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Inhibitory Effects of Lower Aliphatic Alcohols on Electron Transport Phosphorylation Systems

1. Straight-Chain, Primary Alcohols

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Photophosphorylation in isolated spinach chloroplasts and chromatophores from the photosynthetic bacterium *Rhodospirillum rubrum* and respiration and phosphate uptake in isolated rat liver mitochondria were found to be strongly inhibited by lower aliphatic alcohols in rather high concentrations. The inhibitory effects increased with the chain-length of the alcohol. A comparison of the sensitivity of systems with different electron transport pathways in the chromatophores and in the chloroplasts showed that similar alcohol concentrations were always required for inhibition of photophosphorylation. In the one non-cyclic photophosphorylation system tested, namely chloroplasts with electron transport from water to ferricyanide, both electron transport and phosphate esterification were shown to be inhibited.

The alcohols had stronger inhibitory effects on respiration and phosphorylation in mitochondria with NAD-linked * substrates such as β-hydroxybutyrate and glutamate, than with succinate. With the NAD-linked substrates the respiration and phosphorylation rates decreased about simultaneously, *i.e.* with little change in P/O ratio when the concentration of alcohol was increased, but with succinate the phosphorylation was uncoupled from respiration. Thus, of the mitochondrial functions tested, NAD-linked electron transport was most sensitive to the alcohols, presumably due to depletion of intramitochondrial NAD. This interpretation is supported by the demonstration that the sensitivity of the electron transport towards alcohol was considerably lowered in a system with externally added NAD.

It has long been known that homologous series of certain organic compounds inhibit various biological functions. Warburg[1] made an investigation of the effects of several such series on respiration in avian red blood cells, fer-

* Abbreviations: ATP, adenosine triphosphate; ATP-ase, adenosine triphosphatase; NAD, nicotinamide-adenine dinucleotide; FMN, flavin mononucleotide; PMS, phenazine methosulfate; HOQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; DOC, sodium desoxycholate; Tris, tris(hydroxymethyl)aminomethane.

mentation in yeast extracts, and respiration and assimilation of carbon dioxide in *Chlorella* cells. Among the compounds tested were aliphatic alcohols, ketones, and nitriles. It was found that the inhibitory effects of the compounds within each of these series increased with the size of the aliphatic carbon skeleton. In many cases the effects were shown to be reversible. It was suggested that inhibitors such as alcohols (among other types of compounds) acted on surface structures, and that this action was of a physical, rather than a chemical nature. A correlation was found in each homologous series tested, between the extent of depression in surface tension caused by a compound and its potency as an inhibitor.

Recently the inhibitory effects of a series of aliphatic alcohols on photophosphorylation in isolated chloroplasts have been investigated.[2] These studies have now been extended to include other isolated electron transport phosphorylation systems, namely chromatophores from the photosynthetic bacterium *Rhodospirillum rubrum* and mitochondria from rat liver. Several different effects of lower aliphatic alcohols on these electron transport phosphorylation systems have been investigated.

MATERIALS AND METHODS

The alcohols were of analytical grade, except the propanol and pentanol, which were of KEBO purum grade. ATP, PMS, FMN, hexokinase (type III or IV), and Tris were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Other reagents were of analytical grade.

Spinach was obtained as whole plants and washed leaves were stored at 0 – 4°C. The chloroplasts were prepared according to the method of Allen, Whatley and Arnon.[3] The "whole" chloroplasts obtained after one washing were suspended in a small volume of the isolation medium.

"Whole" chromatophores and chromatophore "fragments" were prepared from *R. rubrum* according to the method described by Baltscheffsky.[4] The chromatophore suspensions could be stored in the dark at 0°C for several weeks without significant loss of activity.

Rat liver mitochondria were prepared in 0.25 M sucrose according to the method of Schneider and Hogeboom [5] as modified by Ernster and Löw.[6] The pellet was finally suspended in a small volume of 0.25 M sucrose and stored at 0°C.

