KINETICS ANALYSIS OF THE DECAY OF PHENYLHYDRAZONO-PROPANEDINITRILE ADDITION PRODUCTS WITH THIOLS IN AQUEOUS SOLUTIONS

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The reactions of phenylhydrazonopropanedinitriles with thiols are reversible and with increasing pH-values of aqueous solutions the equilibrium is shifted them to the side of the reactants. A mechanism of the decay of corresponding addition products was proposed and the kinetic description based on this mechanism is in good agreement with the obtained experimental data. From the viewpoint of the decay, the pH-dependent dissociation of the proton of the imino group formed by addition of thiol to the nitrile carbon atom plays a decisive role. The obtained knowledge is useful with respect to the use of phenylhydrazonopropanedinitriles as affinants for selective and reversible sorption of low-molecular thiols and thiol-proteins in affinity chromatography as well as for the study of the mechanism of the effect of these substances in oxidative and photosynthetic phosphorylation and biological systems in general.

The biological effect of phenylhydrazonopropanedinitriles — PHPDs (called carbonyl cyanide phenylhydrazones in biochemistry) may be suppressed by addition of low-molecular thiols[1-3]. However, for the time being this phenomenon has not been satisfactorily explained in spite of frequent use of PHPDs as tools for studying the oxidative and photosynthetic phosphorylation, as well as other bioenergetic processes taking place in living systems[4-11]. Moreover, the problem of molecular basis of the role of PHPDs in the mechanisms of biotransformation of energy in cells has been the topic of primary interest of biochemists for many years. The information about the chemical reactivity of the mentioned compounds[12-14] is important for interpreting the inhibitory effect of thiols at biological activity of PHPDs as well as for explaining the mechanism of their biological activity in general. The corresponding reactions proceeding in the presence of an excess of thiols have been characterized with regard to their molecular mechanism as well as the reactivity and sensitivity

to the conditions of the medium[15,16]. It was found that the reaction proceeds as a nucleophilic addition of the thiolate anion to the electrophilic carbon atom of one nitrile group of PHPD (Eq. (A)). The rate of the reaction is high and significantly dependent on pH of the medium. When transferred into a medium without an excess of thiols, the adduct formed decays to give the starting components[17] (Eq. (A)).

$$R^1—C_6H_4—NH—N=C(CN)—C\equiv N + R^2SH \rightleftharpoons$$
$$\rightleftharpoons R^1—C_6H_4—NH—N=C(CN)—C(=NH)—SR^2 \qquad (A)$$
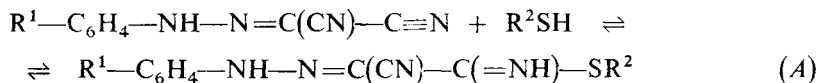
While the bonding of thiols to the activated nitrile group has been sufficiently characterized[13], the decay reaction and its relevant kinetic analysis with respect to reaction conditions has not yet been investigated. Its regognition is important not only from the stand-point of a more detailed elucidation of the basis of the biological effect of PHDPDs but also of further use of their polymer derivatives as selective and reversible sorbents in affinity chromatography of thiols[18-20].

The aim of this study is the determination of the rate constants of the decomposition of addition products of PHPDs with biologically important thiols and the analysis of the mechanism of the reactions involved. The mechanism of base-catalysed cleavage of imido esters to nitriles was studied in general by Gilbert and Jencks[21].

EXPERIMENTAL

Spectrophotometric measurements were made on a UV 3000 (Shimadzu) or an SF spectrophotometer (Applied Photophysics, Model 1705) at 25°C. Mathematical processing of the results was carried out on a IBM PC XT computer by using the Graphad programme (ISI software).

Reduced glutathione was a product of Sigma and coenzyme A was purchased from Calbiochem. Other chemicals were analytical grade reagents (Lachema, Brno).

