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# EFFECT OF STYRENE AND OTHER ALKYL BENZENE DERIVATIVES ON OXIDATION OF FAD- AND NAD-LINKED SUBSTRATES IN RAT LIVER MITOCHONDRIA

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**Abstract**—The effect on energetic metabolism of rat liver mitochondria (RLM) of styrene and other aliphatic benzene derivatives, i.e. toluene, ethylbenzene, α-methylstyrene and butylbenzene, is studied. It is shown that these compounds uncouple oxidative phosphorlyation and this effect is connected with the stimulation of passive entry of protons into mitochondria. The relationship between hydrophobicity of these compounds and their biological activity and mechanism of uncoupling effect are discussed.

The activity of many oxidoreduction systems of the inner mitochondrial membrane depends on its phospholipids' physicochemical state [1]. These phospholipids demonstrate several structural forms as a function of hydratation, temperature, fatty acid composition, polar head groups, perturbing molecules and so on [2]. For some mitochondrial enzymatic activities, i.e. succinate oxidation, ATPase activity ($F_1$) or cytochrome *c* oxidase Arrhenius plotting reveals distinct temperature breaks or transition limits [3–6]. These results may be interpreted as the activity of membrane bound enzymes dependent on the state of phospholipids associated with proteins of these enzymes [7, 8].

Monosubstituted benzene derivatives are well soluble in the lipids, which corresponds to the high logarithm of the partition coefficient in the octanol–water system ($\log P$) [9]. Lipophilic properties of these compounds might show their ability to bind with various kinds of mitochondrial inner membrane phospholipids.

Styrene is one of the principal precursors in the industrial production of artificial polymer materials. Its wide use poses a potential occupational hazard of significant magnitude for a great number of workers [10]. The influence of this compound on cellular metabolism is described in many papers, and it is concluded that styrene is mainly metabolised in the liver microsomal fraction [11–13]. No paper has been found concerning the influence of styrene and other monosubstituted benzene derivatives on oxidation processes in mictochondria. These compounds may cause injury of the enzymatic systems in the mitochondrial membrane. This investigation is concerned with the effect of styrene on the enzymatic processes taking place in the mitochondria, i.e. on the oxidation of FAD- and NAD-linked substrates and on the process of oxidative phosphorylation connected with it. The results obtained for styrene were compared with those for other monosubstituted benzene derivatives differing in hydrophobicity.

## MATERIALS AND METHODS

Rat liver mitochondria were prepared by the method of Weinbach [14]. White male Wistar rats (200–250 g weight) were used.

The respiration rate was measured at 25° with a Clark-type oxygen electrode [15]. The reaction mixture (2 ml) contained 0.25 M sucrose or 0.12 M KCl supplemented with 10 mM Tris–HCl (pH 7.4), 5 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$ and 2 mM EDTA. Oxygen uptake was recorded after the addition of 50 μl of mitochondrial suspension (4 mg of protein) with glutamate or succinate as the substrates. Other experimental details are given in the figures and tables.

ATPase activity in RLM was measured as described previously [16]. Three milligrams of proteins were added to the 1 ml reaction mixture containing 0.2 M sucrose, 10 mM Tris–HCl (pH 7.4) and 5 mM $MgCl_2$. After incubation at 30° for 5 min the reaction was stopped with 0.5 ml 10% (w/v) trichloroacetic acid, and inorganic phosphate ($P_i$) was determined according to Gomori [17]. The results are expressed in nmoles liberated $P_i$ at 5 min of 1 mg mitochondrial protein.

Proton permeability of rat liver mitochondria was measured according to the Mitchell and Moyle procedure [18] in the modification of Haslam *et al.* [19]. The pH was measured by using a combined electrode CK 2401 C connected to Radiometer PHM 84 Research pH Meter. Mitochondria (4 mg protein) were incubated at 30° in 2 ml medium containing KCl (125 mM), glycylglycine buffer (2 mM, pH 6.2), EDTA (0.5 mM, pH 6.2) and antymycin A (5 μg).

Protein determination was carried out using the biurette method [20].

An analytical grade of commercial reagents was employed.

The ethanol solution of redistilled toluene, styrene, ethylbenzene, α-methylstyrene and butylbenzene were used.

