ask whether dissociation of hydrophobic bonds in the mitochondrial membranes may be revealed by functional criteria which can give better clues on the localization of such bonds in the membrane, and might possibly establish specific correlations between enzymic activities and phospholipid association. For this reason we have investigated the effect of monohydric alcohols on phospholipid extraction from beef heart mitochondria and on certain key mitochondrial activities (succinoxidase, ATPase, and oxidative phosphorylation).

## *Methods*

Beef heart mitochondria (BHM) were prepared by a small-scale procedure.[10] Phospholipid extraction by alcohols was accomplished at 30° in the following way: the mitochondria (10 mg of mitochondrial protein) were added to alcoholic solutions of the desired concentration in water.

After 10 min the tubes were quickly removed and centrifuged at 30,000 rpm in the No. 40 rotor of the Spinco model L ultracentrifuge. The residue was suspended in a volume of 2 ml and assayed for protein and phosphorus.

Succinoxidase activity was assayed with a Braun respirometer in a medium containing in a total volume of 3 ml: 0·5 μmoles of sucrose, 0·1 μmoles of Tris-Cl pH 7·5, and the required volumes of the alcohol to be tested; 2·5 mg of mitochondrial protein were added and succinate was mixed from the side arm after 5 min of preincubation; the assay temperature was 30°. When oxidative phosphorylation was measured the method of Lenaz and Beyer[11] was followed. ATPase was measured according to Margolis *et al.*[12]

Protein was assayed with the biuret procedure of Gornall *et al.*[13] and phosphorus according to Marinetti.[14]

## *Results and Discussion*

Figure 1 shows the effect of monohydric alcohols having 1 to 4 carbon atoms on the phospholipid extraction at 30° from BHM. There is a clear correlation between the chain length of the alcohol and the efficacy of phospholipid extraction, as also shown in a linear

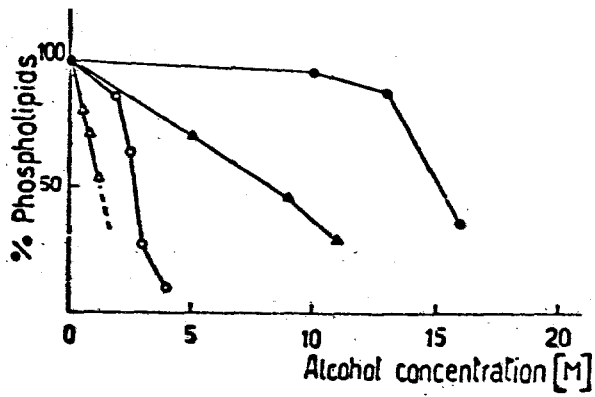


Figure 1. Effect of monohydric alcohols on phospholipid extraction from beef heart mitochondria. ●—●, methanol; ▲—▲ ethanol; ○—○, n-propanol; △—△, n-butanol. The values are expressed as per cent of controls incubated without alcohol.

relationship in Fig. 2. Amyl alcohol (n-pentanol) does not extract phospholipids at the concentrations used, but this may be an effect of insolubility of the phospholipids at the very low concentrations of n-pentanol which are miscible with water. Zwaal and Van Deenen[15] have obtained soluble lipoproteins by treatment of erythrocyte membranes with n-pentanol, but free phospholipids after treatment with n-butanol.

The effect of alcohols on succinoxidase activity of BHM is shown in Fig. 3. Here again

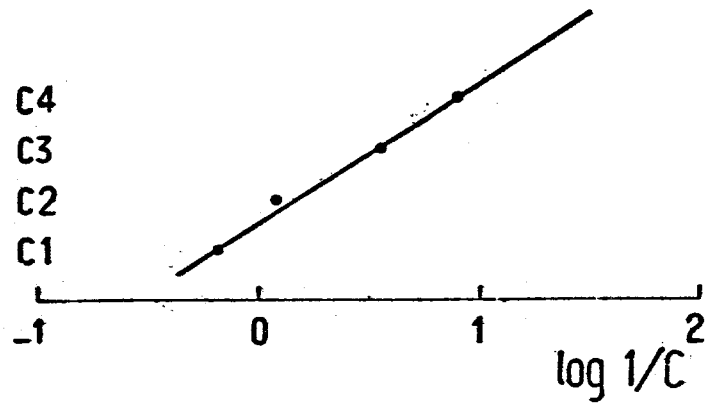


Figure 2. Relationship between the logarithm of the reciprocal of the concentration of alcohol required to extract 50% of the phospholipids and alcohol chain length. The values are calculated from Fig. 1.

there is a correlation between the length of the alkyl chains of the alcohols and the efficacy of the inhibition of succinoxidase (Fig. 4). Small amounts of alcohols appear to enhance the activity, and this may be an effect of an "opening" of the membrane by alcohols, with resulting better availability of the substrate to its site. This "opening" effect appears more evident with the longer chain alcohols.