The experiments with chromatophores were carried out as in Ref. 4 with or without PMS and HOQNO present. In the restoration experiments the concentration of alcohol was decreased by dilution, *i.e.* by adding the preincubated chromatophore suspension to the reaction mixture.

The experiments with the mitochondria were made with the Warburg technique in 1 ml vessels, each containing 25 μmoles sucrose, 50 μmoles KCl, 25 μmoles Tris/HCl pH 7.4, 8 μmoles $MgCl_2$, excess hexokinase, 25 μmoles $KH_2{}^{32}PO_4$, 0.5 μmoles ATP, 30 μmoles glucose, 10μmoles of the sodium salts of succinic, glutamic, or β-hydroxybutyric acids, and 1 – 3 mg mitochondrial protein unless otherwise indicated. The time for incubation was 20 – 30 min, and the temperature was 30°C.

In the experiment with externally added NAD the mitochondria were subjected to an osmotic shock before incubation. This consisted of a treatment with distilled water at 0°C for 2 min, according to the method of Lehninger.[10] Such a treatment depletes the mitochondria of intramitochondrial NAD and makes them accessible to externally added NAD.

In the restoration experiments the mitochondria were preincubated with alcohol in the original 0.25 M sucrose suspension for 20 min at 0°C. The alcohol was subsequently removed by sedimenting the mitochondria at 4500 *g* and resuspending them in alcohol-free sucrose.

Table 1. Approximate alcohol concentration ranges for 50 % inhibition of cyclic photophosphorylation in the "physiological system" of chromatophores, and oxidative phosphorylation in mitochondria with succinate as substrate. Experimental conditions are given in Methods. (Corresponding values for cyclic photophosphorylation in spinach chloroplasts are given in Ref. 2).

Alcohol	Concentration in % (v/v)	
	Chromatophores	Mitochondria
Methanol	7.5—9	13—15
Ethanol	6—8	10—12
Propanol	1.6—2.2	3.5—4.5
Butanol	0.5—1.3	1.8—2.2
Pentanol	0.3—0.5	0.4—0.6

Protein was determined with the Biuret method and the esterification of inorganic phosphate was assayed according to the method recommended by Lindberg and Ernster.[7] The ATP-ase activity was determined according to Ernster *et. al.*[8] Reduction of ferricyanide was determined with the method used by Jagendorf.[9]

RESULTS

Cyclic photophosphorylation in the "physiological system" of chromatophores and respiration and phosphorylation in mitochondria with succinate as substrate, were inhibited in such a way, that the concentrations of alcohols

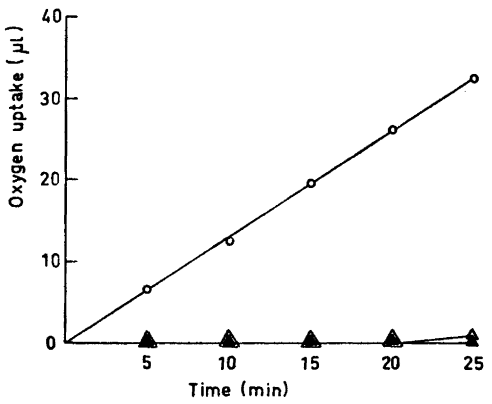

Fig. 1. Effect of ethanol on mitochondrial oxygen uptake as a function of time. O: 20 µmoles of succinate; ▲ : 4 % ethanol, no succinate; △ : no succinate, no ethanol. 1.75 mg mitochondrial protein/ml incubation mixture. Other conditions are given in Methods.

