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Effects of Lower Aliphatic Alcohols on Mitochondrial Structure

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Lower aliphatic alcohols caused swelling of isolated rat liver mitochondria at concentrations similar to those which have been found to inhibit respiration and phosphorylation. The ability of an alcohol to cause swelling increased with the lipid solubility of the alcohol.

The swelling induced by an alcohol occurred in two phases, an initial, rapid phase of rather low amplitude and a slower phase of higher amplitude.

In the presence of ATP* and Mg^{2+}, or of serum albumin, only the initial, rapid swelling occurred. Addition of ATP and Mg^{2+} to a system where swelling had been induced by a moderate concentration of alcohol, caused contraction of the mitochondria, both when the additions were made during the slow swelling and after its completion. Mitochondria in which swelling had been induced by a higher concentration of alcohol did not contract upon addition of ATP and Mg^{2+} unless the alcohol was partly removed.

A detailed interpretation of the effects of alcohol on mitochondrial structure and structure-linked function is given.

Isolated mitochondria are known to swell in response to a decrease in osmolarity of the medium in which they are suspended. [1–4] As was first demonstrated by Raaflaub [1] there also exists another type of swelling, which occurs spontaneously in an isosmotic medium. This swelling is dependent on respiration, it is much slower than the momentaneous swelling of the osmotic type, and it can be greatly accelerated by a number of substances, *e.g.* Ca^{2+}, [3,5,6] phosphate,[1] thyroxine,[6,7] fatty acids,[6,7] *etc.* It can be reversed by addition of ATP or, under phosphorylating conditions, of ADP.[8–10] These thoroughly investigated reactions have rather recently been discussed in great detail in a comprehensive review by Lehninger.[11]

* Abbreviations: ADP, adenosine diphosphate; ATP, adenosi ne triphosphate; NAD, nicotinamide-adenine dinucleotide; Tris, tris(hydroxymethyl) aminoetha ne.

It has been demonstrated that the swelling and contraction of mitochondria are correlated with an uptake and extrusion, respectively, of water from the part of mitochondrion which is normally not accessible to water soluble substances (nonpenetrating substances according to the nomenclature used by Tedeschi and Harris [4]).

In this paper the results from an investigation of the effects of some aliphatic alcohols on mitochondrial structure are presented and discussed, with particular emphasis on comparisons of effects of alcohols on structural and functional properties of mitochondria.[12, 13]

MATERIALS AND METHODS

ATP and Tris were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. The other reagents were of analytical grade except the 1-propanol, 1,5-pentanediol and the 1-pentanol, which were of KEBO purum grade.

Rat liver mitochondria were prepared as in Ref. 12 and stored at 0°C for no longer than 2-3 h before use.

The swelling of the mitochondria was measured as decrease in absorbancy at 510 mμ [14] in a Beckman model B spectrophotometer. The path of light through the cuvette was 1 cm. The studies of swelling were performed at room temperature. The concentration of mitochondrial protein in the suspensions was roughly 0.1 mg/ml unless otherwise stated. The mitochondria were added at zero time and the alcohols were either present from the beginning of the experiment or added at times indicated on the figures.

In the short-time experiments on the time course of the inhibition of phosphorylation and respiration, the pH-technique [15] and the polarographic oxygen electrode technique [16] were used.

RESULTS

Lower aliphatic alcohols cause swelling of rat liver mitochondria, at concentrations similar to those which have been found to inhibit respiration and phosphorylation.[12, 13] The concentration of alcohol required to induce swelling decreases with its chain-length. This is illustrated in Fig. 1, where the effects of methanol, propanol and butanol are compared. Fig. 2 shows the effects of different concentrations of butanol. The initial rate of the swelling increases with the concentration of alcohol in the medium, whereas the extent of the swelling is not much affected.

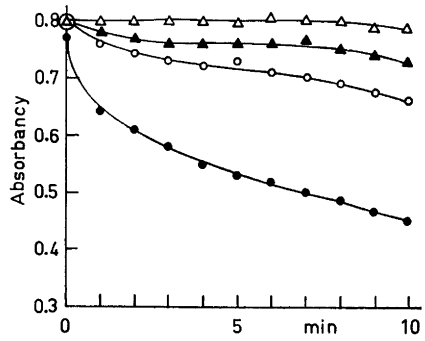

Fig. 1. Swelling induced by methanol, propanol, and butanol. The alcohols were present from the beginning of the experiment. The medium contained 0.115 M KCl and 0.02 M Tris/HCl, pH 7.5. Other conditions are given in Methods. △, no alcohol present; ▲, 7 % methanol; ○, 1.25 % propanol; ●, 1.0 % butanol.