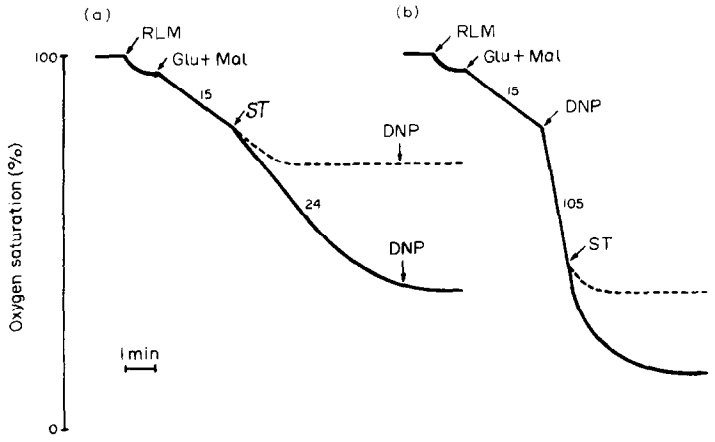


Fig. 1. Effect of styrene on glutamate (+L-malate) oxidation by RLM in the sucrose medium. Oxygen consumption was measured polarographically. As indicated, the following additions were made: 5 mM glutamate (Glu), 5 mM malate (Mal), 0.1 mM DNP, 3 mM styrene (ST)—solid line and 6 mM ST—dotted line. The numbers on slope represent oxygen consumption in nat oxygen/min mg protein.

In all experiments the control contained the same value of solvent (5–20 $\mu$l of ethanol). The concentration of ethanol did not effect the activities assayed.

## RESULTS

### *The influence of styrene and other aliphatic benzene derivatives on glutamate oxidation in RLM*

Figure 1 represents the experiments showing the effect of styrene on glutamate oxidation (in presence of L-malate). $3 \times 10^{-3}$ M styrene accelerates the oxygen consumption for a few minutes and then inhibits this process. This effect is not abolished by 2,4-dinitrophenol (DNP). At the higher concentration the inhibition is observed immediately after the addition of styrene (Fig. 1a). This compound also inhibits DNP stimulated glutamate oxidation (Fig. 1b), thus it may be concluded, that the stimulation of respiration immediately after styrene addition is connected with the uncoupling effect of mitochondria and followed by the inhibition of the activity of respiratory chain.

The other monosubstituted aliphatic benzene derivatives influence oxygen consumption by rat liver mitochondria in a similar way. Their stimulatory effect on respiration depends on aliphatic chain length. The maximal influence is observed for butylbenzene (four carbon chain), when toluene (one carbon chain) is without effect (Fig. 2a).

In DNP uncoupled mitochondria only styrene, ethylbenzene and $\alpha$-methylstyrene (two and three carbon chain) inhibit glutamate oxidation. Butylbenzene (four carbon) and toluene (one carbon) in $3 \times 10^{-3}$ M concentration are practically without effect (Fig. 2b). In KCl medium results are similar to those represented in Figs 1 and 2.

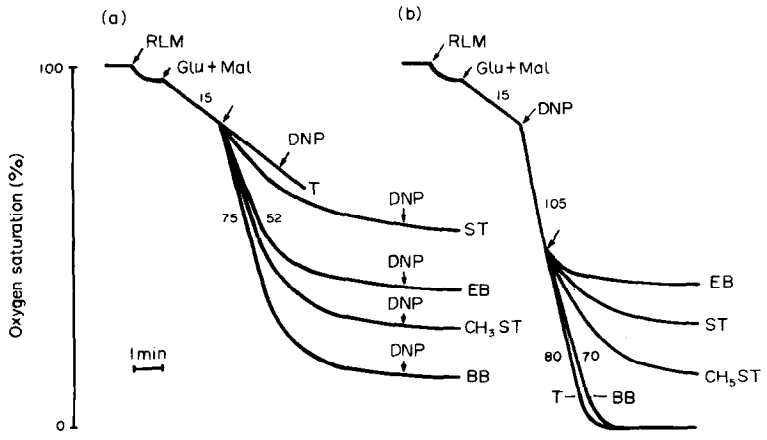


Fig. 2. Comparison of effects of monosubstituted benzene derivatives on glutamate oxidation in RLM in the sucrose medium: (a) before the addition of DNP (0.1 mM); (b) after DNP (0.1 mM). Arrows indicate the styrene derivatives addition. Final concentrations of ST, ethylbenzene (EB), $\alpha$-methylstyrene ($CH_3ST$) and butylbenzene (BB) were 3 mM.

