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ANALYSIS OF THE EFFECTS OF INHIBITORS ON NADPH-DEPENDENT ELECTRON TRANSPORT IN RAT LIVER MICROSOMES

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Abstract—Analysis of the effects of inhibitors on NADPH-oxidation in rat liver microsomes shows that the rate of total oxygen consumption parallels the rate of peroxidation of unsaturated fatty acids but not the rate of electron transfer within the chain. The addition of EDTA, 1 mM, to the incubation medium inhibits peroxidation and does not affect NADPH-dependent electron transfer. There are at least three sites for oxygen reduction within the NADPH-oxygenase complex. Oxygen reduction in the chain may occur both at the flavoprotein level and at the level of cytochrome P-450. In addition, Fe^{2+} ions bound with the functional groups of electron carriers and catalyzing the peroxidation of unsaturated fatty acids also participate in oxygen reduction. The fact that there are several points of activation of molecular oxygen within the NADPH-dependent electron transport chain precludes the evaluation of the effectiveness of action of inhibitors on cytochrome P-450 by measurement of the total oxygen consumption. NADH oxidation inhibited by EDTA is activated by addition of Ca^{2+}.

The oxidation of NADPH and NADH by membranes of the endoplasmic reticulum is accompanied by the reduction of molecular oxygen [1, 2]. Oxygen activated in the NADPH-dependent electron transport chain is utilized in the formation of peroxides of unsaturated fatty acids (UFA), of hydrogen peroxide and of hydroxyl groups in xenobiotic hydroxylation reactions [3–5]. The functional role of reduction of oxygen in the NADH oxidation chain is still obscure. The fact that there are different modes of utilization of molecular oxygen by the membranes of the endoplasmic reticulum prevents us from considering the rate of oxygen consumption as a definitive index for electron transfer rates within the NADPH-dependent chain.

The purpose of the present investigation was to elucidate the effects of inhibitory agents acting at different sites of the electron transport chain on the total rate of oxygen consumption. It was hoped that such an analysis of inhibition would make it possible to clarify rates of oxygen utilization in each of the oxygen-consuming reactions.

MATERIALS AND METHODS

Male rats, 150–200 g, fed a standard diet, were used. The microsomal fraction was obtained as described previously [6].

Enzyme assays. Oxygen consumption was measured by means of an LP-60 polarograph (Lab. Instr. Praha) with a fixed, exposed platinum electrode. One ml of the incubation mixture contained 100 mM Tris–HCl buffer, pH 7.4, and 4 mg of microsomal protein. The reaction was initiated by adding 1 mM NADPH or NADH. Measurements were made at 30° for 3–4 min.

The rates of demethylation of dimethylaniline (DMA) and of UFA peroxidation were determined as previously described [6, 7].

Microsomal protein was measured by the method of Lowry *et al.* [8] in the presence of 0.1% sodium deoxycholate, with crystalline bovine albumin as the standard.

Chemicals. NADPH and NADH were obtained from C. F. Boehringer, Mannheim, Germany. Mersalyl (sodium salt), EDTA (sodium salt), rotenone, albumin (V fraction), *n*-propylgallate and Tris (hydroxymethyl aminomethane) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Parachlormercuribenzoate (PCMB) and NaCN were obtained from Chemapol, Czechoslovakia. 2-Diethylaminoethyl-2,2-diphenylpropylacetate (SKF-525A) was purchased from Smith, Kline & French Laboratories, Philadelphia, Pa., U.S.A. 2-Methyl-1-di-3-pyridylpropanone (metyrapone) was obtained from Serva, Heidelberg, Germany; sodium azide from E. Merck, Darmstadt, Germany; and vitamin K_3 from Berlin-Chemi, Berlin, Germany. Amobarbital (amytal) and 1-phenyl-2,3-dimethyl-4-dimethylaminopyrazolon-5 (aminopyrine) were analytical grade (Sojuzkhemreactiv, Moscow, U.S.S.R.).