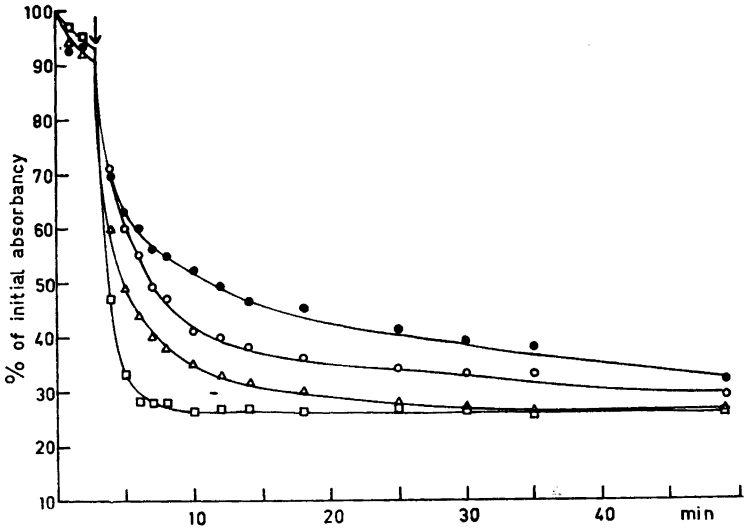

Fig. 2. Swelling induced by different concentrations of butanol. Arrow at 3 min indicates addition of butanol. The medium contained 0.115 M KCl and 0.02 M Tris/HCl, pH 7.5. 100 % absorbancy corresponds to an absorbancy of approximately 0.6 at 510 mμ. Other conditions are given in Methods. ●, 1 % butanol; ○, 1.5 % butanol; △, 2 % butanol; □, 3 % butanol.

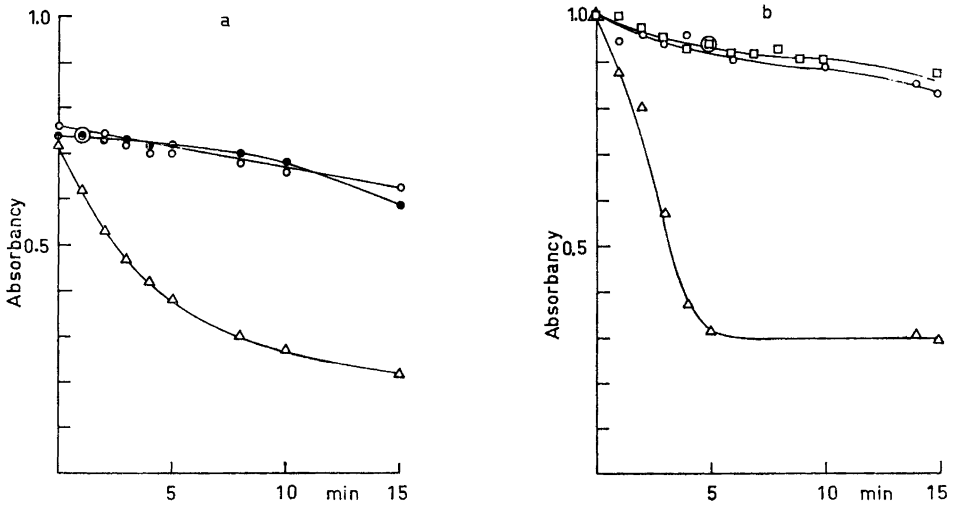

Fig. 3, a. The effect of butanol and 1,4-butanediol on mitochondrial swelling. The alcohols were present from the beginning of the experiment. The medium contained 0.125 M KCl and 0.02 M Tris/HCl, pH 7.5. Other conditions are given in Methods. ●, no alcohol present; ○, 1.5 % 1,4-butanediol; △, 1.5 % butanol. *b.* The effect of pentanol and 1,5-pentanediol on mitochondrial swelling. The alcohols were present from the beginning of the experiment. The medium contained 0.115 M KCl and 0.02 M Tris/HCl pH 7.5. Other conditions are given in Methods. ○, no alcohol present; □, 1.5 % 1,5-pentanediol; △, 1.0 % pentanol.

