REACTIONS OF N-TRICYANOVINYLAMINES WITH THIOLS IN AQUEOUS SOLUTIONS

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Kinetics of reactions between N-substituted tricyanovinylamines and thiols, where primarily reduction of the C=C double bond takes place, was investigated under conditions adequate to physiological ones. The reaction rates did not depend either on the type of the reacting thiol, or on the pH of the medium, but they decreased with the increasing pK_a value of the imino group. A quantitative conversion of the thiol peptide glutathione to its oxidized form was evidenced.

Compounds with a tricyanovinyl substitution react in alkaline media with thiols even at elevated temperatures to give derivatives of pyrrole. The reaction started with reduction of the double bonds, nonetheless, intermediates have not been isolated in spite of the fact that their identification would contribute to the knowledge of their reduction mechanism. N-Substituted tricyanovinylamines (NTCVA) were shown to be biologically active disclosing a multitarget effect on mitochondria[1]. They uncouple the oxidative phosphorylation even at a 10^{-7} mol l^{-1} concentration and decrease the concentration of glutathione present up to 30% at the same time. This paper presents kinetic data of the reaction between NTCVA and thiols at conditions corresponding to experiments in which their biological activity was proved.

EXPERIMENTAL

Chemicals and apparatuses. Following chemicals were employed: reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase [E.C. 1.6.4.2], (Calbiochem), dithiothreitol, 3-mercaptopropionic acid, 2-mercaptoethanol, N-acetylcysteine, cysteine methyl ester, cysteine (Sigma), and thioglycolic acid (Reanal). N-Tricyanovinylamines were prepared from the corresponding amines (Lachema) and tetracyanoethylene (Fluka) according to ref.[2]. 1,2,2-Tricyano-1-benzylaminoethane was obtained by a sulfane reduction of N-benzyl-TCVA at pH 6·0 followed by crystallization. Structure of this product corroborated the presence of methine proton signals at δ 6·45 and 6·80 in the ^{1}H NMR spectrum. The UV spectra and kinetic measurements

were recorded with a Superscan 3 (Varian) spectrophotometer, the pH of solutions was monitored with an OP 208/1 (Radelkis) apparatus.

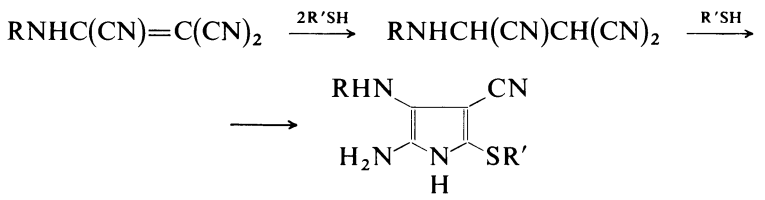
Kinetic measurements. The reaction of NTCVA with thiols was monitored in a 0·2M citrate-phosphate buffer at pH 4·0–8·5 and 25°C under the pseudofirst order condition. Concentrations of the reacting compounds were as follows: NTCVA $2{\cdot}5 \cdot 10^{-5}$ mol l^{-1}, thiols $2{\cdot}5 \cdot 10^{-3}$ mol l^{-1}; concentrations of methanol, in which NTCVA were dissolved did not exceed 1% in the medium. The pK_a values of NTCVA derivatives were obtained from the UV spectra measured in a 0·2 mol l^{-1} McIlvain buffer ($I = 0{\cdot}5$ mol l^{-1}, 1% methanol, 25°C). The pK_a values of SH groups of thiols were taken from the literature[3]. The second order rate constants k ($mol^{-1}\ s^{-1}$) were obtained spectroscopically by the method of initial velocities measurement.

Oxidation of GSH. Samples in which concentrations of GSH and GSSG were estimated by means of glutathione reductase[4] in the pre-set time intervals were withdrawn from the reaction medium consisting of GSH ($2{\cdot}5 \cdot 10^{-3}$ mol l^{-1}), N-cyclohexyl-TCVA (0·2 mol l^{-1}) and McIlvain buffer solution in EDTA (1 mmol). No enzyme inhibition was observed up to a 50 μmol $\cdot\ l^{-1}$ of N-cyclohexyl-TCVA concentration.