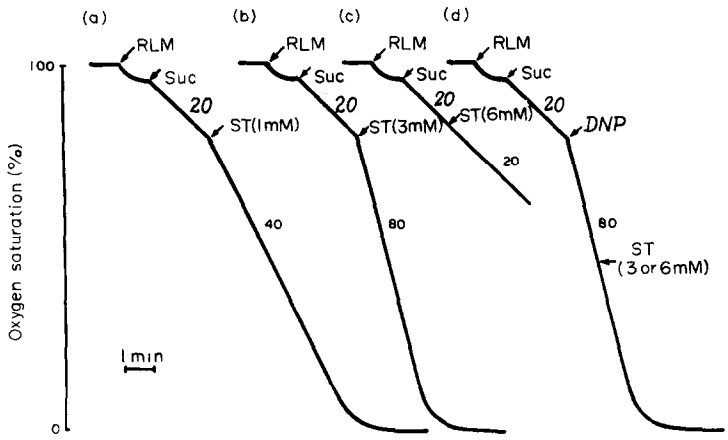


Fig. 3. Effect of styrene on succinate oxidation in RLM in the sucrose medium. The reaction mixture was supplemented by 10 μg rotenone. The other addition: succinate 5 mM (Suc), DNP 0.1 mM.

Table 1. Concentration of aliphatic benzene derivatives giving maximal uncoupling effect in RLM with succinate as the substrate in the sucrose medium

| | Concentration (mM) |
|---|---|
| Benzene (B) | without effect up to 10 mM |
| Toluene (T) | 5.0 ± 1.73 |
| Styrene (ST) | 2.3 ± 0.01 |
| Ethylbenzene (EB) | 1.8 ± 0.02 |
| α-Methylstyrene ($CH_3ST$) | 0.73 ± 0.09 |
| Butylbenzene (BB) | 0.54 ± 0.07 |

The values represent the mean ± SD for N = 7.
All conditions the same as in Fig. 3.

*The influence of aliphatic benzene derivatives on the succinate oxidation in RLM*

The stimulation of succinate oxidation by $3 \times 10^{-3}$ M styrene in metabolic state 4 is observed (Fig. 3b). DNP acts in the same manner (trace 3d). At a higher concentration of styrene ($6 \times 10^{-3}$ M) the acceleration of oxygen uptake is not observed. This species in both concentrations is without effect on DNP stimulated succinate oxidation (trace 3d). Similarly to experiments with glutamate, the stimulation of respiration by styrene is therefore probably connected with the mitochondria uncoupling effect. The other aliphatic derivatives of benzene also stimulate respiration in a similar way to styrene (Table 1). The same results are obtained in the KCl medium.

This uncoupling effect is positively correlated with side chain length of the investigated aliphatic derivatives of benzene, i.e. with hydrophobicity of these compounds [9]. A linear relationship is found between the hydrophobicity measured by logarithm of partition coefficient in the octanol–water system (log $P$) and logarithm of reciprocal concentration of compounds which causes the maximal uncoupling effect (log $1/c$). The relation is shown by the equation counted by the method of least squares.

$$\log 1/c = 1.34 + 0.45 \log P$$

$$N = 12 \quad r = 0.992 \quad s = 0.015$$

*The influence of styrene on ATPase activity*

The uncoupling effect of styrene in mitochondria is supported by the results of the studies of ATPase activity (Table 2). The maximal simulatory effect is observed at styrene concentration in which maximal stimulation of succinate oxidation occurs. It depends