In Fig. 3 a is shown that 1,4-butanediol, which has practically no effect on mitochondrial respiration and phosphorylation[13] has only a very slight effect on mitochondrial swelling as compared to the strong swelling induced by the same concentration of butanol. Fig. 3 b shows that whereas pentanol is a very potent swelling agent, 1,5-pentanediol does not induce any swelling, even at concentrations which have been found to completely uncouple the mitochondria.[13]

The alcohol-induced swelling has been found to occur in two phases, an initial rapid phase of rather low amplitude and a slower phase of higher amplitude. In the presence of ATP and Mg^{2+}, or of serum albumin, only the initial, rapid swelling occurred. Fig. 4 shows the swelling of mitochondria in the

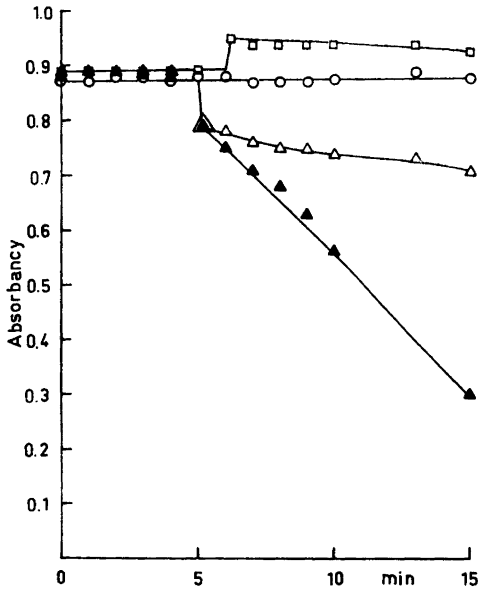

Fig. 4. The effect of butanol and potassium chloride on mitochondrial swelling in the presence and absence of ATP and Mg^{2+}. 1.6 % (v/v) butanol (0.175 M) was added at 5 min, and 20 mg KCl (0.088 M) at 6 min as indicated below. The medium contained 0.104 M KCl and 0.02 M Tris/HCl, pH 7.5. Other conditions are given in Methods. ○, no additions; △, 1.6 % butanol added 0.75 mM ATP and 6.0 mM $MgCl_2$ present; ▲, 1.6 % butanol added, no ATP or $MgCl_2$ present; □, 20 mg KCl added, ATP and $MgCl_2$ present.

absence and presence of ATP and Mg^{2+}. The effect of a change in osmolarity of the medium, similar to that which resulted when alcohol was added, is illustrated in a control in which an amount of KCl, isosmolar with the alcohol was added.

Fig. 5 shows that in the presence of serum albumin only the initial rapid, and not the slower, secondary swelling occurred.

Experiments have been carried out in order to obtain a correlation between changes in absorbancy and changes in mitochondrial volatile liquid content. This was done with determinations of the weight of mitochondrial pellets before and after drying at 105°C, according to the method of Werkheiser and Bartley.[17] The results did show a correlation between changes in absorbancy at 510 mμ and the ratio wet weight/dry weight as is seen in Fig. 6. The substance responsible for these changes may have been water, or the alcohol employed, or a combination of these two components.

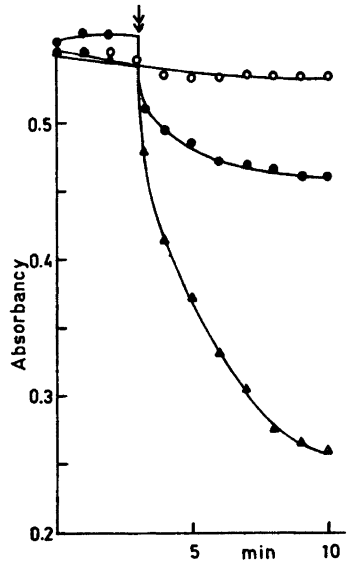

Fig. 5. Swelling induced by butanol in the presence and absence of serum albumin. Arrows at 3 min indicate addition of 1.6 % butanol. The medium contained 0.115 M KCl and 0.02 M Tris/HCl, pH 7.5. Other conditions are given in Methods. ○, no additions; ●, 0.067 % serum albumin present, butanol added; ▲, no serum albumin present, butanol added.

