Effects of N-tricyanovinylamines on Oxidative Phosphorylation and Level of SH-groups in Rat Liver Mitochondria

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Abstract. The effects of N-substituted tricyanovinylamines on oxidative phosphorylation as well as on glutathione and total SH group concentrations in rat liver mitochondria was studied. The N-TCVA derivatives studied (N-cyclohexyl; N-isobutyl; N-benzyl; N-phenyl; N-4-Br-phenyl; N-3-nitrophenyl) had an uncoupling effection on the oxidative phosphorylation. They stimulated the respiration of mitochondria and influenced their membrane potential. In their property as SH agents,the N-TCVA derivatives reduced the level of TSH groups of the mitochondria present in concentrations of 2 μmol/mg protein. The activity of succinate dehydrogenase was decreased by N-TCVA by 13%. N-TCVA derivatives changed the redox state of glutathione in mitochondria. This effect was observed at the concentration 0.3 μmol/mg protein. The results obtained in the present study support the view that the glutathione status is more sensitive than the total level of SH groups to incubation of mitochondria with SH agents such as N-TCVA derivatives.

Key words: Glutathione — SH groups — Uncouplers — Mitochondria — N-tricyanovinylamines

Introduction

A number of research centers have been dealing with problems concerning the role of the SH-groups of proteins and low-molecular thiols in processes of oxidative phosphorylation. Attention has been focused on the essential SH-groups of enzymes of the respiratory chain, in particular the ATP-ase complex (Yagi and Hatefi 1984, 1987; Ježek 1987; Guerieri and Papa 1982; Beharry and Bragg 1989). Some specific agents are frequently used to investigate the relationship between the process of oxidative phosphorylation and the sulfhydryl groups of mitochondria. From this point of view N-tricyanovinylamines (R-NH-C(CN)=C(CN)$_2$; N-TCVA) represent

a perspective group of substances. Some derivatives of N-TCVA prepared in our laboratory significantly stimulated the respiration of rat liver mitochondria (Mihalovová and Podhradský 1986). In the present paper further experimental observations are presented which allow to classify these substances among protonophoric uncouplers. N-TCVA derivatives are able to react with thiols (Podhradský et al. 1990). In view of a possible combined effect of N-TCVA on processes in mitochondria, an attempt was made to investigate the possibilities of influencing the redox state of glutathione as well as the total SH groups in mitochondria.

Materials and Methods

The derivatives of N-TCVA were synthesized in our laboratory from tetracyanoethylene and primary amines (McKusick et al. 1958). The structure of the derivatives was checked by UV, IR and NMR spectroscopy. Reduced glutathione (GSH), oxidized glutathione (GSSG), N-ethylmaleimide (NEM) and glutathione reductase from yeast (EC 1.6.4.2) were supplied by Calbiochem (La Yolla, USA). ADP and safranine (SAF) were obtained from Serva (Heidelberg, GFR). NADPH was a product of Reanal (Budapest, Hungary). 5,5-Dithio-bis(2-nitrobenzoic) acid (DTNB; Ellman's reagent) was supplied by Sigma Chemie (München, GFR). Other chemicals of analytical grade were products of Lachema (Brno, CSFR).

The mitochondria were isolated from the liver of rats (RLM; Gazzoti et al. 1979) using a solution which contained sucrose (0.25 mol/l), tris (hydroxymethyl) aminomethane (Tris; 10 mmol/l), and EDTA (0.5 mmol/l) (pH 7.2). The respiration of the mitochondria was measured in 3 ml of the respiration solution containing sucrose (0.25 mol/l), Tris (10 mmol/l), EDTA (0.05 mmol/l), KH_2PO_4 (2.5 mmol/l), succinate (7.5 mmol/l) or glutamate (5 mmol/l), and malate (5 mmol/l); the measurements were performed using a Clark cell at 25 °C. The pH of the solution was 7.4. The stimulating effect of the substances tested was characterized by parameter SD_{50}, i.e. by the concentration of the respective substance inducing half maximal stimulation of respiration.

The effect of N-TCVA derivatives on RLM membrane potential were investigated by the method according to Akerman et al. (1976). Changes in absorbance of SAF ($\Delta A_{511-533}$) induced by the derivatives of N-TCVA were measured in a solution containing mannitol (0.3 mol/l), EDTA (0.5 mmol/l), Tris (10 mmol/l), malate (7.5 mmol/l), glutamate (7.5 mmol/l), SAF (25 mmol/mg protein) and RLM suspension (pH 7.4).