RESULTS AND DISCUSSION

NTCVA can react with biological systems within pH 5–9. The reactivity of various derivatives was compared with that of the tripeptide glutathione (γ-glutamylcysteinylglycine), which is the most frequented thiol peptide with a medium acidic SH group (pK_a 8·56). Results of kinetic measurements (Table I) indicate the reaction rates to rise with the increasing pK_a value of NTCVA. Generally speaking, the higher is the concentration of the deprotonized imino groups, the lower is the reactivity of NTCVA.

Thiols do not afford α-thiol derivatives with NTCVA, but primarily reduce the C=C double bond. In alkaline medium and in the presence of a catalyst at elevated temperatures also the nitrile groups of NTCVA enter the reaction forming substituted derivatives of pyrrole[5] (Scheme 1).



SCHEME 1

Under physiologic conditions only the first step could be expected as evidenced by the synthesis of 1,2,2-tricyano-1-benzylaminoethane, the UV spectrum of which (Fig. 1) was identical with that of the final product of the kinetically investigated reaction of N-benzyl-TCVA with GSH proceeding at a condition adequate to terms physiological to the mitochondrion.

Oxidation of thiols by oxidation agents, or spontaneously in alkaline medium were reported[6] to follow a radical mechanism. Provided a suitable acceptor of hydrogen radicals is at hand, the oxidation can proceed even under neutral or mild acidic conditions. Such acceptors can be e.g. derivatives of NTCVA, the C=C double bond, reduction of which proceeds through an anion-radical[7]. The radical

TABLE I

Values of k and pK_a of NTCVA, and $t/2$ for the reaction of $RNHC(CN)=C(CN)_2$ (c $2{\cdot}5 \cdot 10^{-5}$ mol l^{-1}) with GSH (c $2{\cdot}5 \cdot 10^{-3}$ mol l^{-1}) in 0·2M McIlvain buffer (pH 6·0) at 25°C

R	pK_a	$t/2$, min	$k \cdot 10^2$, $mol^{-1}\ s^{-1}$
3-Nitrophenyl	3·45	10·0	4·6 ± 0·4
4-Carboxyphenyl	4·20	8·2	5·6 ± 0·4
4-Bromophenyl	4·45	3·9	11·7 ± 0·5
Butyl	5·30	0·9	51·1 ± 0·5
Cyclohexyl	6·01	0·5	98·0 ± 0·9
Isobutyl	6·35	0·4	107·2 ± 1·2

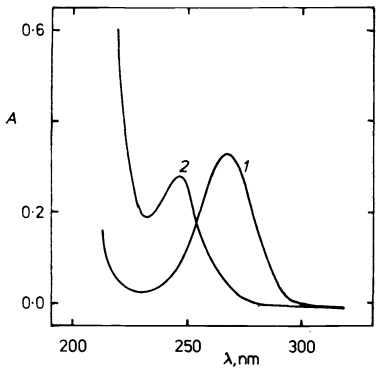


FIG. 1

The UV spectra of N-benzyl-TCVA (*1*) and 1,1,2-tricyano-1-benzylamino-ethane (*2*). Concentration of compounds $2{\cdot}5 \cdot 10^{-5}$ mol. l^{-1}, pH 7·0, 0·2M citrate-phosphate buffer containing 1% of methanol. Spectrum of the kinetically investigated product is identical with that of (*2*)

