LIPOPHILIC THIOUREA AND THIOURACIL AS INHIBITORS OF OXIDATIVE PHOSPHORYLATION

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1. Introduction

In various model reactions for oxidative phosphorylation, P_i as well as ADP were activated by the oxidation of thiol groups in waterfree solution [1]. Sulfenyl phosphates, $RSOPO_3H_2$, were supposed to be the reactive intermediates [2]. A quite stable β-lactoglobulin sulfenyl iodide, RSJ, reacted very rapidly with thiourea and thiouracil, two antithyroid agents, to form the corresponding mixed disulfides [3]. N-Mono-alkyl-thioureas (I) and 6-alkyl-2-thiouracils (II) of increasing chain length showed drastic rate enhancement after a rate minimum with $R = C_4H_9$ in (I) and with $R = C_2H_5$ in (II), supporting the idea that the
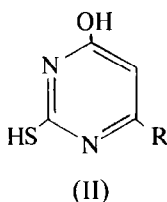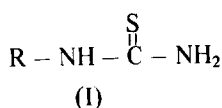
sulfenyl group was located in a lipophilic environment [4]. To test our working hypothesis that oxidized sulfur function, such as sulfenyl groups RS^+, could be involved in the energy transfer from the electron transport chain to the synthesis of ATP, the action of lipophilic thioureas and thiouracils has been studied. This report describes some properties of N-mono-nonyl-thioureas† (MNT; I, $R = C_9H_{19}$) and 6-nonyl-2-thiouracil (NTU; $R = C_9H_{19}$), two new inhibitors of oxidative phosphorylation.

2. Materials and methods

2.1. Isolation of mitochondria

Intact ox heart mitochondria were isolated according to the method of A.L. Smith [5], modified by the use of subtilisin. The mitochondria were separated into light and heavy layer fractions. The protein concentration was determined by the biuret method in the presence of 0.33% desoxycholate [6].

2.2. Measurement of respiration

Oxygen uptake was measured with a Clark-type oxygen electrode. All mitochondrial preparations were checked for structural integrity using the criterion of respiratory control [7].

2.3. Chemicals and reagents

6-Alkyl-2-thiouracils were synthesized according to Anderson et al. [4,8] and N-mono-alkyl-thioureas according to Nair [4,9]. 6-Nonyl-uracil and N-mono-nonyl-urea were prepared in our laboratory. Dihydro-lipoic acid was synthesized according to Gunsalus et al. [10]. The sources of the other reagents were: Merck, Darmstadt: TMPD, lipoic acid, DCCD; Biomol: Dithiothreitol; Fluka, Buchs.: 1.4-Dithiobutane; Serva: CCCP, bovine serum albumin; Schuchhardt:

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**Part of the dissertation work.

†*Abbreviations:* TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCCD, dicyclohexylcarbodiimide; MNT, *n*-mono-nonyl-thiourea; CCCP, *m*-chlorocarbonylcyanidephenylhydrazone; DNP, 2,4-dinitrophenol; NTU, 6-nonyl-2-thiouracil; DIC, dicoumarol.

Dicoumarol; Sigma: Rotenone; Novo Industries, Mainz: Subtilisin; all other reagents were obtained from Boehringer, Mannheim. All N-mono-alkyl-thioureas and 6-alkyl-2-thiouracils as well as 6-nonyl-uracil and N-mono-nonyl-urea were dissolved in ethanol.

3. Results

3.1. The effects of N-mono-nonyl-thiourea (MNT) on the coupled respiration of mitochondria

Of the N-mono-alkyl-thioureas (I; R = H, C_3H_7, C_5H_{11}, C_7H_{15}, C_9H_{19}) tested, only the most lipophilic one, MNT, showed effects on mitochondrial respiration. This is shown in fig.1, with glutamate + malate (experiment A), succinate (experiments B) and