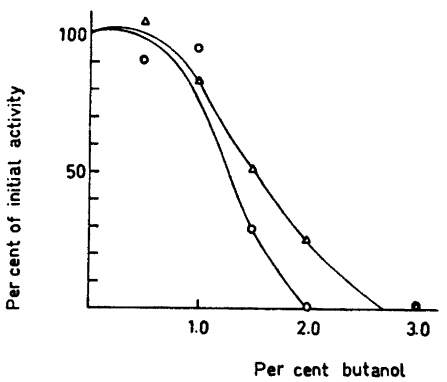

Fig. 2. Effect of increasing amount of mitochondria on the butanol inhibition of phosphate uptake with succinate as substrate. Incubation time 20 min. Other conditions are given in Methods. 100 % activity was 5.7 and 4.3 µmoles phosphate esterified/mg mitochondrial protein/h in O, and △, respectively. O : 0.5 mg mitochondrial protein/ml reaction mixture; △: 3.0 mg mitochondrial protein/ml reaction mixture.

required for 50 % inhibition decreased with increasing chain-length of the alcohol. The results are given in Table 1.

In Fig. 1 it is shown that ethanol was not oxidized in the mitochondrial system. With chloroplasts and chromatophores neither methanol nor ethanol are metabolized in any significant amounts (*cf.* Ref. 2).

The amount of mitochondria present had very little effect on the amount of alcohol required for a certain degree of inhibition, as can be seen in Fig. 2.

In the chloroplasts, photophosphorylation coupled to electron transport, cyclic in the presence of added PMS and „pseudocyclic" in the presence of added FMN, has been shown to require the same concentrations of alcohol for 50 % inhibition.[2] In order to be able to distinguish between alcohol effects on electron transport and on phosphorylation, the non-cyclic system water-ferricyanide was tested. As is shown in Fig. 3 not only phosphorylation but also electron transport from water to ferricyanide was inhibited. Thus it may

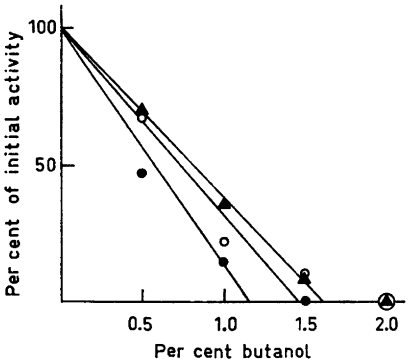

Fig. 3. Influence of butanol on photophosphorylation. 1) Cyclic photophosphorylation in the presence of PMS: ▲; 2a) noncyclic photophosphorylation with ferricyanide as electron acceptor: ●; and 2b) electron transport from water to ferricyanide: O. In system 2 were added per ml: Tris/HCl pH 8.0, 160 μmoles, $MgCl_2$ 20 μmoles, ATP 0.5 μmoles, glucose 30 μmoles, excess hexokinase, $KH_2{}^{32}PO_4$ 10 μmoles, and $K_3Fe(CN)_6$ 10 μmoles. 100 % activity was 230 μmoles phosphate esterified/mg chlorophyll/h in the PMS-system, and 185 μmoles ferricyanide reduced and 42 μmoles phosphate esterified/mg chlorophyll/h in the non-cyclic system.

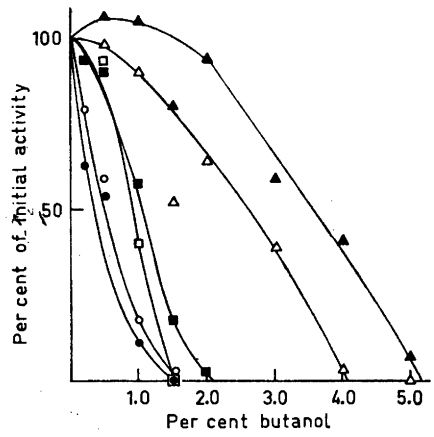

Fig. 4. Inhibition by butanol of electron transport and phosphate esterification in mitochondria. Substrates: succinate, β-hydroxybutyrate, and glutamate. Filled symbols represent oxygen uptake, open symbols represent phosphate esterification. ▲, △: 20 μmoles of succinate, 2.0 mg mitochondrial protein/ml incubation mixture, incubation time 22 min; ■, □: 20 μmoles of β-hydroxybutyrate, 2.4 mg mitochondrial protein/ml incubation mixture, incubation time 27 min; ●, O: 20 μmoles of glutamate, 2.0 mg mitochondrial protein/ml incubation mixture, incubation time 24 min. Other conditions are given in Methods. 100 % activity was 5.2, 2.4, and 3.5 μmoles oxygen uptake/mg mitochondrial protein/h, and 12.6, 5.6, and 9.4 μmoles phosphate esterified/mg mitochondrial protein/h with succinate, β-hydroxybutyrate, and glutamate, respectively.