Comparison of Figs. 1 and 3 shows that a correlation between inhibition of succinoxidase activity and phospholipid extraction is roughly present: the concentrations of the alcohols required to inhibit succinoxidase by 50% are about half of those concentrations necessary to extract 50% of the phospholipids. The same types of bonds, i.e. hydrophobic bonds, must be broken in the two cases: this lets us exclude that the pattern of phospholipid extraction depends exclusively upon their solubility in the alcohol-water mixtures employed.

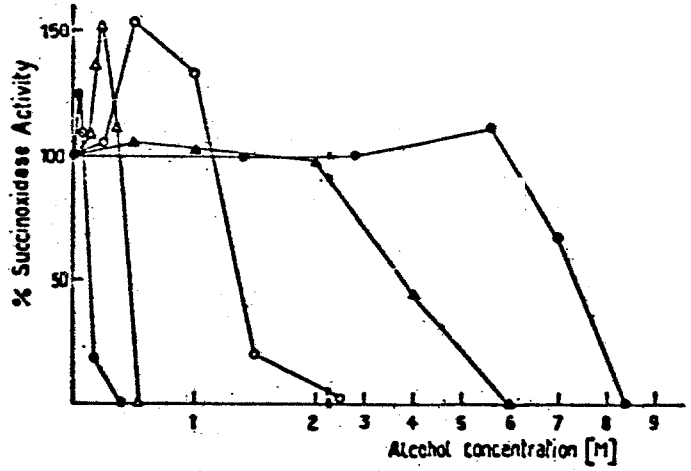


Figure 3. Effect of monohydric alcohols on succinoxidase activity of beef heart mitochondria. ●—●, methanol; ▲—▲, ethanol; ○—○, n-propanol; △—△, n-butanol; ⊙—⊙, n-pentanol. The values are expressed as per cent of controls incubated without alcohols.

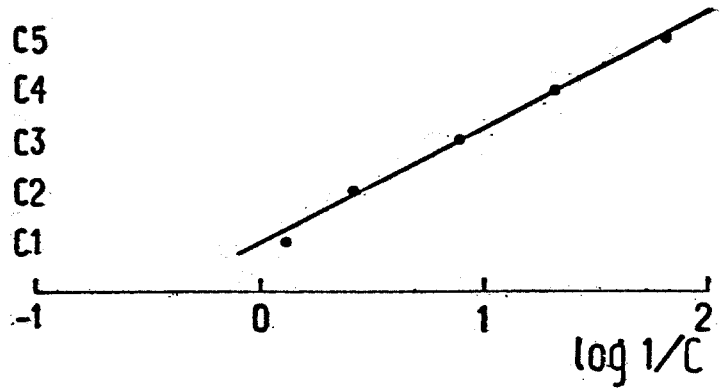


Figure 4. Relationship between the logarithm of the reciprocal of the concentration of alcohol required to inhibit succinoxidase activity of 50% and alcohol chain length. The values are calculated from Fig. 3.

Figure 5 shows that the ATPase activity is progressively inhibited by alcohols and their efficacy is again dependent on the length of the alkyl chains (Fig. 6); with the lower alcohols there is a progressive decrease of activity beginning at low concentrations of alcohols, which is not comparable with the sudden fall after an initial increase in the case of succinoxidase. Table I and Figs. 7 and 8 show that oligomycin sensitivity decreases by increasing the concentration of the alcohol, and this decrease also depends on the chain length of the alcohol. Apparently alcohols first functionally detach the headpiece from the membrane, and make the ATPase oligomycin insensitive; then the ATPase complex is disorganized and the activity is completely lost. Higher chain alcohols (n-butanol and n-pentanol) even enhance activity in the presence of oligomycin; it is known that alcohols protect the oligomycin-insensitive ATPase $F_1$[16] from subunit dissociation.[17]