Table 2. Effect of styrene on ATPase activity in RLM

| | $P_i$ (nmoles/mg protein) |
|---|---|
| Mit* | 84 |
| Mit + $MgCl_2$ | 130 |
| Mit + $MgCl_2$ + DNP | 797 |
| Mit + $MgCl_2$ + DNP + olig | 150 |
| Mit + $MgCl_2$ + $1.5 \times 10^{-4}$ M ST | 90 |
| Mit + $MgCl_2$ + $3.0 \times 10^{-4}$ M ST | 140 |
| Mit + $MgCl_2$ + $6.0 \times 10^{-4}$ M ST | 190 |
| Mit + $MgCl_2$ + $1.2 \times 10^{-3}$ M ST | 850 |
| Mit + $MgCl_2$ + $3.0 \times 10^{-3}$ M ST | 820 |
| Mit + $MgCl_2$ + $1.2 \times 10^{-3}$ M ST + olig | 140 |
| Mit + $1.2 \times 10^{-3}$ M ST* | 190 |
| Mit + $MgCl_2$ + $6.0 \times 10^{-3}$ M ST | 150 |

Olig—1 μg oligomycin was added.
* In this experiment 5 mM $MgCl_2$ was omitted.

Table 3. Comparison of various concentrations of monosubstituted benzene derivatives on $Mg^{2+}$ dependent ATPase activity in rat liver mitochondria

| Concentration (mM) | T | ST | EB | $\alpha$-$CH_3$ST | BB |
|---|---|---|---|---|---|
| | | $\Delta P_i$ (nmoles/mg protein) | | | |
| 0.05 | — | — | 70 | 0 | 0 |
| 0.15 | 0 | 0 | 95 | 0 | 250 |
| 0.30 | 0 | 50 | 250 | 250 | 635 |
| 0.60 | 0 | 100 | 440 | 780 | 225 |
| 1.20 | 60 | 780 | 930 | 565 | 110 |
| 3.00 | 300 | 750 | — | — | — |

$\Delta P_i$—the increase of $P_i$ after the substraction of control value (without addition of benzene derivatives).

on the presence of magnesium ion in the medium and is oligomycin sensitive. The other aliphatic benzenes also stimulate ATPase activity (Table 3). As in the experiment shown in Table 1, the correlation between the hydrophobicity of compounds and their concentration, which maximally stimulate ATPase activity, is observed. Butylbenzene, which is the most hydrophobic, stimulates the activity in a smaller concentration than toluene, which is the least hydrophobic. At higher concentration, styrene and also other aliphatic benzenes (Table 3) show much less effect on ATPase activity. As indicated in Table 2, at $6 \times 10^{-3}$ M styrene concentration (i.e. 2 times higher than optimal) the ATPase activity is the same as in the control.

*The effect of styrene on proton permeability in RLM*

The uncoupling effect of styrene is probably connected with the increased permeability of protons in mictochondria. Intact well coupled mitochondria are highly impermeable to protons [18]. If acid is added to non-respiring mitochondria suspended in a lightly buffered medium, pH falls abruptly and then partially recovers in a time-dependent fashion. Thus, the time-dependent alkalinization of the medium is a measure of passive entry of protons into the mitochondrial matrix. As shown in Fig. 4, a large overshoot in acidity after the addition of HCl and followed by a very slow alkalinization of medium was observed. The addition of uncoupler (DNP) markedly increases this process (half-life of alkalinization 90 sec). $3 \times 10^{-3}$ M styrene acts in the same way (the same half-life). The addition of $6 \times 10^{-3}$ M styrene decreases this parameter to 9 sec, whereas within 60 sec the effect of acidification was abolished completely.

## DISCUSSION

The results presented in this paper indicate that there is a high correlation between the effect of monosubstituted benzene derivatives on the oxidative phosphorylation and their hydrophobicity. It was found that the logarithm of the reciprocal of optimal uncoupling concentration and log *P* indicates a strong linear relation to correlation coefficient, $r = 0.992$.

The data in Table 3 also indicate that $Mg^{2+}$-dependent ATPase activity was stimulated by alkyl benzene derivatives, and this stimulation was also correlated with their hydrophobic properties. Similar connections between biological activity and hydrophobicity were found for many substances [9, 21, 22].