RESULTS AND DISCUSSION

NADPH oxidation by the microsomal fraction is coupled with UFA peroxidation. The stoichiometry of this process is such that from 4 to 10 moles of oxygen are consumed during the oxidation of 1 mole of NADPH [9, 10]. Consequently, the contribution of this reaction to the total oxygen consumption rate may be significant. Thus, an attempt was made to estimate the proportion of oxygen utilized for peroxidation of UFA to the total oxygen consumption rate during the course of NADPH oxidation by means of the addition of EDTA, an inhibitor of UFA peroxidation (Fig. 1A). It was found that the DMA hydroxylation reaction was much more resistant to

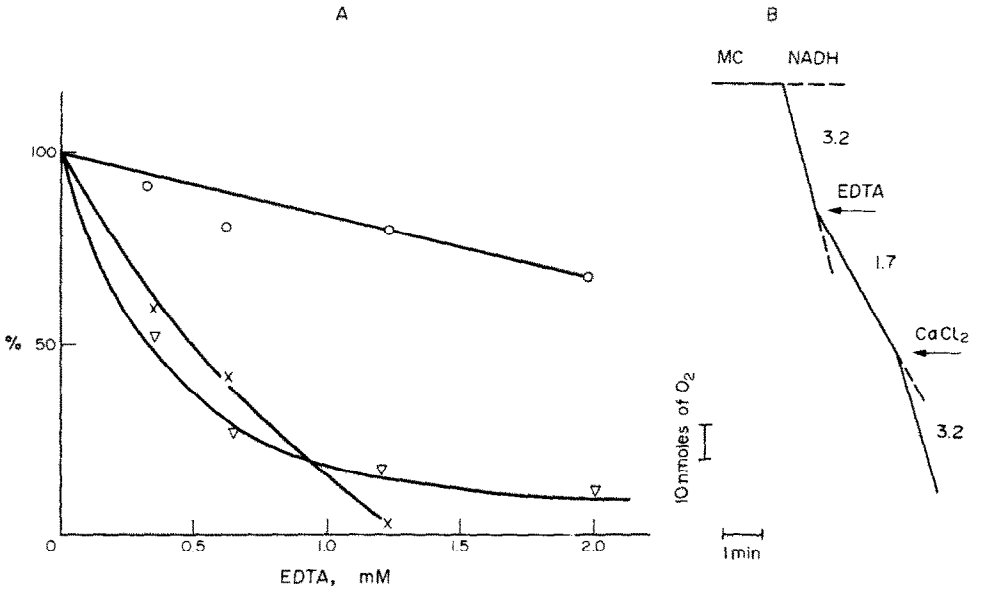

Fig. 1. Effect of EDTA on the rate of NADPH oxidation, DMA hydroxylation, and UFA peroxidation (A) and NADH oxidation (B). (A) NADPH oxidation (–×–×–). One ml of the incubation mixture contained 100 mM Tris–HCl buffer, pH 7.4, and 4 mg of microsomal protein. The oxygen consumption rate in the absence of EDTA (equal to 6.5 nmoles O_2/min/mg of microsomal protein) is assigned a value of 100 percent. Hydroxylation of DMA (–O–O–). One ml of the incubation mixture contained 40 mM Tris–HCl buffer, pH 7.4; 16 mM $MgCl_2$; 3 mM NADPH; 6 mM DMA; and 1.5 to 2 mg of microsomal protein. The rate of formation of formaldehyde in the absence of EDTA (equal to 8 nmoles/min/mg of microsomal protein) is assigned a value of 100 percent. Peroxidation of UFA (–∇–∇–). One ml of the incubation mixture contained 25 mM Tris–HCl buffer, pH 7.4; 0.012 mM Fe^{2+}; 0.2 mM NaPP; 3 mM NADPH; and 0.5 to 1 mg of microsomal protein. The rate of formation of malonic dialdehyde in the absence of EDTA (equal to 1.5 nmoles/min/mg of microsomal protein) is assigned a value of 100 percent. (B) NADH oxidation. One ml of the incubation mixture contained 100 mM Tris–HCl buffer, pH 7.4. Additions: microsomes (MC), 4 mg; NADH, 1 mM; EDTA, 0.6 mM; and $CaCl_2$, 1 mM. The units refer to the oxygen consumption in nmoles/min/mg of microsomal protein.

EDTA inhibition than was UFA peroxidation or free oxidation of NADPH. At the concentration of EDTA utilized (2 mM), the decrease in the DMA demethylation rate was only 25 per cent, whereas at an EDTA concentration of 0.3 mM, peroxidation of UFA and total oxygen consumption was inhibited by 50 per cent. The similarity in sensitivity of UFA peroxidation and of NADPH oxidation to EDTA inhibition suggests that the oxygen consumption in the system containing no EDTA reflects the rate of peroxidation processes rather than the electron transfer rate in the chain. In the presence of 0.6 mM EDTA, the oxygen consumption rate reflects the rate of electron transfer within the NADPH oxidation chain, as peroxidation of UFA is almost completely inhibited at this level of EDTA.