To estimate the effects of substances tested (N-TCVA derivatives and substrates of oxidative phosphorylation) on the mitochondrial glutathione concentration and TSH levels, the mitochondrial suspension was incubated at 25 °C for 5 min. in the incubation medium (pH 7.4) containing sucrose (0.25 mol/l), Tris (10 mmol/l) and EDTA (0.5 mmol/l). The respective agents were added after a preincubation (at 25 °C for 3 min.) of the mitochondrial suspension in the incubation medium.

The levels of total SH groups were determined using the Ellman's agens (Sedlák and Lindsay 1968). The incubation medium (1 ml) containing the respective substances was centrifuged for 10 min. at 5300 × g, and the sediment was resuspended in 1 ml of a solution (pH 8.0) containing Tris (0.2 mol/l) and EDTA (20 mmol/l). Then, it was left to react with 0.1 ml of DTNB solution (10 mmol/l) at 25 °C for 10 min., and 4ml of chilled

Table 1. Effects of the N-substituted derivatives of N-TCVA on the mitochondrial membrane potential. DD_{50} - concentration of N-TCVA derivatives (μmol/l) producing 50 % depolarization of mitochondrial membrane. For techniques see Methods.

derivatives N-TCVA	DD_{50} μmol/l
N-isobutyl	12.30
N-cyclohexyl	6.62
N-phenyl	2.50
N-4-Br-phenyl	25.40
N-3-nitrophenyl	152.00
N-benzyl	1.06

methanol were subsequently added, followed by centrifugation for 5 minutes ($530 \times g$) and reading of absorbance of the supernatant 412 nm.

GSH and GSSG were determined by the HPLC method (Reed et al. 1980) after derivatization with monoiodoacetic acid and 1-fluoro-2,4-dinitrobenzene, or by the spectrophotometric method (Eyer and Podhradský 1986) using DTNB and glutathione reductase. Proteins in 1 ml of the incubation medium were precipitated with $HClO_4$ (1 mol/l). After 10 minutes, the pH was adjusted with KH_2PO_4 (1.8 mol/l) to 6. After centrifugation (15 min., $530 \times g$) the supernatant was used for the determination of glutathione using one of the methods mentioned above.

The activity of succinate dehydrogenase (EC 1.3.99.1; SDH) was determined spectrophotometrically at 600 nm using 2,6-dichlorphenolindophenol and phenazine metasulfate (Krivchenkova 1977).

The protein content of the primary suspension of mitochondria was determined from the view point of according to Lowry et al. (1951).

In all experiments methanolic solution of N-TCVA and NEM were used. The final concentration of methanol did never exceed 1%.

Results

Some derivatives of N-tricyanovinylamines stimulate the respiration of mitochondria (Mihalovová and Podhradský 1986). The assumption that these N-TCVA derivatives affect oxidative phosphorylation as protonophoric uncouplers was verified by measuring the rat liver mitochondria membrane potential. Only mitochondria with RCR (respiration control rate) higher than 6.0 were used for the measurements. Depolarization of mitochondrial membrane was measured after addition of the respective N-TCVA derivatives in terms of $\Delta A_{511-533}$ changes of safranine. Changes in membrane potential of RLM produced by N-benzyl TCVA are shown in Fig. 1. A change in membrane potential already occurred after the addition of 0.176 μmol/l N- benzyl TCVA. DD_{50} values (the concentration of a compound