The uncoupling effect of styrene and other benzene derivatives is probably dependent on gradient proton abolishment between matrix and medium. Uncoupling styrene concentration stimulates passive entry of protons into mitochondria (Fig. 4). Similar results were obtained with the classical uncoupler DNP [18]. According to the chemiosmotic theory, dissipation of membrane potential is generally regarded as decisive for exhibition of uncoupling action. With a protonophoric uncoupler, dissipation of membrane potential is caused by shuttling protons across the membrane with an acid dissociable group within the molecules [23]. However, styrene does not possess an acid dissociable group. Therefore, it dissipates membrane potential by a different mechanism than that of protonophoric uncouplers.

On the other hand, studies of uncoupler binding to mitochondria led Weinbach and Garbus to conclude that uncoupling reagents induce structural changes in mitochondria, which may be interpreted as reflecting

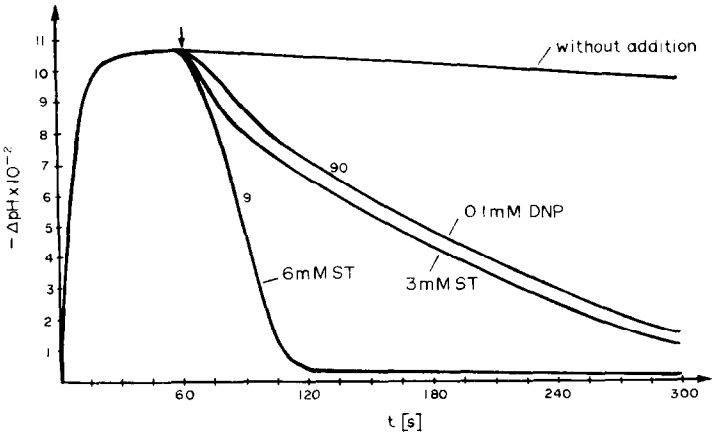


Fig. 4. Effect of styrene on the rate of passive entry of protons into RLM. The initial pH was 6.2 ± 0.1. At zero time 5 $\mu$l 25 mM HCl solution was added. Arrow indicates the addition of DNP or styrene. The numbers on slope represent half-life of alkalinization in seconds.

conformational transition in the lipoprotein complex [24]. The good positive correlation between the uncoupling ability of reagents and their binding parameters to phospholipids of mitochondria confirm this assumption [25].

As indicated in the experimental part, the most hydrophobic butylbenzene uncouples completely in $5 \times 10^{-4}$ M concentration, i.e. only 10 times higher concentration than the classical uncoupler DNP. Because it was indicated that mitochondria can bind aromatic uncouplers to the specific site in a higher amount than the minimum necessary for uncoupling [26, 27], it is possible that alkyl benzene derivatives bind to the same site.

It is likely that alkyl benzene derivatives also inhibit an initial segment of the respiratory chain (Complex I acc. to Hatefi) [28]. Inhibition of glutamate oxidation and parallel stimulation of succinate oxidation would confirm this idea.

As can be seen in Fig. 2b, there is no correlation between hydrophobic properties and their inhibitory effect on glutamate oxidation. The alkyl benzene derivatives with two carbon aliphatic chain, i.e. ethylbenzene and styrene, are the most effective. This effect might be explained in terms of specific interaction between the two carbon alkyl group of benzene derivatives and the inhibitory site. A similar explanation was given in the paper concerning the effects of phtalate esters on the rat liver mitochondria respiration [29].

Styrene and other monosubsituted benzene derivatives stimulate $Mg^{2+}$-dependent ATPase activity. Similar results were obtained for many compounds such as halothane [16] or carbon tetrachloride [30]. In these cases increase of ATPase activity ($Mg^{2+}$ stimulated) was probably connected to a direct effect at the level of this enzyme in the membrane or to an indirect effect of the benzene derivatives on the protonopermeability across the mitochondrial membrane.

Many pharmacologically active species containing alkylbenzene fragments in their molecules would act unspecifically on mitochondrial energetic metabolism, particularly in overdoses. The following drugs could be included in this group: phenformin—hypoglucaemic biguanide, cinnamedrine $\beta$—adrenoreceptor agonist, flunarizine—vasodilatator drug, amphetamines—anorectic drugs.

There is no information as to the fact that *in vivo* concentration of alkylbenzenes achieve the values which are needed to uncouple oxidative phosphorylation.

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