Addition of EDTA to the incubation mixture also inhibits the NADH-specific electron transfer chain, possibly as a consequence of the chelation of Ca^{2+} ions. Addition of Ca^{2+} ions to the incubation mixture containing the complex restores the NADH oxidation rate (Fig. 1B). When NADH is used as substrate, peroxidation of UFA is undetectable; as a consequence, ferrous ions cannot play an essential role in the processes of oxygen consumption when this reduced pyridine dinucleotide is oxidized.

For studying the action of inhibitors and activators of NADPH and NADH-dependent electron transfer on oxygen consumption, two types of agents were used: those which can interact with the flavoproteins of electron transfer complexes and those which can interact with cytochrome P-450.

Additions of amytal, rotenone and antimycin A (inhibitors blocking mitochondrial electron transfer from flavoprotein to cytochrome *b*) [11] do not produce inhibition of NADPH oxidation in microsomes. The inhibiting effect of amytal on NADH oxidation (Fig. 2, curve 1) cannot be explained by the presence of mitochondrial contamination [12], since other inhibitors of the mitochondrial respiratory chain (rotenone, and antimycin A) did not produce such an effect (Fig. 2, curves 2 and 3).

Studying the action of vitamin K_3 on the respiration rate in microsomes, we found that this agent, which according to the data of Nishibayashi *et al.* [13, 14] is an electron acceptor at the level of the NADPH-specific flavoprotein, sharply accelerates the oxidation rates of both NADPH and NADH (Fig. 2, curves 4 and 5).

The results obtained allow us to state that microsomal electron transfer chains are not sensitive to the action of specific mitochondrial inhibitors (amytal, rotenone, and antimycin A). At the same time, the oxygen consumption may be sharply stimulated at the flavoprotein level by vitamin K_3.

According to the data of Torielli and Slater [15] and Archakov *et al.* [16], addition of *n*-propylgallate to the incubation medium causes a marked inhibition

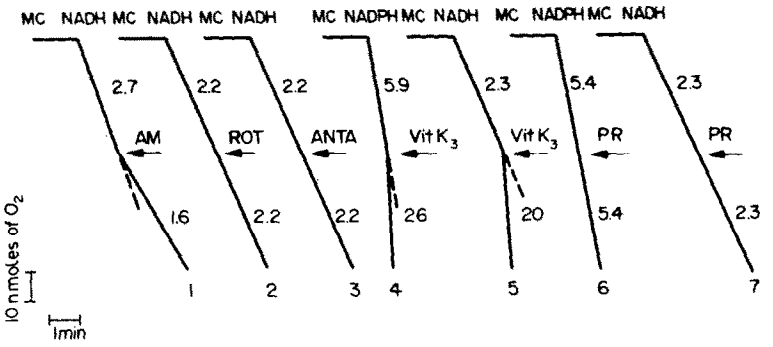

Fig. 2. Effect of compounds affecting the flavoprotein site of electron transfer chains on the NADPH oxidation rate in microsomes. One ml of the incubation mixture contained 100 mM Tris–HCl buffer, pH 7.4, and 0.6 mM EDTA. Additions: microsomes (MC, 4 mg; NADPH, 1 mM; NADH, 1 mM; amytal (AM), 3 mM; rotenone (ROT), 2×10^{-6} M; antimycin A (ANT A), 2×10^{-4} M; vitamin K_3 (Vit K_3), 1 mM; and *n*-propylgallate (PR), 0.2 mM. The units indicate the oxygen consumption rate in nmoles/min/mg of microsomal protein.

of hydroxylation reactions as a consequence of its interaction with NADPH-specific flavoprotein. The results illustrated in Fig. 2, curves 6 and 7, show that inhibition of hydroxylation reactions by *n*-propylgallate is not directly related to its action on the oxygen consumption rate.

In studying the effect of substrates and inhibitors of hydroxylation reactions, e.g. SKF-525A and metyrapone, it was observed that they produce different effects on the oxygen consumption rate. Aminopyrine and SKF-525A accelerate and metyrapone inhibits this reaction (Fig. 3, curves 1, 2, and 3). In the case of the NADH-specific chain, all these compounds exert an inhibitory effect, the mechanism of which remains obscure (Fig. 3, curves 4, 5, and 6).