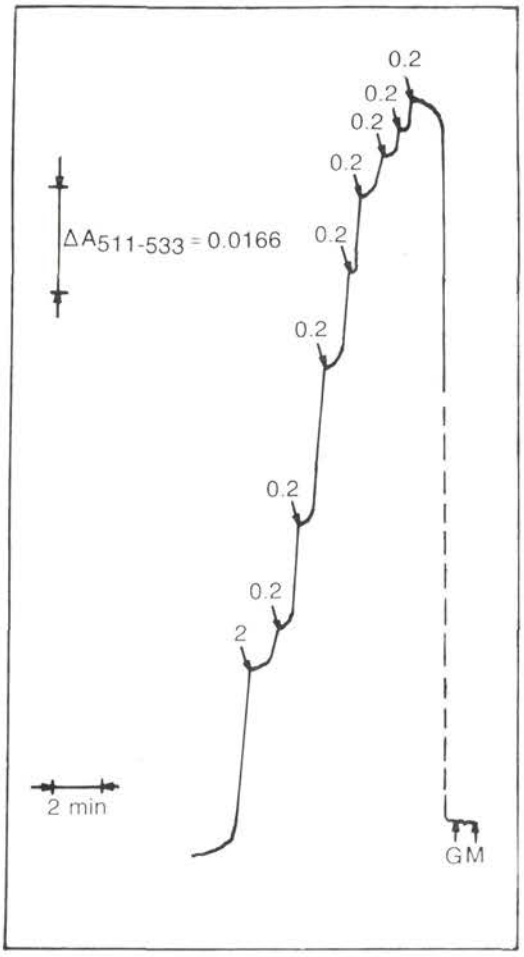

Figure 1. Changes in the RLM membrane potential produced by the N-benzyl TCVA tested, in terms of $\Delta A_{511-533}$ of safranine. Mitochondria (0.6 mg prot./ml) were resuspended in a solution containing glutamate (G), malate (M) and safranine. For the composition of the solution and the concentrations of the substances, see Materials and Methods.The quantities of the N-benzyl TCVA added are expressed in μmol/l of methanolic stock solution (1 mmol/l).

producing 50% depolarization of the mitochondrial membrane) were determined for six N-TCVA derivatives (Table 1).

N-TCVA derivatives react with thiols (Podhradský et al. 1990). We attempted to abolish the effects of N-TCVA on the respiration by thiols (mercaptoethanol and GSH). As seen from Figs. 2*a* and 2*b*, mercaptoethanol could restore the respiration,

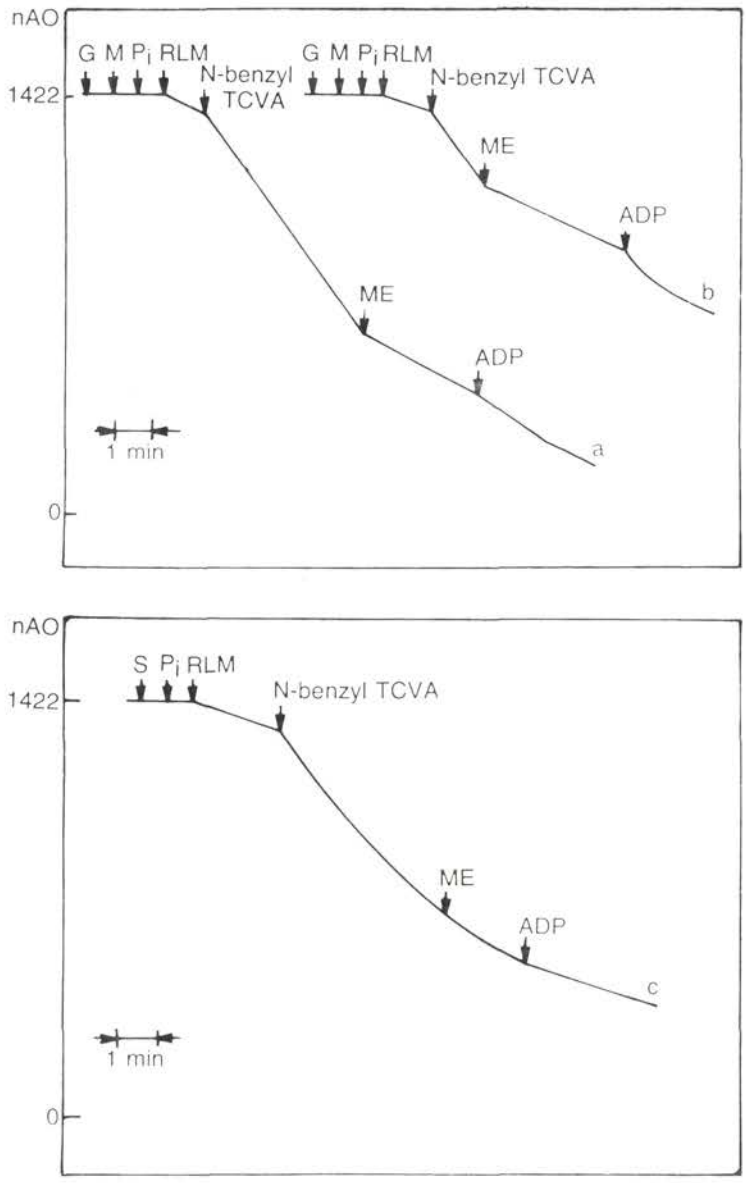

Figure 2. Effect of N-benzyl TCVA on RLM respiration rate in the presence of glutamate (G), malate (M) (*a*,*b*), and succinate (S) (*c*). For the composition of the solution, see Materials and Methods. N-benzyl TCVA, mercaptoethanol and ADP were added in concentrations of 5 μmol/l, 10 mmol/l and 150 μmol/l, respectively. Characteristics of mitochondria: RCR=3.0 in the presence of succinate, RCR=5.4 in the presence of glutamate and malate, final protein concentration 1.2 mg/ml.