well be that an inhibition of the electron transport is the primary action of alcohols on other photophosphorylation systems which have been investigated. Fig. 3 also shows that the sensitivity of photophosphorylation towards alcohol is approximately the same in the cyclic PMS-system as in the non-cyclic ferricyanide system, which by analogy gives some substantiation to the view presented above. Also in the bacterial chromatophores several control experiments have shown that the same concentration of alcohol was required for 50 % inhibition of cyclic photophosphorylation in the "physiological system" and in the "PMS-system".

In the mitochondria the NAD-linked electron transport and phosphorylation with β-hydroxybutyrate or glutamate as substrates were more sensitive to alcohol than with succinate as substrate, as is shown with butanol in Fig. 4. This figure also shows that with the NAD-linked substrates, butanol inhibited electron transport about as strongly as phosphorylation, but with succinate as substrate phosphorylation was more strongly inhibited than electron transport. Consequently, the P/O-ratio with glutamate as substrate is kept fairly constant, even when the inhibition of mitochondrial phosphorylation and electron transport is very strong, as is shown in Fig. 5. It should be observed that this is also true for a preparation with an as low initial P/O-ratio as that shown in this figure.

Table 2 shows that in a system depleted of intramitochondrial NAD by hypotonic treatment [10] as described under Methods, and provided with externally added NAD, the sensitivity of the NAD-linked electron transport from β-hydroxybutyrate is lowered, approaching the insensitivity of the succinate-linked electron transport. This strongly suggests that the greater sensitivity of the NAD-linked electron transport is due to a leakage of intramitochondrial NAD, as a consequence of the treatment with alcohol.

In Fig. 6 is shown that butanol has a strong stimulatory effect on the Mg^{2+}-activated ATP-ase and that the initially high ATP-ase activity in the DOC-treated mitochondria was not further stimulated by butanol.

It has been shown that preincubation of chloroplasts with an inhibitory concentration of butanol and subsequent centrifugation did not result in inhi-

Table 2. Decreased effect of butanol on NAD-linked electron transport in a reconstituted mitochondrial system with externally added NAD. Each vessel contained 2 mg of mitochondrial protein. Incubation time was 25 min. Substrate: 20 μmoles of β-hydroxybutyrate. The mitochondria in the two vessels containing NAD had before incubation been subjected to an osmotic shock, as described in Methods, where also the other conditions are given.

Additions	μmoles oxygen uptake/ mg protein/h	% inhibition with alcohol	μmoles phosphate uptake/ mg protein/h	% inhibition with alcohol
None	1.75		5.46	
2 % Butanol	0	100	0.18	97
5 μmoles NAD	2.56		0	
5 μmoles NAD + 2 % butanol	1.08	58	0	

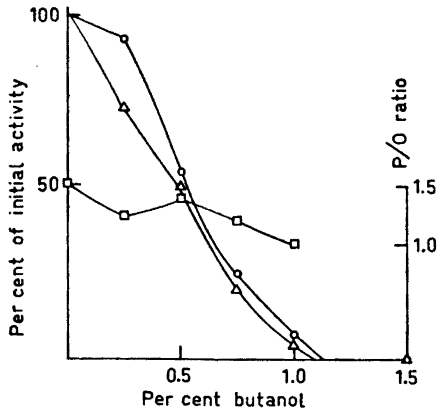

Fig. 5. Influence of butanol on P/O ratio in mitochondria. Substrate: glutamate. 2 mg mitochondrial protein/ml incubation mixture, incubation time 25 min. O: oxygen uptake; △; phosphate esterification; □: P/O ratio. Conditions are given in Methods. 100 % activity was 5.5 μmoles oxygen uptake and 8.7 μmoles phosphate esterified/mg mitochondrial protein/h.

