REVERSIBILITY OF REACTIONS OF PHENYLHYDRAZONOPROPANEDINITRILES WITH THIOLS

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S-Benzyl (2-phenylhydrazono)cyanothioacetimidate, as a model product of the reactions of phenylhydrazonopropanedinitriles with thiols, is decomposed in physiological medium into the original reactants, *i.e.* benzyl mercaptan and phenylhydrazonopropanedinitrile. The decomposition rate increases with increasing pH value of the medium. The found reversibility of the reactions of phenylhydrazonopropanedinitriles with thiols is discussed with respect to elucidation of bioactivity of phenylhydrazonopropanedinitriles.

Phenylhydrazonopropanedinitriles, which are incorrectly called carbonylcyanide phenylhydrazones in biochemical literature, are typical uncouplers of oxidative and photosynthetic phosphorylation[1-3] and are used as efficient tools in studies of energy transformation processes in biosystems[4-7]. Due to the presence of acid-base centre in the molecule (the imino group with pK_a in the interval of 5·5–7·0) and to suitable lipophilic-hydrophilic properties, these molecules can transfer protons through a phase interface and thereby remove the pH gradients generated on biological membranes[8-10]. Another bioactivity-determining property of phenylhydrazonopropanedinitriles consists in their chemical reactivity with SH groups of physiologically important lowmolecular thiols and thiolproteins[11-13]. The reactions with thiols belong to bimolecular nucleophilic addition reactions[14-15], the attack by the thiolate anion taking place at the carbon atom of some of the cyano groups present in the heterocumulene grouping in molecules of these compounds[16].

The present paper deals with problems of reaction equilibria of phenylhydrazonopropanedinitriles with thiols, because several biological papers[12,17,18] contained indirect hints about their importance.

EXPERIMENTAL

The syntheses of phenyldrazonopropanedinitrile and its reaction product with benzyl mercaptan were carried out according to Sulo and coworkers[16]. The Ellman ragent, 5,5′-dithiobis(2-nitro-

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benzoic acid) (DTNB), was a commercial product (Fluka, Switzerland). Tris(hydroxymethyl)-methanamine (TRIS), adenosine diphosphate (ADP), rotenone, oligomycin, morpholinopropanesulphonic acid (MOPS), and succinic acid were also commercial products (Sigma, St. Louis, U.S.A.).

The spectrophotometric measurements were carried out by means of a Specord UV VIS (Zeiss, Jena, G.D.R.) and an SP 30 Unicam (Cambridge, England) apparatus. The kinetics of both formation and decomposition of S-benzyl (2-phenylhydrazono)cyanothioacetimidate were followed spectrophotometrically at 25°C in buffers of various pH values.

The formation of benzyl mercaptan after the decomposition of S-benzyl (2-phenylhydrazono)-cyanothioacetimidate was proved by means of the Ellman detection method for thiols[19] and that of phenylhydrazonopropanedinitrile by means of TLC of the reaction mixture on Silufol plates (Kavalier, Czechoslovakia) with benzene–ethyl acetate (4 : 1) as the eluent. Besides that we also used the extraction method with spectrophotometric detection of the decomposition products, making use of the fact that S-benzyl (2-phenylhydrazono)cyanothioacetimidate is extracted with hexane practically quantitatively whereas the ionized form of phenylhydrazonopropanedinitrile only negligibly. The decomposition of the compound ($5 . 10^{-5}$ mol dm^{-3}) was followed in a borate buffer of pH 10·0. After a one minute incubation at 25°C, two 2 ml samples were taken from the reaction mixture. One of them was acidified with concentrated hydrochloric acid (0·1 ml) to pH 4·0 and extracted with 2 ml hexane, the other was extracted without acidification. The UV and VIS spectra of the two samples were compared with those of the reactants themselves.

The decomposition of S-benzyl (2-phenylhydrazono)cyanothioacetimidate was also followed in the presence of cysteine. The borate buffer of pH 10·0 containing 1 mmol l^{-1} cysteine was mixed with methanolic solution of the compound followed to make its final concentration equal to $5 . 10^{-5}$ mol l^{-1}. After various incubation periods (1—45 min), 2 ml samples were taken and acidified with concentrated hydrochloric acid to pH 4·0. After extraction with 2 ml hexane, electronic absorption spectra were measured in both the phases and compared with those of pure reactants.

The mitochondria were isolated from the rat liver by the procedure given in ref.[20]. Their protein content was determined by the Lowry method[21]. The respiration of mitochondria was followed in a medium containing (*per* 1 l): 10 mmol TRIS, 0·5 mmol EDTA, 0·3 mol saccharose, 2 mmol KH_2PO_4, 2 mmol $MgCl_2$, 2·5 μmol rotenone, and 7·5 mmol succinate (pH 7·4) by means of a Clark oxygen electrode in a temperated vessel (25°C) with stirring. The substances investigated were added in the form of fresh solutions in dimethyl sulphoxide (the resulting solvent concentration never exceeded 1 vol. %). After addition of basic mitochondria suspension to the final concentration of 0·4 mg ml^{-1} proteins and stabilization of the respiratory rate (the state 4), a check of functional integrity (the respiration check) of the mitochondria was carried out by addition of ADP in a concentration of 0·2 mmol dm^{-3} (the state 3).