The synthesis of phenylhydrazonopropanedinitrile and 4-nitrophenylhydrazonopropanedinitrile as well as the synthesis of their addition products with mercaptoacetic acid, benzylmercaptan and thiophenol was carried out using the method described in ref.[13]. The addition products of phenylhydrazonopropanedinitrile with glutathione (γ-glutamyl-cysteinyl-glycine) and coenzyme A were prepared in aqueous solutions as follows: An ethanolic solutions of phenylhydrazonopropanedinitrile (15 mg in 6 ml) was added to 5 mg of reduced glutathione (or 5 mg of coenzyme A) dissolved in 2 ml of 0·05M Britton–Robinson buffer, pH 4. After 12 hours standing at 25°C the reaction solution was transferred into a chromatographic column (150 × × 10 mm) containing Spheron-Thiol 300 (Lachema, Brno) and then eluted with the same buffer. Immediately after separating the unreacted phenylhydrazonopropanedinitrile the solution of the addition product (the first outflowing yellow fraction, volume 250 μl, spectrophotometrically determined concentration $2 . 10^{-4}$ mol l^{-1}) was added to 2·25 ml of constant ionic strength buffer solution of Miller and Golder with a higher pH value (up to 12) and the kinetics of the decay reaction was investigated spectrophotometrically. When the reaction was over the resulting pH-value of the reaction solution was measured.

The spectrophotometric measurement of the kinetics by the stopped flow technique was performed in the following manner: First, a fresh solution of the addition products of phenylhydrazonopropanedinitrile with benzylmercaptan or thiophenol ($c\ 4\ .\ 10^{-5}\ mol\ l^{-1}$) in distilled water adjusted to pH 4 with hydrochloric acid, was prepared. Then it was mixed in a 1 : 1 ratio with a buffer solution of higher pH in a stopped flow equipment and the pertinent kinetics of the decay was investigated spectrophotometrically by using the wavelenght region (400—700 nm) where the change in absorbance of the reaction solution before and after reaction was the greatest. After termination of the kinetic measurement the resulting pH-value of the solution was determined. The kinetics of the decay of the addition products of phenylhydrazonopropanedinitrile with 2-mercaptoacetic acid (i.e. (2-(4-nitrophenylhydrazono)-3-iminopropanedinitrile)-S-thioacetic acid) was investigated spectrophotometrically after mixing ethanolic solutions (10 μl) of these products with buffer solutions of known pH-value or with an NaOH solution containing a known concentration of hydroxyl ions. The electronic absorption spectra of (2-(4-nitrophenylhydrazono)-3-iminonitrile)-S-thioacetic acid were measured in aqueous NaOH solutions in the presence of 0·02M sodium 2-mercaptoacetate. The hydroxyl ion concentration was determined by titration with oxalic acid.

The dissociation curve of the addition product of 4-nitrophenylpropanedinitrile with 2-mercaptoacetic acid was obtained from absorbance values of this product at 460 nm. These values were read immediately after adding the addition product into the aqueous medium containing 0·02M sodium 2-mercaptoacetate and hydroxyl ions in a known concentration. The dissociation constant was determined from the inflexion point of the dissociation curve.