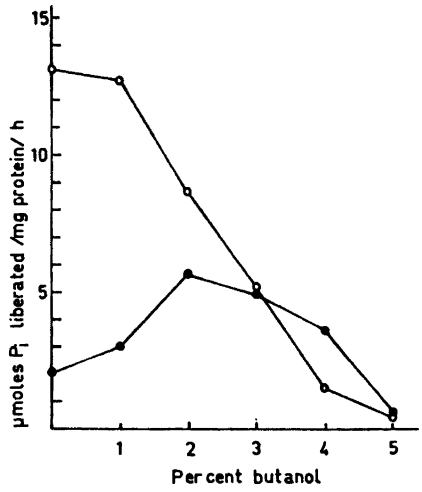

Fig. 6. Influence of butanol on Mg^{2+}-activated ATP-ase with and without pretreatment of the mitochondria with DOC. The incubation mixture contained in a total volume of 2 ml the following reagents in μmoles: Tris/HCl pH 7.5 100, sucrose 500, $MgCl_2$ 8, ATP 10. O: mitochondria pretreated with 0.1 % DOC in 0.25 M sucrose for 2 min at 0°C; ●: mitochondria not pretreated. The hydrolysis of ATP took place at 30°C. Incubation time was 20 min. The liberated inorganic phosphate was assayed as described in Methods.

bition of photophosphorylation in the resuspended chloroplasts.[2] A similar experiment with mitochondria is shown in Table 3 (preincubation with butanol of a strongly inhibiting concentration and subsequent centrifugation). ATP--formation and P/O-ratios with β-hydroxybutyrate, glutamate, or succinate as substrates are only slightly influenced in the resuspended mitochondria.

In Table 4 a strong inhibition of cyclic photophosphorylation in chromatophores by methanol, ethanol, or butanol is seen to be almost completely relieved by a 15-fold dilution of the alcohol-incubated preparation.

DISCUSSION

The concentrations of alcohols required to inhibit phosphorylation and electron transport are rather high. Warburg [1] proposed that the inhibitory effects on various biological functions caused by narcotic agents such as alcohols, was the consequence of a depression of surface tension caused by these agents. The inhibition would accordingly be of a general physical, rather than a specific chemical character. It was suggested by Baltscheffsky [2] that the inhibition of cyclic photophosphorylation in chloroplasts might be due

Table 3. Restoration of butanol-inhibited phosphorylation and respiration in mitochondria with succinate, β-hydroxybutyrate, or glutamate as substrates. Conditions are given in Methods.

Substrate	Conc. of alcohol in reaction mixture %	μmoles phosphate esterified/mg protein/h	% of initial activity	P/O ratio
Succinate	—	9.7	100	2.0
	3.0 [1]	0	0	—
	—	8.5	88	1.7
Glutamate	—	20.0	100	2.3
	2.5 [1]	0	0	—
	—	17.0	85	2.7
β-Hydroxybutyrate	—	5.7	100	3.0
	3.0 [1]	0	0	—
	—	4.6	81	2.4

[1]) Mitochondria preincubated 20 min in 0.25 M sucrose at 0°C with 3.0, 2.5 and 3.0 % of butanol respectively. To remove the alcohol the mitochondria were centrifuged at 4500 $\times g$ for 10 min and resuspended in alcohol-free sucrose.

Table 4. Restoration of alcohol-inhibited cyclic photophosphorylation in the "physiological system" of chromatophores by dilution of the alcohols. Conditions are given in Methods.