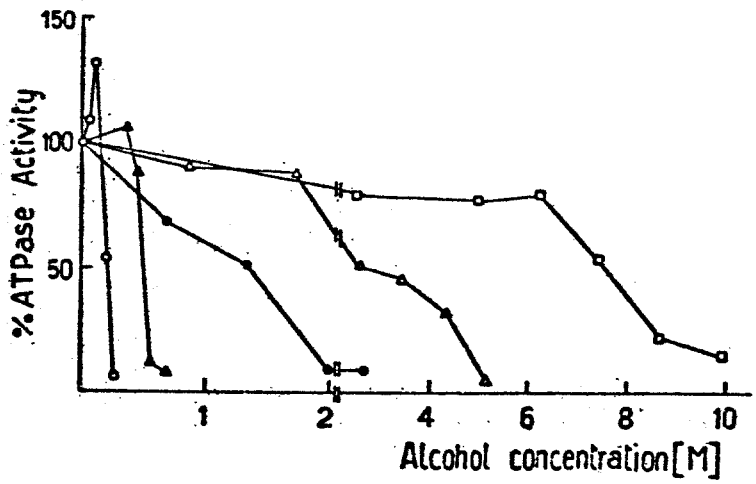


Figure 5. Effect of monohydric alcohols on total ATPase activity of beef heart mitochondria. □—□, methanol; △—△, ethanol; ●—●, n-propanol; ▲—▲, n-butanol; ○—○, n-pentanol. The values are expressed as per cent of controls incubated without alcohols.

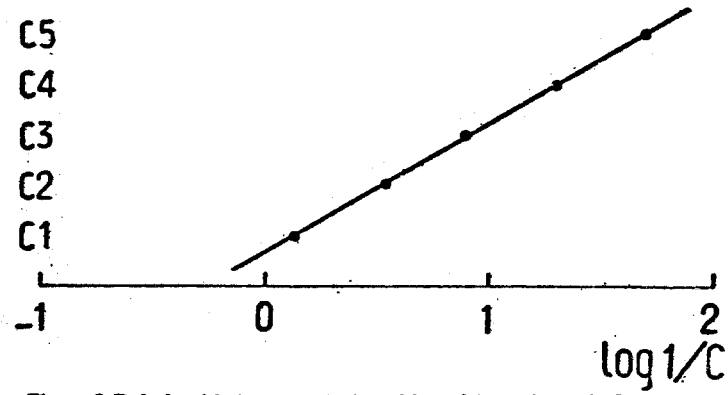


Figure 6. Relationship between the logarithm of the reciprocal of the concentration of alcohol required to inhibit ATPase activity of 50% and alcohol chain length. The values are calculated from Fig. 5.

TABLE I. Oligomycin sensitivity of ATPase in presence of monohydric alcohols*

| Alcohol | Concentration [M] | ATPase activity | % inhibition by oligomycin |
|---|---|---|---|
| — | — | 0·481 | 85 |
| Methanol | 4·95 | 0·285 | 54 |
| Ethanol | 2·58 | 0·248 | 28 |
| n-Propanol | 0·67 | 0·342 | 67 |
| n-Butanol | 0·34 | 0·535 | <0 |
| n-Pentanol | 0·09 | 0·618 | 25 |

* In this table are reported only some representative values. Inhibition by oligomycin at other alcohol concentrations may be calculated from the values of Fig. 8.

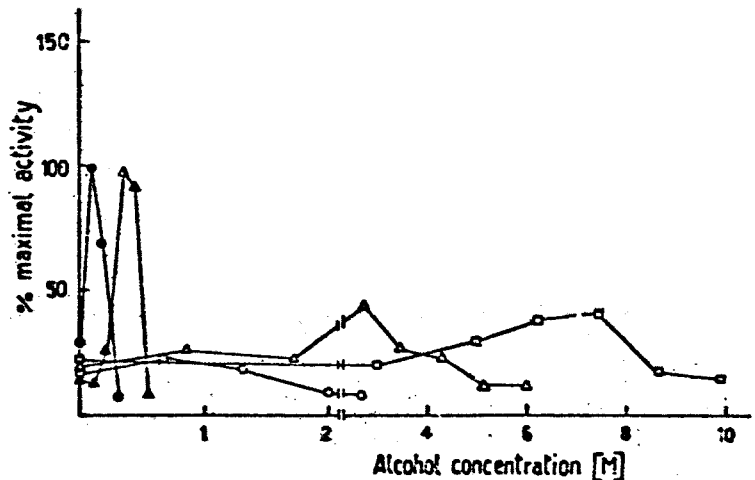


Figure 7. Oligomycin-insensitive ATPase in presence of monohydric alcohols. □—□, methanol; △—△, ethanol; ○—○, n-propanol; ▲—▲, n-butanol; ●—●, n-pentanol. Oligomycin was added at the concentration of 1 μg per 1 ml assay. The values are expressed in per cent of the ATPase activity without oligomycin and in the absence of alcohols.