RESULTS AND DISCUSSION

In our preceding paper[17] it was shown that S-benzyl-(2-phenylhydrazono)cyanoacetamide (an addition product of phenylhydrazonopropanedinitrile with benzylmercaptan) decomposes in aqueous solutions to give the original reactants. This decay can be also observed in aqueous solutions of other addition products of phenylhydrazonopropanedinitrile with thiols. The pH dependence of the observed rate constants of the S-benzyl-(2-phenylhydrazono)cyanoacetamide and S-phenyl-(2--phenylhydrazono)cyanoacetamide decay is shown in Fig. 1. The observed values
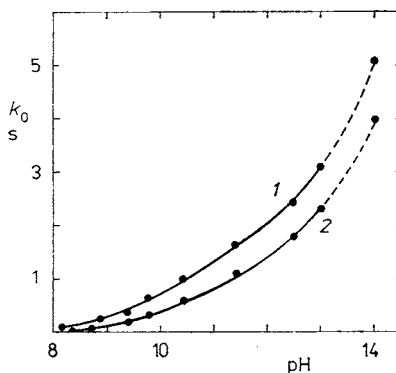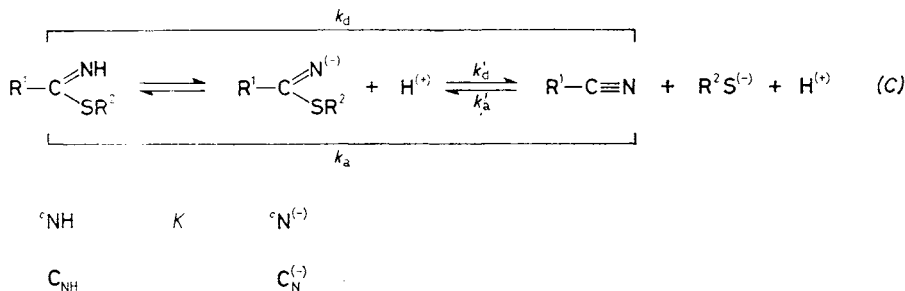

FIG. 1

Dependence of the observed rate constants of decay k_0 (s^{-1}) of the addition product of phenylhydrazonopropanedinitrile with thiophenol (1) and benzyl mercaptan (2) on pH of the reaction medium. Initial concentration of the addition products $2\ .\ 10^{-5}$ mol . l^{-1}

were obtained by evaluating the corresponding decomposition kinetics, measured by the stopped flow technique according to the conditions valid for first order reactions. In both cases, the presence of the formed thiol was proved by the Ellman method[22] after the reaction end. From Fig. 1 it results that an increase in concentration of the hydroxyl ions in the reaction medium brings about an increase in the values of the observed rate constants. This dependence indicates the main role of the dissociable proton in molecules of the addition products of PHPDs with thiols in the decay of these products. As evident from Eq. (B), the nucleophilic addition of a thiol to the activated carbon atom of the nitrile group results in binding the proton to the adjacent nitrogen.

$$R^1\!-\!C\!\equiv\!N \ + \ R^2SH \ \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \ R^1\!-\!C\!\!\underset{SR^2}{\overset{NH}{\big<}} \qquad (B)$$
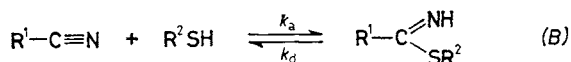
Provided the proton is bonded to this nitrogen atom (nonaqueous medium or aqueous medium the pH-value of which is very low when compared with the pK-value of the imino group), the reverse reaction does not practically proceed $(k_d \ll k_a)$. On increasing pH, the dissociation of this proton results in the generation of a negative charge on the imino nitrogen atom in the vicinity of the carbon–sulphur bond, owing to which the molecule can decay and give rise to a thiolate and a stable neutral nitrile group in the PHPD molecule (Eq. (C)).

$$R\!-\!C\!\!\underset{SR^2}{\overset{NH}{\big<}} \ \overset{k_d}{\underset{k_a}{\rightleftharpoons}} \ R^1\!-\!C\!\!\underset{SR^2}{\overset{N^{(-)}}{\big<}} \ + \ H^{(+)} \ \underset{k_a'}{\overset{k_d'}{\rightleftharpoons}} \ R^1\!-\!C\!\equiv\!N \ + \ R^2S^{(-)} \ + \ H^{(+)} \qquad (C)$$

$$^cNH \qquad K \qquad ^cN^{(-)}$$
$$C_{NH} \qquad \qquad C_N^{(-)}$$

Assuming a zero concentration of R^1CN and R^2S^-, we may write Eq. (1) for the rate of decay of the addition product which exists, at a given pH and at the reaction start, both in dissociated and non-dissociated form (according to the pK value of the iminogroup)