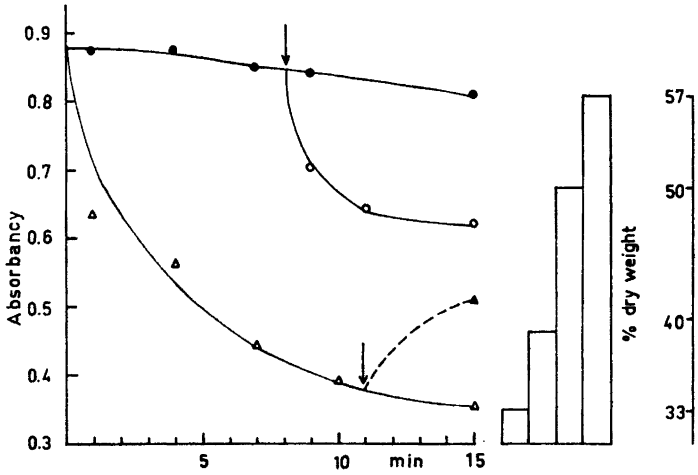

Fig. 6. Correlation between absorbancy and mitochondrial dry weight. The medium contained 0.115 M KCl and 0.02 M Tris/HCl, pH 7.5. Arrow at 8 min indicates addition of 2 % butanol, arrow at 11 min addition of 0.75 mM ATP and 6.0 mM $MgCl_2$. Other conditions are given in Methods. ●, ATP and $MgCl_2$, but no butanol present at the beginning of the experiment; ○, ATP and $MgCl_2$ present at the beginning of the experiment, 2 % butanol added at 8 min; △, 2 % butanol, but no ATP or $MgCl_2$ present at the beginning of the experiment; ▲, 2 % butanol present at the beginning of the experiment, ATP and $MgCl_2$ added at 11 min.

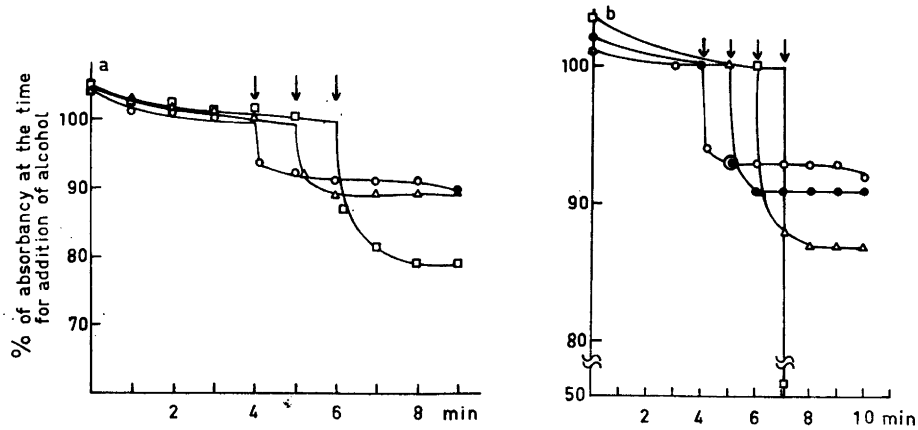

Fig. 7 a and *b*. Rapid swelling in the presence of 0.75 mM ATP and 6.0 mM $MgCl_2$, induced by (*a*.) different concentrations of butanol and (*b*.) equal concentrations of different alcohols. Arrows indicate addition of alcohol. The medium contained 0.115 M KCl and 0.02 M Tris/HCl, pH 7.5. Other conditions are given in Methods. (*a*) O, 0.5 % butanol added at 4 min, △, 1.0 % butanol added at 5 min, □, 1.5 % butanol added at 6 min. 100 % absorbancy corresponds to an absorbancy of approximately 0.8 at 510 mμ. (*b*) O, 0.15 M ethanol added at 4 min, ●, 0.15 M propanol added at 5 min; △, 0.15 M butanol added at 6 min; □, 0.15 M pentanol added at 7 min. 100 % absorbancy corresponds to an absorbancy of approximately 0.8 at 510 mμ.