RESULTS AND DISCUSSION

Our study of the decomposition of the reaction products of phenylhydrazonopropanedinitriles with thiols was carried out with S-benzyl (2-phenylhydrazono)cyanothioacetimidate, which is formed by the addition of benzyl mercaptan to the parent derivative[22]. As it is seen in Fig. 1, the decomposition rate can be easily followed by means of spectrophotometry. The absorption spectrum of the reaction mixture after complete decomposition is identical with that of phenylhydrazonopropanedi-

nitrile. The observed decomposition rate constants determined from the time dependence of $\log(A_t - A_\infty)$ are increased with increasing pH value of the medium (Fig. 2), which indicates that the decomposition reaction is preceded by the ionisation of S-benzyl (2-phenylhydrazono)cyanothioacetimidate.

For a more detailed characterization of the decomposition products formed from S-benzyl (2-phenylhydrazono)cyanothioacetimidate the extraction method was used which is described in Experimental. The adduct of benzyl mercaptan and phenylhydrazonopropanedinitrile ($pK_a = 10{\cdot}5$) is extracted practically quantitatively with hexane, whereas the ionized form of phenylhydrazonopropanedinitrile ($pK_a = 6{\cdot}5$) is extracted negligibly. The decomposition of the addition product was followed in a borate buffer (pH 10·0). The results of spectrophotometrical analysis of the extracted samples showed that S-benzyl (2-phenylhydrazono)cyanothioacetimidate was decomposed rapidly, because it was not found in the hexane phase. The phenylhydrazonopropanedinitrile formed remained in the aqueous phase. With the sample extracted after acidification to pH 4·0 it was possible to deduce from the absorption spectrum of the hexane phase that the latter contained a part of the extracted phenylhydrazonopropanedinitrile, the rest being in the aqueous phase.

The results obtained by following the decomposition of S-benzyl (2-phenylhydrazono)cyanothioacetimidate in the presence of high concentrations of cysteine as

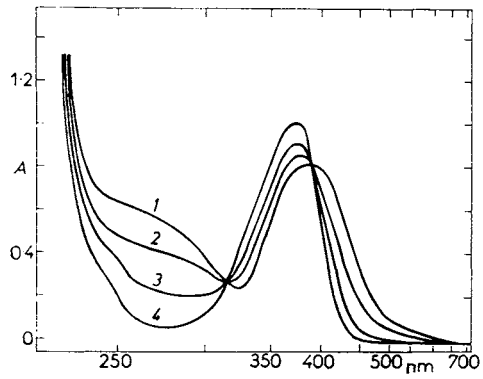

FIG. 1

Decomposition of S-benzyl (2-phenylhydrazono)cyanothioacetimidate ($5 \cdot 10^{-5}$ mol . . dm^{-3}) in phosphate buffer (pH 7·0) at 25°C. Curve *1* represents the spectrum immediately after the start (the recording time 2·2 min), curve *2* after 10 min, *3* after 40 min, and *4* after 240 or 340 min from the start

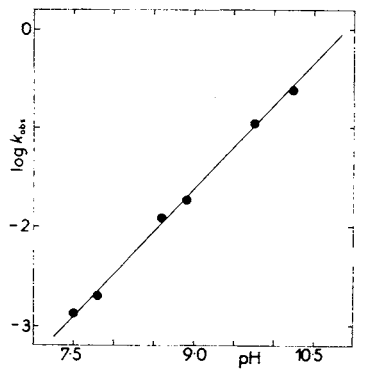

FIG. 2

The pH dependence of the decomposition rate constant of the first order (k_{obs}, s^{-1}) for S-benzyl (2-phenylhydrazono)cyanothioacetimidate. The reactions were folowed spectrophotometrically at 417 nm in buffers at 25°C. The initial concentration of the reactant was $7{\cdot}5 \cdot 10^{-5}$ mol dm^{-3}

a model thiol in borate buffer (pH 10·0) showed, too, that the S-benzyl (2-phenylhydrazono)cyanothioacetimidate was rapidly decomposed into benzyl mercaptan and phenylhydrazonopropanedinitrile, whereupon the latter substance reacted slowly with the cysteine present. This fact is indicated by the absorbance increase in aqueous phases and decrease of the absorption maxima in the spectra of hexane fractions. The reaction product from phenylhydrazonopropanedinitrile and cysteine cannot be extracted with hexane due to the presence of ionized carboxylic group of the attached cysteine.

Besides the extraction method, chromatography was also used to prove the decomposition of the adduct into benzyl mercaptan and phenylhydrazonopropanedinitrile (R_F: benzyl mercaptan 0·66, phenylhydrazonopropanedinitrile 0·50, adduct 0·30). The formation of benzyl mercaptan during the decomposition was also verified with application of the Ellman reagent (yellow product).