ascorbate + TMPD (experiment C) as substrates. In all experiments ADP was first added to establish that the mitochondria exhibited respiratory control. In experiment A, when 140 nmol MNT/mg protein were added, one could observe some stimulation of state 4 respiration, but subsequent addition of ADP did not increase the rate of respiration, i.e. the transition state 4 → state 3 was prevented; 1.25 μM CCCP and 12.5 μM DNP resp. could not relieve this inhibition. In experiment B 170 nmol MNT/mg protein enhanced state 4 respiration to 70% of the initial state 3 respiration after the first addition of ADP as well as before it. No additional stimulation was found with 200 nmol ADP or with 1.25 μM CCCP. In experiment C maximal respiration rate was obtained with 125 nmol MNT/mg protein 83 μM dicoumarol enhanced the respiration
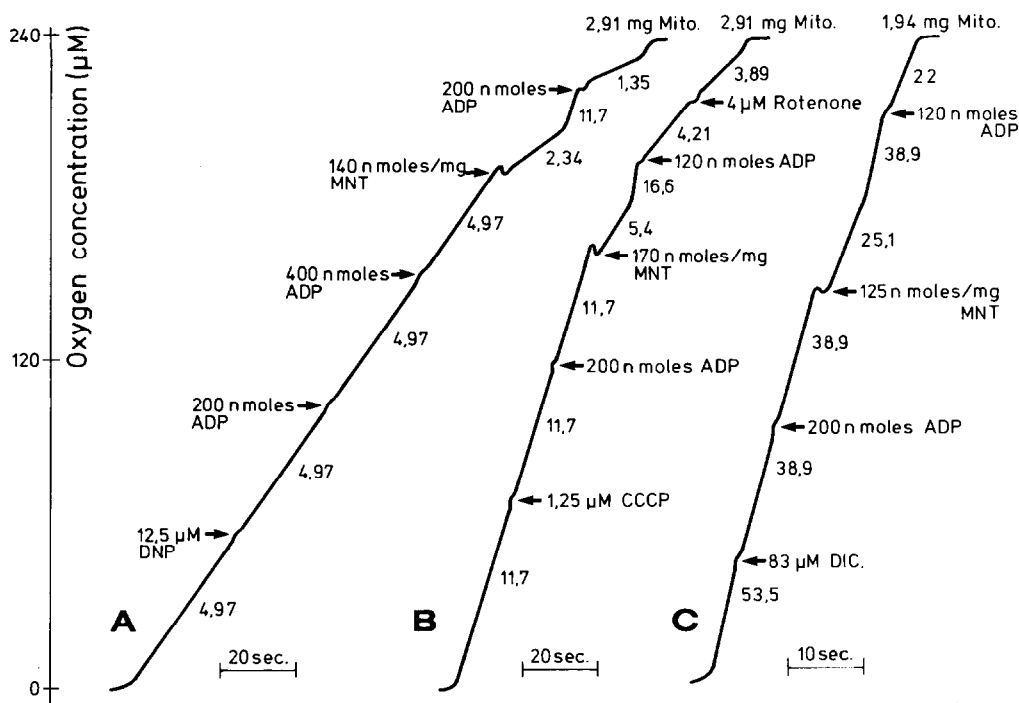

Fig.1. Effect of MNT on the couples respiration of mitochondria. The lines represent the output from an oxygen electrode. The numbers on the lines are respiration rates, μmoles of oxygen mg^{-1} protein h^{-1} at 25°C. Experiment A: ox heart mitochondria (2.91 mg) were added to a reaction mixture consisting of 2.4 ml 0.25 M sucrose containing 2.5 mM glutamate, 2.5 mM D, L-malate, 5 mM malonate, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate and 20 mM Tris–HCl, pH 7.4. Experiment B: ox heart mitochondria (2.91 mg) were added to a reaction mixture consisting of 2.4 ml 0.25 mM sucrose containing 10 mM succinate, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate and 20 mM Tris–HCl, pH 7.4. Experiment C: ox heart mitochondria (1.94 mg) were added to a reaction mixture consisting of 2.4 ml 0.25 M sucrose containing 2.5 mM ascorbate, 0.25 mM TMPD, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate and 20 mM Tris–HCl, pH 7.4.

rate to 140% of state 3 respiration. If the mitochondria were preincubated, in 0.25 M sucrose only with 50 nmol MNT/mg protein for 12 h at 5°C, state 4 → state 3 transition was inhibited completely with malate + glutamate as substrates. None of these effects of MNT was produced by equimolar amounts of the oxygen analogue, the N-mono-nonyl-urea ($C_9H_{19}NHCONH_2$). Neither bovine plasma albumin (2.5 mg/3.4 mg mitochondria), dihydrolipoic acid (160 μM), dithiothreitol (80 μM) nor 1.4-dithiobutane (400 μM) could relieve the inhibition of state 3 or the stimulation of state 4 respiration, described above.

3.2. The effects of 6-nonyl-2-thiouracil (NTU) on the coupled respiration of mitochondria

Using the same substrates as previously, the hetero-cyclic thiol compounds, the 6-alkyl-2-thiouracils (II), showed the same effects on mitochondrial respiration as the lipophilic thiourea MNT, when their alkyl chains were at least as large as an n-heptyl group. With the n-nonyl group lowest amounts, i.e. 240 nmol NTU/mg

protein, were necessary for the inhibition of state 4 → state 3 transition (experiment D, fig.2) instead of 140 nmol MNT/mg protein. In both, experiment E and F (fig.2), higher amounts of NTU as of MNT had to be used too for maximal stimulation of state 4 respiration, whereas in the preincubation experiments, exactly as for MNT, 50 nmol NTU/mg protein were enough to inhibit completely state 4 → state 3 transition with the substrates malate + glutamate. Equimolar amounts of the oxygen analogue of NTU, the 6-nonyl-uracil were inactive likewise after direct addition as well as after preincubation.