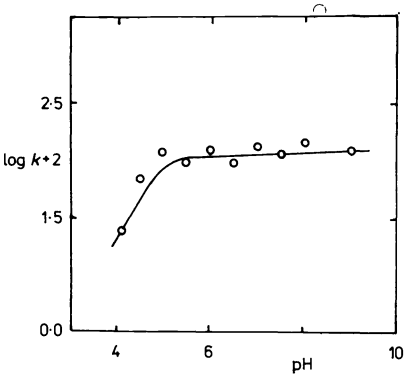


FIG. 2

The pH dependence of reactions between N-cyclohexyl-TCVA ($2{\cdot}5 \cdot 10^{-5}$ mol l^{-1}) and GSH ($2{\cdot}5 \cdot 10^{-3}$ mol l^{-1}) in 0·2M McIlvain buffer ($I = 0{\cdot}5$ mol l^{-1})

mechanism was corroborated by results of the kinetics measurement of the reaction between N-cyclohexyl-TCVA and GSH in relation to pH (Fig. 2) showing that *a*) the rate constants k (mol l^{-1}) recorded in the pH 6–9 range were virtually constant ($k \sim 1$); *b*) constants of a series of thiols do not depend on the pK_a values of SH groups (Table II); *c*) analyses proved the quantitative conversion of GSH into GSSG (Fig. 3).

The reduction of NTCVA followed by an immediate oxidation of glutathione, examined with the N-cyclohexyl derivative, could be illustrated by equations:

$$2\,GSH \rightarrow GSSG + 2\,e^- + 2\,H^+ \qquad (A)$$

TABLE II

Rate constants k for reactions of thiols (c 2·5 . 10^{-3} mol l^{-1}) with N-cyclohexyl-TCVA (c 2·5 . . 10^{-5} mol l^{-1}) in 0·2M citrate-phosphate buffer (pH 7·0, I 0·5 mol l^{-1})

Compound	pK_a[a]	k, mol^{-1} s^{-1}
Cysteine methylester	6·50	3·69 ± 0·4
Dithiothreitol	8·30/9·50	1·72 ± 0·1
Cysteine	8·53	2·32 ± 0·5
Glutathione	8·56	1·05 ± 0·5
N-Acetylcysteine	9·52	1·17 ± 0·1
2-Mercaptoethanol	9·72	4·56 ± 0·3
3-Mercaptopropionic acid	10·27	2·07 ± 0·2
Thioglycolic acid	10·40	2·98 ± 0·3

[a] Taken from ref.[3].

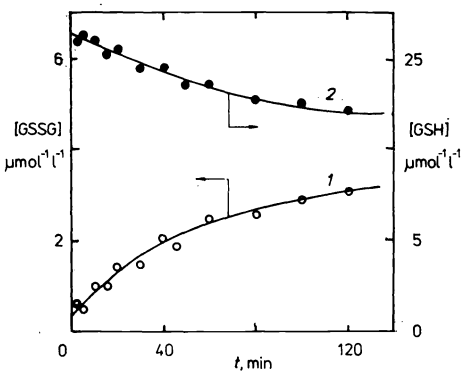


FIG. 3

Formation of GSSG (*1*) during oxidation of GSH (*2*) with N-cyclohexyl-TCVA (5 . 10^{-5} mol l^{-1}) at pH 6·02

$$C_6H_{11}NHC(CN)=C(CN)_2 + 2\,H^+ + 2\,e^- \rightarrow$$

$$\rightarrow C_6H_{11}NHCH(CN)CH(CN)_2\,. \qquad (B)$$

The rate constants k_{obs} estimated enzymatically for reaction (*A*) and spectrophotometrically for reaction (*B*) are identical (k_{obs} = 0·0225). Considering that reaction (*A*) does virtually not occur in the absence of the NTCVA derivative then the rate of GSH oxidation is subject to the affinity of the C=C bond towards hydrogen radicals.

NTCVA has uncoupling and oxidative effects on mitochondria. The highest uncoupling effect disclosed N-isobutyl and N-cyclohexyl derivatives, in other words compounds showing the highest rate constant on reaction with GSH. Similar multitarget properties were already described with phenylhydrazonopropanedinitriles, which react reversibly with thiols[8,9]. NTCVA appear to be more perspective as reagents suitable for investigating the role of SH-aminoacids and peptides in uncoupled mitochondria.

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