$$v = k_d c_{NH} + k_d' c_{N^-} . \qquad (1)$$

If the rate constant of the non-dissociated form decay, k_d is small in comparison with the rate constant k_d', the first term in Eq. (1) can be omitted and thus we may

write:

$$v = k'_d c_{N^-} . \tag{2}$$

Provided the rate constants of the dissociation of the proton from the imino group are by several orders of magnitude higher than the rate constant of decay of the dissociated form of the addition product, the concentration of this form has to be calculated from the total analytical concentration of the addition product c_A by using the dissociation constant K

$$c_A = c_{N^-} + c_{NH} \tag{3}$$

$$c_{NH} = (c_{N^-}c_{H^+})/K \tag{4}$$

$$c_{N^-} = Kc_A/(c_{H^+} + K) . \tag{5}$$

By inserting c_{N^-} in Eq. (2), we obtain for the rate of decay

$$v = k'_d Kc_A/(c_{H^+} + K) \tag{6}$$

$$v = k_o c_A \tag{7}$$

$$k_o = Kk'_d/(c_{H^+} + K) , \tag{8}$$

where k_o is the observed rate constant of a first order reaction dependent on pH of the reaction medium. Provided the hydrogen ion concentration is several times smaller than the value of the dissociation constant of the imino group, the observed dissociation constant is identical with the real rate constant of a first order reaction which is independent of pH of the reaction medium. Unfortunately, the maximum rate constants of decay of numerous addition products of SHPDs with thiols are out of the range of measurability by the stopped flow technique and moreover, they can be determined only in media with high concentration of hydroxyl ions, where some parallel non-specific reactions appear.

From Eq. (8) it results that the rate constant k'_d which is independent of the pH reaction medium may be calculated from the observed rate constants k_o only by using the known value of the dissociation constant K by which the concentration of the decaying form of the addition product is maintained. In some cases a convenient choice of conditions in the presence of a high concentration of thiol permits the determination of the corresponding dissociation constant. Among substances investigated by us, it was the addition product of 4-nitrophenylhydrazonopropane-dinitrile with 2-mercaptoacetic acid which fulfilled these conditions. This is due to

the fact that this addition product, i.e. (2-(4-nitrophenylhydrazono)-3-iminopropane-nitrile)-S-thioacetic acid, has the lowest decay rate in comparison with other investigated products, and owing to its high solubility 2-mercaptoacetic acid may be applied in high concentration. On the other hand, the nitro group in position 4 of the benzene ring causes marked differences in the optical spectrum of the dissociated and non-dissociated form of this compound. Fig. 2 shows the spectra of the addition product of 4-nitrophenylhydrazonopropanedinitrile with 2-mercaptoacetic acid. They were measured by recording the rapid change in wavelength (1 200 nm min) immediately after addition of an ethanolic solution of the investigated substance into aqueous solutions of sodium 2-mercaptoacetate with a known concentration of the hydroxyl ions. Dissociation of proton from the molecule produces a considerable shift of the maximum in the long-wave region. The dissociation curve constructed from the absorbance values of the substance at 460 nm is also drawn in Fig. 2. The inflexion point corresponding to the pK-constant has the value 13·7.

Fig. 3 represents the experimental rate constants of (2-(4-nitrophenylhydrazono)--3-iminopropanedinitrile)-S-thioacetic acid decay k_o for varying concentrations of hydroxyl ions at constant ionic strength ($\mu = 5$ mol l^{-1}, attained with the help of NaCl). By using the spectrophotometrically determined value of the dissociation constant $K = 1·99 \cdot 10^{-14}$ mol l^{-1} and the fitted rate constant of decay $k'_d = 1·25$.
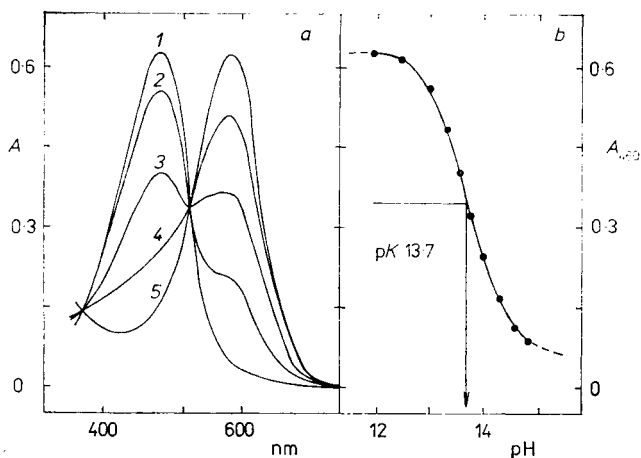