4. Discussion

MNT and NTU inhibited state 3 of respiration with the substrates glutamate + malate and this inhibition could not be relieved by CCCP or DNP. They stimulated state 4 respiration with succinate in the presence of rotenone to 70%, and with ascorbate + TMPD to the
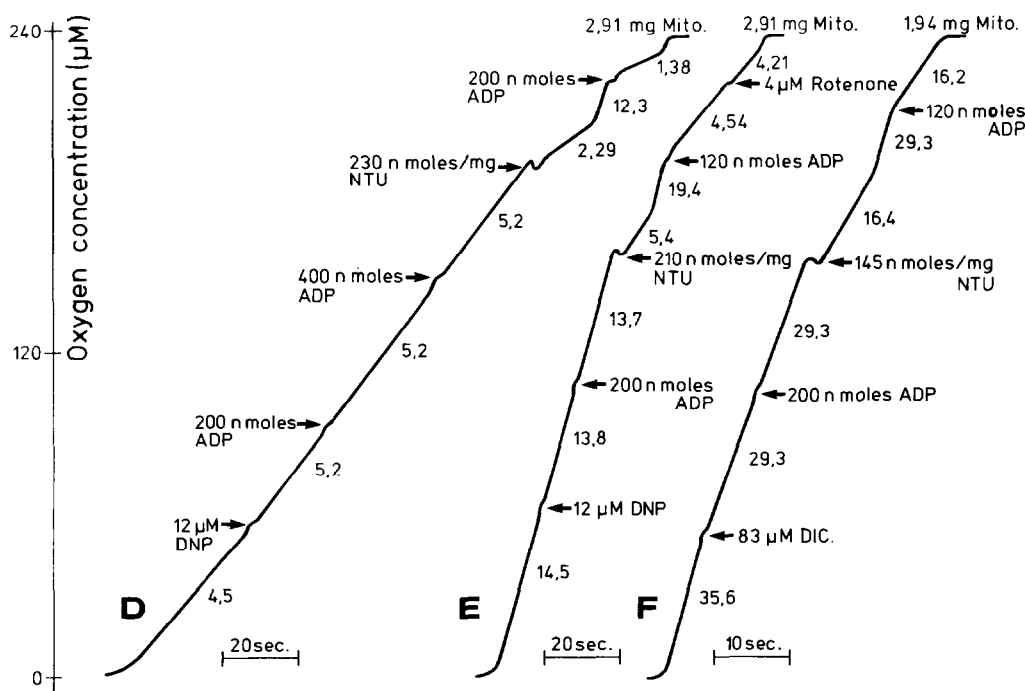

Fig.2. Effect of NTU on the coupled respiration of mitochondria. The conditions were the same for the experiments D, E and F in the same sequence as for the experiments A, B and C in fig.1. The numbers on the lines are respiration rates, μmoles oxygen mg^{-1} protein h.$^{-1}$ at 25°C.

maximum of state 3 respiration. For the latter system only, additional enhancement of the respiration rate could be shown by its specific uncoupler dicoumarol. From these findings one could derive that both substances were acting on the three coupling sites in a different manner. They probably affected the coupling process before the phosphorylating step, as state 3 was inhibited completely after preincubation with NTU in the absence of P_i, and because the stimulation of state 4 occurred without the addition of ADP. In no case was inhibition of the electron transport found. Thus, a reaction with the hypothetical, nonphosphorylated high-energy intermediate $X \sim I$ appeared a possibility. In terms of our working hypothesis, a sulfenyl group (RS^+) could be such an intermediate, which would react with MNT and NTU to form the corresponding mixed disulfide. A merely lipophilic interaction could be excluded, for equimolar amounts per mg protein of the oxygen analogues of MNT and NTU were inactive.

In a second type of reaction, thiourea in strongly acidic solution split cystine into the mixed disulfide and cysteine [11], i.e. a protonized disulfide reacted like an

$$ R - \underset{\oplus}{\overset{\overset{\displaystyle H}{|}}{S}} - \overset{X^{\ominus}}{S} - R \; \rightleftarrows \; RSH + RSX \qquad (1) $$

activated thioester of the sulfenic acid (RSOH) [12]. The inhibitory effect of MNT and NTU could therefore be related to such a protonized disulfied which would be the functional group for an energy transfer in mitochondria and this could be the mechanistic linkage to a proton-driven ATP synthesis, $X \sim I$ being the protonized disulfide and the protonation the coupling step. With the formation of the proton-induced sulfenyl group a thiol group was liberated (equation 1), which was assumed to react in mitochondria with low concentrations of lipophilic thiol reagents [13]. This mechanism may explain the findings of Sabadie-Pialoux and Gautheron [14], that the

total of thiol groups in whole mitochondria increased in state 3 respiration.

The unexpected stimulation of state 4 respiration by MNT and NTU with succinate or ascorbate + TMPD as substrates may be interpreted as hydrolysis or thiolysis of the mixed disulfide. Martius and Hess [15] may have observed a similar stimulation with 6-methyl-2-thiouracil preincubation experiments during the course of their studies on thyroxine-mitochondria interaction. Yet further work with radioactively labelled MNT and NTU has to be done to elucidate the action of the two substances, above all, if a covalent bound is really formed and if thiol or disulfide groups, for example, of the mitochondrial ATPase, are involved.

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