Roelofsen *et al.*[18] have found that alcohols and alcohol mixtures inactivate ATPase activity of erythrocyte ghosts at critical values of dielectric constants of the media and have related this effect to the breaking of lipid–protein bonds.

Figure 9 finally shows the effect of alcohols on oxidative phosphorylation. In this case much lower concentrations of alcohols are required to disrupt the phosphorylative capacity. Figure 10 shows the correlation between the chain length of the alcohol and the specific activity of phosphorylation in comparison with that of succinate oxidation.

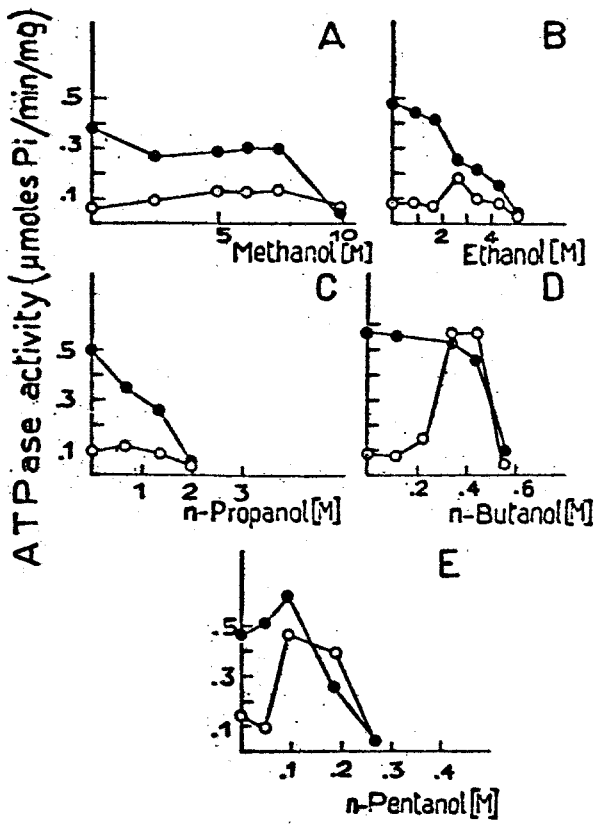


Figure 8. ATPase activity of beef heart mitochondria in the presence of monohydric alcohols. A, methanol; B, ethanol; C, n-propanol; D, n-butanol; E, n-pentanol. The values are expressed as ATPase specific activities in μmoles of Pi released per min per mg of protein at 30°. ●—●, activity without oligomycin; ○—○, activity with oligomycin (1 μg/ml).

The values for phosphorylation lay on a straight line (compare with Figs. 2, 4 and 6), but values for succinoxidase are largely anomalous in comparison with the values in Fig. 4: the shorter-chain alcohols appear more effective than predicted from theory in inhibiting respiration; this discrepancy may be due to different experimental conditions in comparison with those for Fig. 4.

Table II shows that 5·6 M methanol is much more effective in inhibition of succinoxidase under phosphorylative conditions than in a sucrose-Tris medium. The reason cannot be apparently ascribed to respiratory control since the rate of oxidation without

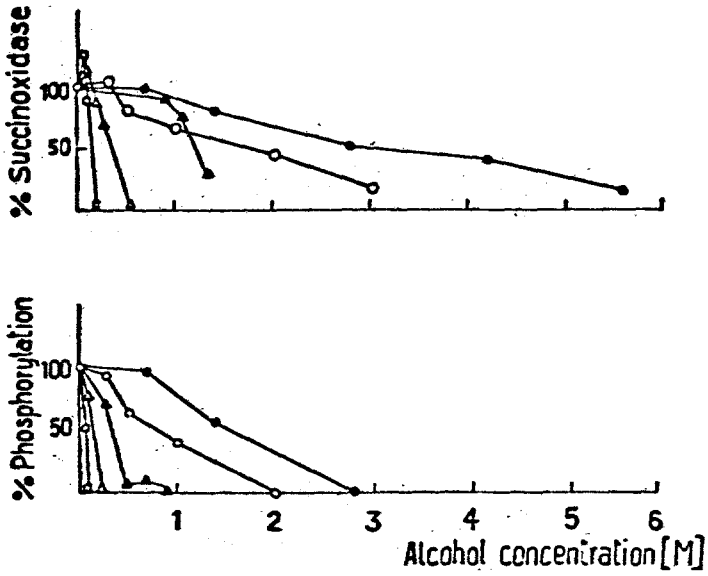


Figure 9. Effect of monohydric alcohols on oxidative phosphorylation in fresh mitochondria. Upper graph: succinate oxidation, % of control activity; lower graph: phosphorylation specific activity, % of control. ●—●, methanol; ○—○, ethanol; ▲—▲, n-propanol; △—△, n-butanol; □—□, n-pentanol.

alcohol is the same under the two conditions; the presence of salts (phosphate, $Mg^{++}$, ADP) could perhaps enhance an effect of the more polar alcohols on extracting or inactivating cytochrome *c* (cf.: the effect of acetone[19]).