FIG. 2

Electronic absorption spectra (a) and dissociation curve (b) of the addition product of 4-nitro-phenylhydrazonopropanedinitrile with 2-mercaptoacetic acid in aqueous solutions with NaOH concentrations 0·01 (1), 0·1 (2), 0·3 (3), 1·0 (4), 5·0 (5) mol l^{-1} in the presence of 0·02M mercapto-acetate; the addition product was applied in resulting concentration of 2 . 10^{-5} mol l^{-1}. A constant ionic strength (μ 5 mol l^{-1}) was adjusted with NaCl

$. 10^{-2} \text{ s}^{-1}$, the theoretical values of the rate constants k_o were calculated by means of Eq. (8). They are plotted as a function of pH together with the experimental values in Fig. 3. From Fig. 3 it results that the measured and calculated values of the decay rate constants of the investigated substance are in good agreement.

For biochemically real conditions (where pH-value varies within the $4-10$ range), i.e. for media where the proton concentration c_{H+} is by several orders higher than the value of the dissociation constant K of the decay rate of the investigated addition products (of PHPDs with thiols) we may write Eqs $(9)-(11)$:

$$k_o = K k'_d c_{H+} \qquad (9)$$

$$\log k_o = X + \text{pH} \qquad (10)$$

$$X = \log k'_d + \log K = \text{const.} \qquad (11)$$

Thus we obtain a linear relationship between the logarithms of the observed rate constants k_o and the pH of the medium. The pH-dependences of the rate constants
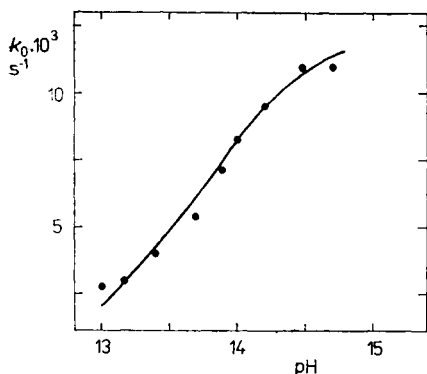

FIG. 3

Dependence of the observed rate constants of decay of the 4-nitrophenylhydrazono-propanedinitrile addition product with 2--mercaptoacetic acid on pH of the reaction medium. Initial concentration of the adduct in aqueous solutions with varying NaOH concentration and constant ionic strength $(\mu \; 5 \text{ mol l}^{-1})$, $2 \cdot 10^{-5} \text{ mol l}^{-1}$. The points indicate the experimental values and the curve shows the data calculated by means of Eq. (8) for pK 13·7 and $k \; 1·25 \cdot 10^{-2} \text{ s}^{-1}$
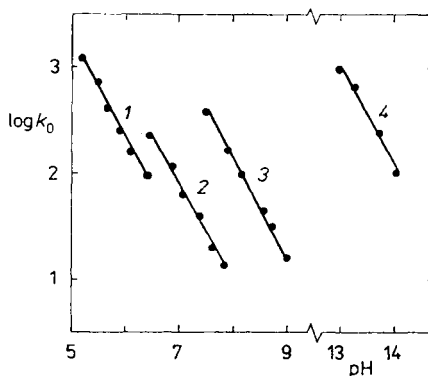
FIG. 4