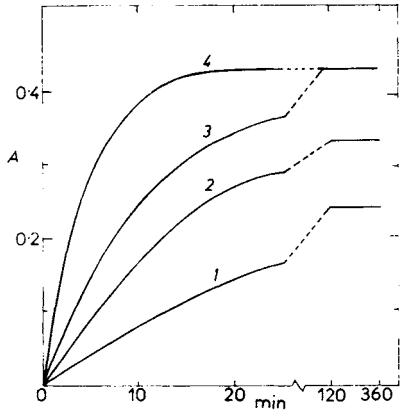

FIG. 3

The reaction kinetics of phenylhydrazonopropanedinitrile (FHPD) with benzyl mercaptan in TRIS buffer (pH 7·5) with dioxane (1 : 1) at 25°C. The initial concentration of FHPD $5 . 10^{-5}$ mol dm^{-3}, that of benzyl mercaptan: *1* $9{\cdot}5 . 10^{-4}$, *2* $1{\cdot}9 . 10^{-3}$, *3* $3{\cdot}8 . 10^{-3}$, *4* $7{\cdot}6 . 10^{-3}$ mol dm^{-3}. The absorbance was measured at 440 nm in a 1 cm cell, the reference cell contained $5 . 10^{-5}$ mol dm^{-3} FHPD

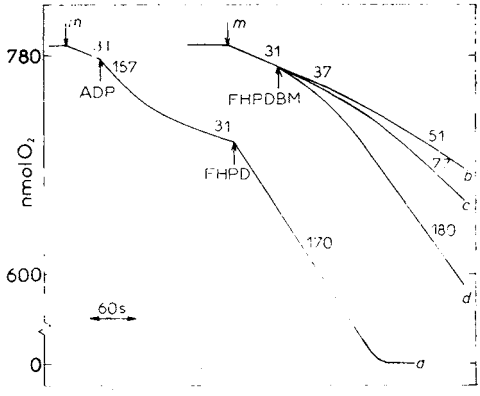

FIG. 4

The oxygen consumed by mitochondria isolated from liver of rats (*m*) in the presence of $5 . 10^{-7}$ mol dm^{-3} phenylhydrazonopropanedinitrile (the record *a*) and of S-benzyl (2-phenylhydrazono)cyanothioacetimidate with the final concentration of *b* $1{\cdot}5 . 10^{-7}$, *c* $3 . 10^{-7}$, and *d* $8 . 10^{-7}$ mol dm^{-3}. The characteristics of mitochondria: RCR = 5·1, ADP/O = 1·6, 0·4 mg prot. ml^{-1}. The numbers above the curves denote the rate of oxygen consumption related to the prot. amount (in nmol min^{-1} mg^{-1})

With the aim of an at least preliminary assessment of the reversibility degree of the reaction of phenylhydrazonopropanedinitrile with benzyl mercaptan as a model thiol, we followed (spectrophotometrically) the course of this reaction in the presence of varying excess of benzyl mercaptan. As both benzyl mercaptan and the adduct are little soluble in water, the reaction was followed in a buffer of pH 7·5 containing dioxane (1 : 1). Under these conditions, phenylhydrazonopropanedinitrile is completely stable. The kinetic dependences obtained are given in Fig. 3. With the benzyl mercaptan concentrations equal to 3·8 and 7·6 mmol dm^{-3} the conversion of phenylhydrazonopropanedinitrile is higher than 99%, which is indicated by equal absorbances of the reaction mixtures after finished reaction, the amount of the adduct formed being practically maximum ($5 . 10^{-5}$ mol dm^{-3}). From Fig. 3 it is also obvious that an about half conversion takes place at the benzyl mercaptan concentration of $9·5 . 10^{-4}$ mol dm^{-3}, *i.e.* with its about twenty fold excess over phenylhydrazonopropanedinitrile.

The decomposition of S-benzyl (2-phenylhydrazono)cyanothioacetimidate can also be observed at physiological level. Its application to respirating mitochondria isolated from the rat liver (in the amount of $1·5 . 10^{-7}$ to $8·0 . 10^{-7}$ mol dm^{-3}) results in a gradual increase of the respiratory rate (Fig. 4). This phenomenon is connected with the fact that only phenylhydrazonopropanedinitrile alone shows the ability to stimulate the respiration, its addition product with benzyl mercaptan being inefficient to mitochodria. As the adduct applied undergoes decomposition into phenylhydrazonopropanedinitrile, the result is an acceleration of the respiration which is proportional to concentration of the decomposition product formed.

The found reversibility of the reactions of phenylhydrazonopropanedinitriles with thiols is important not only from the point of view of explanation of reasons of bioactivity of phenylhydrazonopropanedinitriles (variability of effects with time, removal of the effect by addition of thiols, a complex pH dependence of the activity *etc.*) but also with respect to potential application of the respective polymeric derivatives of these selective reagents[14,15] as reversible sorbents of lowmolecular thiols and thiolproteins[22,23].

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