In studying the effect on the NADPH oxidation rate of inhibitors which are specific for mitochondrial cytochrome oxidase, we found that NaCN and NaN_3 do not produce an inhibiting effect (Fig. 3, curves 7, and 8). At the same time, oxidation of NADPH is sensitive to carbon monoxide (Fig. 3, curve 9). On the contrary, NADH oxidation is sensitive to NaCN and NaN_3 but not to CO (Fig. 3, curves 10, 11, and 12).

These results show that a cyanide or azide-sensitive factor participates in the NADH oxidation reaction. From the data obtained in our laboratory, this component may be cytochrome oxidase which is present in the microsomal fraction due to mitochondrial contamination [12]. According to the data of Oshino *et al.* [17], it is a cyanide-sensitive factor which takes part in the desaturation reactions.

It is more difficult to explain the effect of the inhibitors on NADPH-dependent electron transfer. There is autoxidizable flavin within the redox chain which renders difficult the evaluation of their effects on cyto-

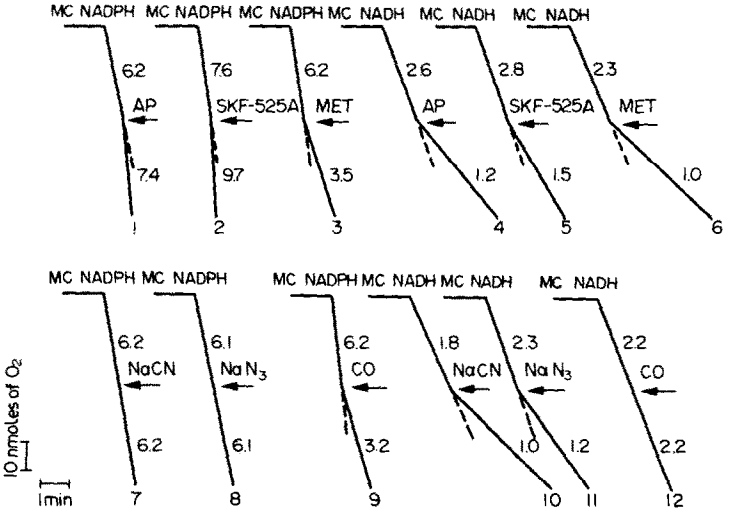

Fig. 3. Effect of compounds interacting with cytochrome P-450 on NADPH and NADH oxidation rates. One ml of the incubation mixture contained 100 mM Tris–HCl buffer, pH 7.4, and 0.6 mM EDTA. Substrates and inhibitors were added in final concentrations of 10^{-3} M. Abbreviations include: microsomes (MC); carbon monoxide (CO); metyrapone (MET); and aminopyrine (AP). CO was blown through the cuvette for 1 min. The units indicate the oxygen consumption rate in nmoles/min/mg of microsomal protein.

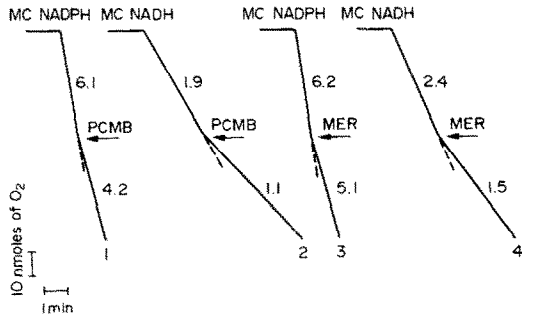

Fig. 4. Effect of sulfhydryl binding agents on NADPH and NADH oxidation rates. The experimental conditions are given in Fig. 3. Abbreviations include: microsomes (MC); and mersalyl (MER).

chrome P-450, since there remains a possibility of direct electron transfer from flavoprotein to oxygen.

These factors should be taken into consideration when the effects of sulfhydryl binding agents are examined (Fig. 4). Treatment of microsomes with PCMB and mersalyl at concentrations of 0.2 and 1 mM, respectively, produces an insignificant inhibiting effect on the oxygen consumption rate in NADPH and NADH oxidation in spite of the fact that the same concentrations completely block hydroxylation and peroxidation reactions [18]. It is noteworthy that the inhibiting effect of sulfhydryl finding agents is much more pronounced on the NADH oxidation chain.

The low effectiveness of CO and sulfhydryl binding agents as inhibitors of NADPH-oxidase when compared to their marked effectiveness as inhibitors of hydroxylation reactions favors the suggestion that inhibition of cytochrome P-450 has an insignificant effect on the overall rate of oxygen consumption within the chain.

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