Table III summarizes all the effect of alcohols on the various parameters considered. The direct dependance of phospholipid extraction upon the hydrophobicity of the alcohols used points out that phospholipids are held in the membrane largely by hydro-

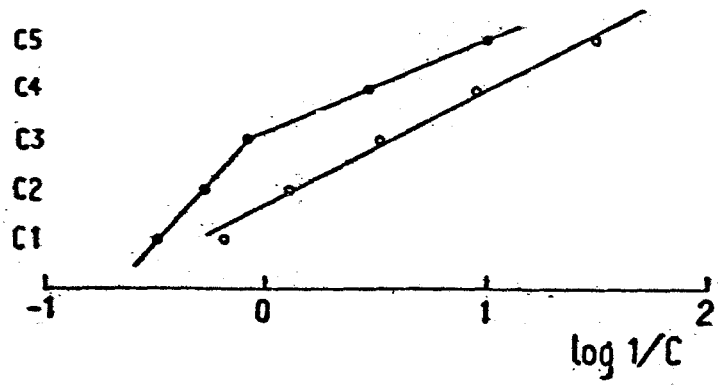


Figure 10. Relationship between the logarithm of the reciprocal of the concentration of alcohol required to inhibit oxidation and phosphorylation and the chain length of the alcohol. The values are calculated from Fig. 9.

TABLE II. Effect of methanol on the succinoxidase activity of beef heart mitochondria under phosphorylative and nonphosphorylative conditions

| Addition | Phosphorylative conditions | | Nonphosphorylative conditions | |
|---|---|---|---|---|
| | Specific activity* | % | Specific activity* | % |
| — | 0·093 | | 0·092 | |
| Methanol, 5·6 M | 0·053 | 57 | 0·021 | 23 |

* μatoms $O_2$/min/mg protein.

phobic forces; the fact that succinoxidase and ATPase are affected in similar ways by alcohols suggests that these enzymes are functionally organized by hydrophobic interactions. The isolated oligomycin-sensitive ATPase appears to be bound to phospholipid micelles hydrophobically and the same interactions could be operative *in vivo* with the "basepiece".[20] A phospholipid requirement for both succinoxidase and ATPase has been described.[21–25] Certain differences of effect of the alcohols on the two activities suggest that other forces may be operative in the organization of the enzymic complexes. The polar end of the alcohols may also have an effect on certain bonds: the hypothesis that other bonds besides hydrophobic interactions are involved in membrane assembly is worth exploring; in other studies we have demonstrated that less polar solvents than alcohols, such as diethyl ether, cannot extract phospholipids from the mitochondrial membranes, unless they are previously treated with diluted HCl or by other means, which detach "soluble" protein and the ATPase complex from the membrane (ref. 3, and unpublished data). This fact may suggest that polar bonds may also be involved in the attachment of the headpiece to the membrane.

TABLE III. Comparison of the effect of different alcohols on phospholipid extraction and various mitochondrial activities

| | Methanol | Ethanol | n-Propanol | n-Butanol | n-Pentanol |
|---|---|---|---|---|---|
| | Concentration (M) | | | | |
| Phospholipid extraction (50%) | 15·2 | 8·2 | 2·7 | 1·25 | — |
| Succinoxidase (50%) | 7·35 | 3·80 | 1·35 | 0·47 | 0·15 |
| Total ATPase (50%) | 7·55 | 2·90 | 1·35 | 0·50 | 0·20 |
| Oligomycin-insensitive ATPase (max) | 7·40 | 2·60 | 0·65 | 0·35 | 0·10 |
| *Oxidative phosphorylation* | | | | | |
| $O_2$ uptake (50%) | 3·0 | 1·85 | 1·22 | 0·34 | 0·10 |
| Phosphorylation (50%) | 1·52 | 0·77 | 0·30 | 0·11 | 0·03 |

All assays were carried out on frozen mitochondria; oxidative phosphorylation was tested on fresh mitochondria.

The results of this investigation reinforce the idea of an important general contribution of hydrophobic bonding to the organization of the inner mitochondrial membrane. The

very low concentrations of alcohols required to inhibit phosphorylation represent further evidence of the marked lability of the coupling process to various agents, like bile salts[26] and its dependance on the integrity of a highly specific hydrophobic membrane.

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