Conc. of alcohol in reaction mixture	μmoles phosphate esterified/mg chlorophyll/h	% of initial activity
—	15.5	100
15 % methanol	3.7	24
10 % ethanol	2.6	17
2 % butanol	0.8	5
1 % methanol	15.0	97
0.67 % ethanol	15.5	100
0.13 % butanol	16.0	103
— [1]	13.2	85
—	11.3	73
—	13.3	86

[1]) Chromatophore suspension preincubated 20 min, at 0°C with 2, 10, and 15 % of methanol, ethanol, and butanol, respectively, and then diluted 15 fold by adding the chromatophores to the reaction mixture.

to an enrichment of added alcohol in the phase boundaries between lipophilic and hydrophilic phases in the chloroplast membranes, where the electron transport and energy transfer reactions are considered to take place.[2,11]

According to the view expressed by Boyer [12] it is essential for the phosphorylation reactions to take place in non-aqueous surroundings. Thus the addition of surface active agents like alcohols, [14] which may well be thought to cause water to leak into the phosphorylating structures, would lead to an inhibition of phosphorylation. A somewhat related idea was advanced by Mitchell,[13] who suggested that any damage causing leakyness of the membranes would be expected to affect the energy coupling system by disturbing an H^+-gradient, which he considers as possibly being essential for the formation of ATP.

The enhanced ATP-ase activity which results from a treatment of the mitochondria with alcohol, indicates that disarrangement of the membranes actually takes place, in the same or a similar way as when mitochondria are treated with DOC, which is well-known as a membrane-destroying agent,[15] and which also stimulates the ATP-ase activity. It can be seen that the effects of DOC and butanol are not additive, *i.e.* after a pretreatment with DOC, the mitochondrial ATP-ase was not further stimulated by butanol. Somewhat related observations were made by Kiessling and Tilander [16] who showed that prolonged feeding of rats with ethanol enhanced the rate of Mg^{2+}-stimulated ATP-ase in the liver mitochondria.

In view of our demonstration that alcohols act not only on phosphorylation but also on electron transport in the subcellular particles investigated, it would appear that the inhibitory effects of higher concentrations of alcohols are due to rather severe damage on the membranes. Such damage will primarily cause a loss of the capacity for phosphorylation, as suggested by several authors,[1,12,13] but also a loss of the capacity for electron transport. Loss of intramitochondrial NAD, resulting from damage of the membranes caused by the alcohols, was a typical example of this latter phenomenon. This conclusion is supported by the demonstration that in a system which has been subjected to hypotonic treatment, and where respiration subsequently has been restored by addition of an excess of external NAD, the sensitivity of electron transport towards alcohol is considerably lowered. Similar results have been obtained with liver mitochondria from rats which had been given ethanol *in vivo*.[16] This treatment strongly decreased the ability of the mitochondria to oxidize pyruvate and glutamate, while the oxidation of succinate was not affected.

Another example of this was the effect on photophosphorylation in chloroplasts in the system where electrons were transported from water to ferricyanide. Here no uncoupling could be observed, *i.e.* electron transport and phosphate esterification were inhibited simultaneously. In this system, as in the NAD-linked systems for oxidative phosphorylation, electron transport thus appears to be the function which is primarily inhibited. The phosphorylation in the cyclic PMS-system where the rate of electron transport cannot be measured is about equally sensitive to alcohol as the phosphorylation in the ferricyanide system, which indicates that also in the cyclic system electron transport may be the function which is primarily inhibited.

The effects of alcohols on various functions of electron transport-coupled phosphorylation systems appear to us, as has been indicated in the discussion above, to be a consequence of their polarity and their lipid solubility, which would seem to cause enrichment of alcohol in the lipid part of the lipid-protein boundary of the membrane structures and inhibitory effects on the membrane-linked functions. The question of whether the inhibitory action of the alcohols is due, in essence, to an induction of leakyness in the membranes, which would destroy the necessary milieu for the reactions in question, may be investigated experimentally. In mitochondria there exists an appreciable body of knowledge on relations between uptake of water, swelling of the structures, and mutual influences between these phenomena and mitochondrial functions. Results from investigations of these interrelationships will be presented in a forthcoming paper.[17]

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