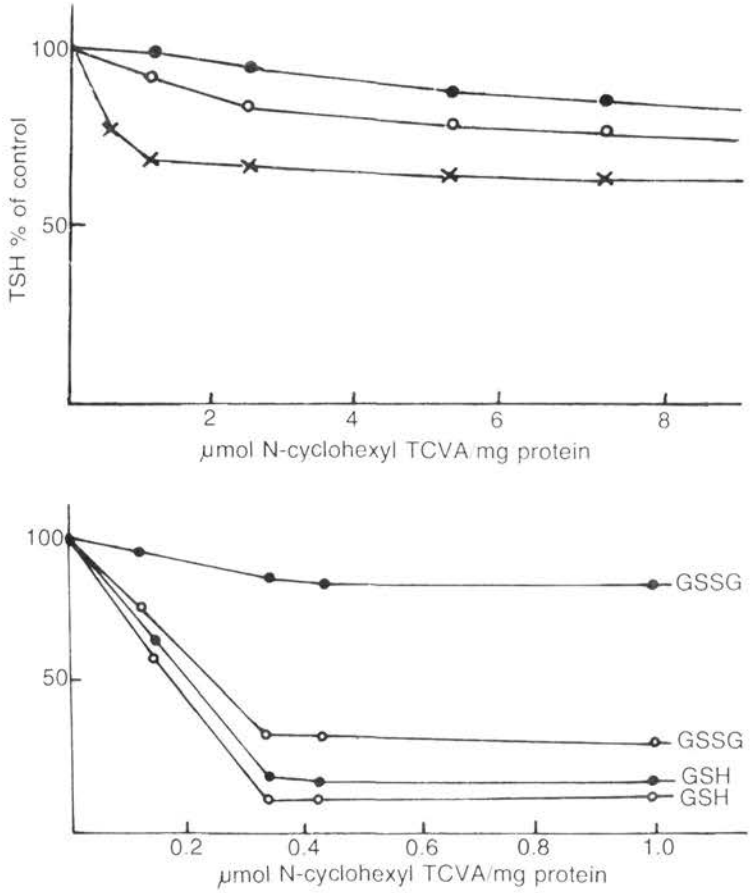

Figure 3. Effects of N-cyclohexyl TCVA on the level of total SH groups (3*a*) and GSH or GSSG concentration (3*b*) in the presence of succinate (– – • – – • ––); without succinate (– – o – – o ––) and in the presence of glutamate and malate (– – × – – × ––). For the technique see Methods. Each value is an average of 3 experiments. Values obtained in the absence of substrates were taken for 100 %: TSH=136 nmol/mg protein, GSH=4.4 nmol/mg protein, GSSG=1.8 nmol/mg protein; succinate (150 μmol/mg protein): TSH=200.39 nmol/mg protein, GSH=5.7 nmol/mg protein, GSSG=0.7 nmol/mg protein; glutamate and malate (100 μmol/mg protein, 100 μmol/mg protein): TSH=306.54 nmol/mg protein.

and this depended on the lenght of action of N-TCVA on the mitochondria. This phenomenon, i.e. abolishment of the N-TCVA effect on respiration, could not be achieved with GSH. The effect of N-benzyl TCVA on the mitochondrial respiration in the presence of succinate is shown in Fig. 2*c*. The respiration was stimulated

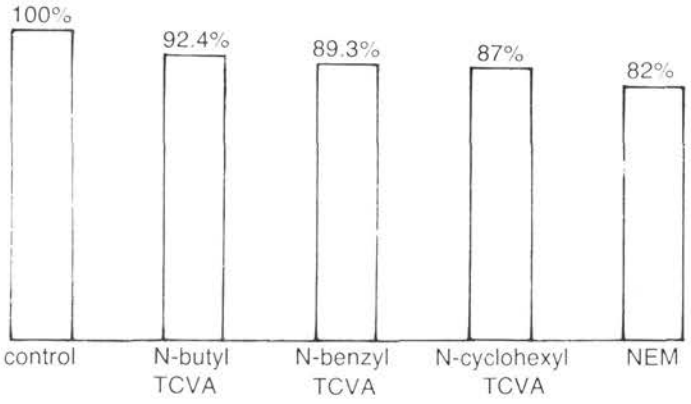

Figure 4. Effects of N-TCVA derivatives and N-etylmaleimide (NEM) on succinate dehydrogenase activity of the mitochondrial suspension. Incubation (0.4 mg protein): 10 min. at 37 °C. The concentrations of N-TCVA and NEM were 12 μmol/l.

by N-benzyl TCVA, but as early as after 2 minutes, it decreased and gradually, irreversible inhibition occurred.