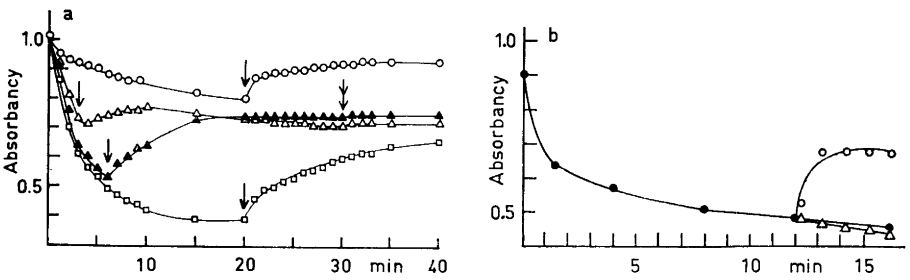

Fig. 8 a and *b*. Reversibility of swelling induced by (*a*) a moderate concentration of butanol and (*b*) a higher concentration of butanol. Arrows indicate addition of 0.75 mM ATP and 6.0 mM $MgCl_2$. (*a*) The medium contained 0.125 M KCl and 0.02 M Tris/HCl, pH 7.5. 1.5 % butanol was present in all cuvettes except the control at the beginning of the experiment. O, no butanol present, ATP and $MgCl_2$ added at 20 min; △, butanol present, ATP and $MgCl_2$ added at 3 and 30 min; ▲, butanol present, ATP and $MgCl_2$ added at 6 and 30 min, □, butanol present, ATP and $MgCl_2$ added at 20 min. (*b*) The medium contained 0.115 M KCl and 0.02 M Tris/HCl, pH 7.5. 3 % butanol was present from the beginning of the experiment in a mitochondrial suspension containing 1.5 mg mitochondrial protein/ml. At 12 min the concentration of butanol was lowered in two samples by diluting the suspension 5 times. To one of the diluted samples and to the original suspension 0.75 mM ATP and 6.0 mM $MgCl_2$ was added. ●, 3 % butanol present, ATP and $MgCl_2$ added at 12 min; O, 3 % → 0.6 % butanol, ATP and $MgCl_2$ added at 12 min; △, 3 % → 0.6 % butanol, no ATP or $MgCl_2$ added.

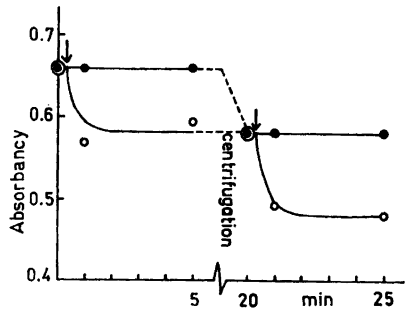

Fig. 9. Alcohol induced swelling in sucrose. The conditions were as in the preincubation experiments in a preceding paper,[12] *i.e.* the swelling took place in a dense (15 mg of mitochondrial protein/ml) suspension of mitochondria in 0.25 M sucrose at 0°C. Aliquots were taken at the times indicated in the figure and the absorbancy at 510 mμ was determined. Other conditions are given in Methods. ●, no alcohol added; O, 3 % butanol added at 15 sec.

In Fig. 7 a and b is shown that the amplitude of the initial rapid swelling increases with the concentration of alcohol and with its chain-length,respectively. Fig. 7 a may be compared with Fig. 2 which shows that in the absence of ATP and Mg^{2+} only the rate of the large amplitude swelling, not its amplitude is affected by the concentration of alcohol employed.

As is shown in Fig. 8 a, addition of ATP and Mg^{2+} to a system where swelling was induced by a relatively low concentration of butanol caused contraction, both when the additions were made during the slow swelling phase, and when they were made after its completion. Fig. 8 b shows mitochondria, in which swelling was induced by a higher concentration of the same alcohol. These mitochondria did not contract upon addition of ATP and Mg^{2+} unless the alcohol was partly removed. As can be seen in the control sample, removal of the alcohol without addition of ATP and Mg^{2+} did not result in contraction.