Negative logarithms of the first order rate constants $k_0 \; (\text{s}^{-1})$ of decay of the addition products of phenylhydrazonopropanedinitrile with glutathione (1), thiophenol (2), coenzyme A (3), and 2-mercaptoacetic acid (4) as a function of pH. The slopes and correlation coefficients: curve (1) $a = 0·953$, $r = 0·996$; curve (2) $a = 0·919$, $r = 0·986$; curve (3) $a = 0·912$, $r = 0·996$; curve (4) $a = 0·9999$, $r = 0·983$

of decay of the addition products of phenylhydrazonopropanedinitrile with mercapto-acetic acid, thiophenol, glutathione and coenzyme A are shown in Fig. 4. The difficulties in the isolation of the last two addition products stimulated as to prepare them in aqueous solution at a low pH and to remove the excess of non-consumed phenylhydrazonopropanedinitrile by affinity chromatography using a column with Spheron-SH. The kinetic measurements were carried out immediately after chromatographic purification of the addition product at a convenient pH-value of the medium. In spite of partial decomposition of the addition product thus prepared, the kinetics obeyed the law for first order reactions. In Fig. 4 it can be seen that the slopes of the logarithm of the rate constant k_o versus pH-value of the reaction medium plots are identical. The values of these slopes for the investigated compounds are close to one, which also results from Eq. (10).

From kinetic analysis of the decay reactions of the addition products of PHPDs with thiols it follows that the rate of these reactions depends on the value of the dissociation constant of the imino group bonded to the carbon atom adjoined to the thiol the influence of which is dependent on the value of its original dissociation constant. For the simple homologous series of alkyl thiols we may thus assume a linear correlation between the dissociation constants of the SH-groups and the rate constants of decay of the corresponding addition products with PHPDs.

As for thiols containing another group with a charge able to interact with the imino group the expected relationship will be more complex. Except for one case, we did not succeed in determining the dissociation constants of the imino group of the addition products and also the real rate constants. Therefore we chose the values of the observed rate constants in the region of the linear dependence of log k_o on pH of the medium as a measure characterizing the differences between the pro-

TABLE I
Negative logarithms of the observed rate constants of decay k_o (s^{-1}) of the addition products of phenylhydrazonopropanedinitrile with different thiols, and dissociation constants of these thiols

Thiol	$-\log k_o$	pK
2-Mercaptoacetic acid	8·5[a]	10·2[c]
Coenzyme A	3·3[a]	9·6[d]
Benzylmercaptan	3·1[b]	9·4[d]
Glutathione	1·4[a]	8·6[d]
Thiophenol	1·8[a]	6·6[d]

[a] Values of k_o read from Fig. 4 after extrapolation to pH 7·0; [b] ref.[17]; [c] ref.[23]; [d] ref.[24].

perties of individual products. Extrapolation of the linear dependence of log k_o on pH of the medium enabled us to estimate the values of the observed rate constants of decay of all derivates for a medium of pH 7 (Table I). This table demonstrates that the values of the observed rate constants k_o thus obtained depend on the dissociation constants of the corresponding thiols. In spite of the relatively low number of tested substances, the data in Table I show that the rate constants of decay increase with the dissociation constant of the thiol bonded to phenylhydrazonopropanedinitrile. Only the thiophenol adduct is an exception, probably due to different properties of aromatic and aliphatic thiols. The observed differences between the values of the rate constants of decay of the addition products of different thiols with phenylhydrazonopropanedinitrile made possible a deliberate use of polymer PHPDs for chromatographic separation of thiols[20]. On the other hand, the high stability of the addition product of phenylhydrazonopropanedinitrile with mercaptoacetic acid indicates that PHPDs can be efficient modification agents for low-molecular thiols and thiol-proteins the SH groups of which exhibit a pK-constant value equal to about 10. This fact has to be taken into consideration in the elucidation of the chemical basis of the biological effects of SHPDs (refs[3−13,25]).

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