As in the presence of succinate the RLM respiration is particularly sensitive to the inhibitory effect of N-TCVA , we investigated the activity of SDH in mitochondrial suspension after incubation with N-TCVA. The decrease of SDH activity after the incubation of the mitochondria with the N-TCVA derivatives studied and/or NEM is illustrated in Fig. 4.

Being SH agent, the N-TCVA derivatives studied are able to interact with the SH groups of enzymes of the respiratory chain and with low-molecular thiols of the mitochondria. After incubation of the mitochondrial suspension, with N-TCVA derivatives the levels of total SH groups and the state of glutathione (reduced and oxidized) were simultaneously studied.

First, the effects of the substrates of oxidative phosphorylation (succinate, glutamate and malate) on the level of TSH groups of the mitochondria and the glutathione status were determined. The level of TSH groups depends on the metabolic state of mitochondria. In the presence of the substrates glutamate and malate or succinate, TSH levels increased by 125% or 51% respectively (Table 2). The redox state of mitochondrial glutathione changed after the addition of succinate (Table 3). The observed changes were due to a lowering of the GSSG level.

The effects of N-cyclohexyl TCVA on the level of TSH groups and glutathione are shown in Figs. 3*a* and 3*b*. N-cyclohexyl TCVA (5.32 μmol/mg protein) reduced the level of TSH groups by 11% after 5 minutes of incubation in the presence of

Table 2. Changes in the total SH group levels (TSH) in mitochondria in the presence of substrates of oxidative phosphorylation. All results are given as percentages of values measured without the substrates (136 nmolSH/mg protein). Characteristics of mitochondria: 1.1 - 1.4 mg protein/ml, RCR=4 in the presence of succinate, and RCR=5.7 in the presence of glutamate and malate.

Substrate	Amount of protein μmol/mg	TSH %
nil		100
succinate	30	131.3
succinate	150	147.3
succinate	300	151.7
glutamate + malate	100+ 100	225.4

Table 3. Effects of succinate, P_i and ADP on the mitochondrial GSH and GSSG concentrations. GSH and GSSG were measured with the method described by Eyer and Podhradský (1986). For techniques see Methods. Each value represents an average of 3 experiments. Characteristics of mitochondria: 1.2 mg protein/ml, RCR=3.5.

Substrate	GSH nmol/mg protein	GSSG
nil	2.30	1.16
succinate + P_i	2.52	0.65
succinate + P_i + ADP	2.23	0.84

succinate (0.15 mmol/mg protein), and by 19 % in the absence of succinate. N-cyclohexyl TCVA changes the redox state of mitochondrial glutathione after 5 minutes of incubation. GSH levels decreased by 87 % in the presence of succinate, and by 82% in its absence, if N-cyclohexyl TCVA was used in a concentration of 0.33 μmol/mg protein. In both cases a simultaneous decrease in GSSG levels occurred.

Discussion

N-substituted tricyanovinylamines were prepared in our laboratory according to McKusick et al. (1958). Some of these derivatives stimulated the respiration of rat liver mitochondria. N-TCVA resemble carbonyl cyanide phenylhydrazones (CCP) in their structure; CCP are known uncouplers of oxidative phosphorylation. The effects of N-TCVA on respiration could be assumed on the grounds of the chemical

structure of these substances, the pK_a-values of the NH groups and the distribution coefficients. The pK_a-values of the NH groups of these structures as well as the distribution coefficients correlate with the measured values of the respiration stimulation (Mihalovová and Podhradský 1986). We investigated the effects of N-TCVA derivatives on the membrane potential (Table 1). The protonophoric effect of N-TCVA was supported by the ability of these agents to depolarize the mitochondrial membrane. The most efficient derivatives, i.e. N-benzyl TCVA, N-cyclohexyl TCVA, and N-butyl TCVA also had the strongest stimulating effect on respiration.