The fact that several correlations were found between the effects of alcohols on structural and functional properties in mitochondria, afforded a possibility to test how a preincubation such as that described in a preceding paper [12] did affect the mitochondria in terms of swelling. The result is shown in Fig. 9. A rapid swelling of low amplitude occurred upon addition of 3 % butanol to the mitochondrial suspension. When the alcohol had been removed by centrifugation and the resulting pellet was resuspended in alcohol-free sucrose, the absorbancy of the resuspended alcohol-pretreated suspension equalled that of a suspension which had been likewise centrifuged and resuspended, but not pretreated with alcohol (the overlapping circles at 20 min). A second addition of alcohol again produced the rapid swelling. Thus the rapid swelling which took place under these conditions was fully reversed by removal of the alcohol. Another experiment, similar to the previous one except that the mitochondria were suspended in a buffered KCl solution instead of sucrose and were protected from the secondary swelling by the presence of ATP and Mg^{2+}, was performed. Exactly the same picture was obtained in this experiment, which brings further support to our conclusion that the primary, rapid swelling can be reversed by washing the mitochondria free from alcohol.

Experiments carried out with the rapid pH-electrode technique,[15] in collaboration with Dr. Saris, as well as with the polarographic oxygen electrode showed that the inhibition of respiration and phosphorylation in the mitochondria was completed within 10—30 sec after addition of the alco-

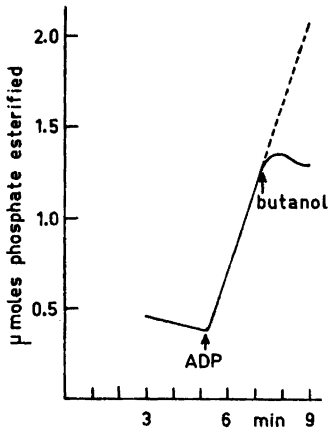

Fig. 10. Inhibition by butanol of phosphorylation, linked to oxidation of succinate. At the beginning of the experiment the mitochondria (3.7 mg mitochondrial protein) were added to a reaction mixture, containing in a total volume of 3 ml: Tris/HCl, pH 7.5, 15 µmoles; KCl, 240 µmoles; Na-succinate, 3 µmoles; KH_2PO_4 6 µmoles; $MgCl_2$, 8 µmoles. At 5 min, 3 µmoles of ADP were added, and at 7 min 3 % butanol. One µmole of phosphate esterified corresponded to one µmole of OH^- produced.

hol, both with succinate and the NAD-linked substrate β-hydroxybutyrate. Thus the inhibitions appear to coincide with the rapid initial swelling phase. It has been suggested in a preceding paper[12] that the greater sensitivity of the NAD-linked respiration compared to the succinate-linked is due to a dissociation of bound NAD from the membranes. These experiments strongly suggest that such a dissociation would occur during the initial stages of the alcohol-induced swelling. The inhibition of phosphorylation linked to oxidation of succinate is shown in Fig. 10.

An experiment was made in order to test in a more direct way whether the inhibition of oxidative phosphorylation was indeed connected with the initial rapid swelling phase. It has been shown previously in this paper that serum albumin does protect against the alcohol-induced large-amplitude swelling, but not against the initial rapid swelling. If the latter is indeed closely linked with the inhibition of oxidative phosphorylation, serum albumin would not be expected to protect against this inhibition. Table 1 shows that serum albumin did not prevent the alcohol inhibition of oxidative phosphorylation in mitochondria.

Table. 1. Inhibition by butanol of succinate-linked electron transport and phosphorylation in mitochondria with and without serum albumin present.

	Per cent of control	
Additions	Phosphorylating activity	Respiratory activity
none	100	100
0.1 % serum albumin	107	100
2 % butanol	24	64
2 % butanol + 0.1 % serum albumin	19	62

100 % activity was 17.2 µmoles phosphate esterified and 8.1 µmoles oxygen uptake/mg mitochondrial protein/h. Experimental conditions are given in Ref. 12.