The N-TCVA derivatives react with thiols, and the effect on oxidative phosphorylation is possible but for relative by high concentrations of low molecular thiols. The successful recovery of RCR mitochondria is dependent on the interval of N-TCVA action on mercaptoethanol concentration. The N-TCVA as SH reagents are able to modify the SH groups of proteins. Complex II of the respiratory chain (succinate: ubiquinone oxidoreductase) is more sensitive to the N-TCVA derivatives studied than is complex II (NADH:ubiquinone oxidoreductase). This may explain the observation that with succinate as the substrate of oxidative phosphorylation, respiration was inhibited after 5 minutes of incubation with N-benzyl TCVA (Fig. 2*c*). N-TCVA modify the essential groups of SDH, they reduce the enzyme activity, and their effect is comparable with that of the alkylating reagent N-ethymaleimide (Fig. 4). N-TCVA are protonophoric uncouplers of oxidative phosphorylation; based on their effect on respiration however, it may be expected that these derivatives react with the SH groups of mitochondria. This assumption is supported by the results of the testing of N-TCVA effects on TSH group levels (Fig. 3*a*). The N-TCVA derivatives studied are able to attach to the SH groups of proteins. A decrease in the concentrations of TSH groups may be achieved with N-TCVA concentrations of 1 μmol/mg protein when an uncoupling effect develops. The levels of TSH groups depend of the metabolic state of mitochondria (Sabadie-Pialoux and Gautheron 1971). The dependence of TSH levels on succinate concentration agrees with their membrane transport.

If N-TCVA interaction with mitochondria results in alkylation of SH groups of the membrane, destabilization of the integrity of the latter may ensue. Le-Quoc and Le-Quoc (1982,1985) have analyzed the role of SH groups in the control of the inner mitochondrial membrane. It is very difficult to establish a causal relationship between modification of thiols and changes of mitochondrial quality. It is not clear whether the thiol reagents attack a specific protein and thus change the permeability, or whether some nonspecific perturbance occurs. Modification of SH groups of the membrane proteins affect the release of metabolites and ions from the matrix so that compounds able to penetrate through the mitochondrial membrane can enter through it. The SH groups play an essential role in the maintenance of the integrity of the membrane (Le-Quoc and Le-Quoc 1985).

The integrity of the mitochondrial membrane, its permeability, and activities of many enzymes depend on the glutathione redox system. In mitochondria there is a pool of glutathione (Griffith and Meister 1985). This thiol has to play some important functions in relation to the SH groups of proteins: GSH is involved in the protection against oxidizing and alkylating agents (Sies and Cadenas 1983; Kappus 1987). The redox state of glutathione influences the dithiol-disulfide reactions of the SH groups of proteins; thus, this "third messenger" is able to increase or decrease the activities of various enzymes (Inoue et al. 1987; Gilbert 1982). The interest in glutathione arose from the study of oxidative phosphorylation, in particular after the hypothesis was developed according to which dithiol-disulfide exchange should play an immediate role in the energy-transducing processes (Robbilard and Konings 1982).

The GSH/GSSG ratio in mitochondria is relatively stable. We observed a decrease of GSSG levels upon changing the metabolic state. The changes in GSSG concentration may have been caused by an increased activity of glutathione reductase. Glutathione reductase plays an important role in the function of glutathione. This enzyme is inhibited by TCVA derivatives as soon as their concentration reaches 10 mmol/l. The effects of N-TCVA on the redox state of glutathione were therefore studied. The levels of GSH and GSSG decreased upon the incubation of mitochondria with N-TCVA derivatives (0.1 μmol/mg protein; Fig. 3*b*).

It may be concluded from the results that the N-TCVA derivatives studied affect mitochondrial SH groups in the following ways:

1. N-TCVA derivatives lower the glutathione level (10^{-7} mol/mg protein). These compounds also influence GSH concentration indirectly by stimulating mitochondrial respiration, i.e. by affecting GSH-peroxidase activity.

2. The numbers of TSH groups decrease in the presence of N-TCVA derivatives in concentrations exceeding 10^{-6} mol/mg protein.

N-TCVA compounds act as uncouplers on the process of oxidative phosphorylation. In addition, they stimulate mitochondrial respiration and depolarize the mitochondrial membrane. Because of their reactivity with thiols, they are capable of interacting with SH-groups of the mitochondrial membrane thus affecting its integrity. A better understanding of these influences is important for the determination the effect of N-TCVA on the process of oxidative phosphorylation.

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