DISCUSSION

The ability of alcohols to induce swelling as a rule runs quite parallel to their inhibitory effects on electron transport and phosphorylation in the mitochondria, in the sense that an alcohol either displays an effect on both these parameters or on none of them. There is, however, one exception to this rule. 1,5-Pentanediol which uncouples electron transport from phosphorylation has no effect on mitochondrial structure, even at concentrations which completely inhibit phosphorylation.[13]

Several data presented above justify our distinction between two phases in the alcohol-induced swelling of the mitochondria, one initial phase and one secondary, which is observed only after the cessation of the initial phase. The initial, rapid swelling seems to be the event which is directly connected with inhibition of phosphorylation and electron transport. This conclusion is supported by our demonstration with the rapid pH-technique,[15] and with the polarographic oxygen electrode technique,[16] that the inhibition is accomplished rapidly enough to allow consideration of the primary swelling as being directly connected with the inhibition. Additional data in line with these views were obtained in the experiments with serum albumin, which was shown to prevent the secondary but not the primary swelling and to be unable to protect the mitochondria against loss of phosphorylating and respiratory activities, which thus also from these data appear to be more connected with the rapid, initial structural changes.

The initial swelling has some properties which suggest that it is a swelling of an osmotic type, similar to that described by other authors.[1-4] It is very rapid, it is not prevented by ATP or serum albumin, and its amplitude is determined by the concentration and kind of alcohol added. It is to be observed that the mitochondria swell, rather than shrink, when the osmolarity of the medium is increased at the addition of alcohol. This circumstance is characteristic for penetrating substances[4] and is suggested to be a reflection of the manner in which alcohols interfere with mitochondrial structure and function. Due to their dipolar character the alcohol molecules are assumed to layer in the phase boundaries between lipid and aqueous phases in the mitochondrial membranes.[18,19] Such an enrichment of alcohols in the lipid/protein interphase might induce a change in permeability of the membranes, leading to a rapid change in mitochondrial volume as shown above, with production of the demonstrated swelling, and with consequences for mitochondrial function. The possible effects of such a disarrangement of the membranes have been discussed in two previous papers.[12,13]

The second swelling phase has the same characteristics as the "active", respiration-dependent swelling, described by other authors,[3,9,11] *i.e.* it is comparatively slow, of large amplitude and can be reversed or prevented by addition of ATP. The ability of serum albumin to protect against the secondary swelling might indicate that a liberation of fatty acids takes place in analogy with the experiments of Lehninger and Wojczak.[6,7] They showed that serum albumin protected against swelling induced by a variety of agents, all of which also induced a liberation of fatty acids, while it was shown to be ineffective in protecting against swelling which was not accompanied by a liberation

of fatty acids, such as that induced by phosphate. The possibility that alcohol-induced swelling may be accompanied by a liberation of fatty acids has, however, not been experimentally tested in the present study.

Our view about structural and functional changes which occur in mitochondria upon addition of alcohol may now be summarized. The primary event is assumed to be the rapid penetration of alcohol into the mitochondrial membranes as reflected by the initial rapid swelling phase. As has been shown above it is accompanied by a change in mitochondrial structure. This brings about an inhibition of the phosphorylation and the electron transport reactions, which can be reversed simply by washing the mitochondria free from alcohol. It has been shown that the very alcohol-sensitive NAD-linked electron transport and the concomitant phosphorylation were inhibited about as rapidly as the succinate-linked reactions. In a preceding paper[12] we suggested that the inhibition of the NAD-linked reactions is mainly due to a dissociation of bound NAD from the electron transport chain. This would imply that the dissociation of bound NAD takes place during the initial stages of the alcohol-induced swelling, *i.e.* during the rapid swelling phase.

After the penetration of alcohol into the mitochondria has taken place, a relatively slow swelling of larger amplitude can be observed. This swelling is similar to that caused by other swelling agents, *e.g.* Ca^{2+},[3,5,6] thyroxine,[6,7] and fatty acids.[6,7] During this swelling phase no effects on mitochondrial function in addition to those which took place during the rapid swelling have been found.

It may be worthwhile to point out the possibility that connections between structural and functional parameters, similar to those which have been observed in mitochondria, exist also when chloroplasts and chromatophores are treated with alcohols. So far, however, the effects of alcohols on these systems have been tested only on functions, *i.e.* on electron transport and photophosphorylation.

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