Reactions of L-Ergothioneine and Some Other Aminothiones with 2,2'- and 4,4'-Dipyridyl Disulphides and of L-Ergothioneine with Iodoacetamide

2-MERCAPTOIMIDAZOLES, 2- AND 4-THIOPYRIDONES, THIOUREA
AND THIOACETAMIDE AS HIGHLY REACTIVE NEUTRAL SULPHUR
NUCLEOPHILES

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1. The reactions of 2,2'- and 4,4'-dipyridyl disulphide (2-Py-S-S-2-Py and 4-Py-S-S-4-Py) with L-ergothioneine (2-mercapto-L-histidine betaine), 2-mercaptoimidazole, 1-methyl-2-mercaptoimidazole, thiourea, thioacetamide, 2-thiopyridone (Py-2-SH) and 4-thiopyridone (Py-4-SH) were investigated spectrophotometrically in the pH range approx. 1–9. 2. These reactions involve two sequential reversible thiol–disulphide interchanges. 3. The reaction of L-ergothioneine with 2-Py-S-S-2-Py and/or with the L-ergothioneine–Py-2-SH mixed disulphide, both of which provide Py-2-SH, is characterized by at least three reactive protonic states. This provides definitive evidence that neutral L-ergothioneine is a reactive nucleophile, particularly towards the highly electrophilic protonated disulphides. 4. A similar situation appears to obtain in the reactions of L-ergothioneine and Py-2-SH with 4-Py-S-S-4-Py and in the reactions of the other 2-mercaptoimidazoles, thiourea and Py-4-SH with 2-Py-S-S-2-Py. The nucleophilic reactivity of Py-4-SH suggests that general base catalysis provided by the disulphide in a cyclic or quasi-cyclic transition state is not necessary to generate nucleophilic reactivity in the other aminothiones whose geometry could permit such catalysis. 5. The existence of a positive deuterium isotope effect in the L-ergothioneine–2-Py-S-S-2-Py system at pH 6–7 provides no evidence for general base catalysis but is in accord with a mechanism involving specific acid catalysis and post-transition-state proton transfer. 6. The pH-dependences of the overall equilibrium positions of the various thiol–disulphide interchanges are described. 7. Reaction of thioacetamide with a stoichiometric quantity of 2-Py-S-S-2-Py at pH 1 provides 2 molecules of Py-2-SH per molecule of thioacetamide and elemental sulphur; these findings can be accounted for by thiol–disulphide interchange to provide a thioacetamide–Py-2-SH mixed disulphide followed by fragmentation to provide CH_3CN , S and Py-2-SH. 8. Provision of high reactivity in the neutral forms of the members of this series of sulphur nucleophiles by electron donation by the amino group is compared with the well known α effect that provides enhanced nucleophilicity in compounds containing an electronegative atom adjacent to the nucleophilic atom. 9. The decrease in the u.v. absorption of L-ergothioneine at 257 nm consequent on transformation of its aminothione moiety into an S-alkyl-2-mercaptoimidazole moiety provides a convenient method of following the alkylation of L-ergothioneine by iodoacetamide. 10. The pH dependence of the extinction coefficient of L-ergothioneine at 257 nm is described by $\epsilon_{257} = \{8 \times 10^3 / (1 + K_a/[H^+])\} + 6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in which $\text{p}K_a = 10.8$. 11. In the pH range 3–11 the reaction is characterized by two reactive protonic states (X and XH). 12. The X state, reaction of the ionized 2-mercaptoimidazole moiety of the L-ergothioneine dianion with neutral iodoacetamide, is characterized by the second-order rate constant $4.0 \text{ M}^{-1} \cdot \text{s}^{-1}$ (25.0°C, $I = 0.05$). The XH state, characterized by the second-order rate constant $0.03 \text{ M}^{-1} \cdot \text{s}^{-1}$, is interpreted as reaction of the thione form of the neutral 2-mercaptoimidazole moiety of the L-ergothioneine monoanion with neutral iodoacetamide. 13. The XH state of the alkylation reaction does not exhibit a deuterium isotope effect.

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As part of a study on the reactions of aromatic disulphides with thiol groups perturbed electronically either through space or through the molecule, we examined the reactions of 2,2-dipyridyl disulphide [2-Py-S-S-2-Py, (I)] and 4,4-dipyridyl disulphide [4-Py-S-S-4-Py (II)] with L-ergothioneine (III), the betaine of 2-mercapto-L-histidine.

L-Ergothioneine is widely distributed in nature and is the only naturally occurring compound discovered to date that contains the 2-mercaptoimidazole moiety (Stowell, 1961).

One factor that has hindered the elucidation of the role (if any) of L-ergothioneine (see Carlsson *et al.*, 1974) is the lack of a convenient and specific assay method for this compound. The present paper reports that L-ergothioneine reacts very rapidly with both 2-Py-S-S-2-Py and 4-Py-S-S-4-Py in acidic media. This is in marked contrast with the analogous reactions of simple thiols (Brocklehurst & Little, 1972, 1973). Studies on the mechanisms of these rapid reactions in acidic media are described in the present paper and a convenient assay for L-ergothioneine in blood based on its rapid reaction with 2-Py-S-S-2-Py at pH1, where the reaction producing Py-2-SH is characterized by a relatively favourable equilibrium constant, is reported in the following paper (Carlsson *et al.*, 1974).

Materials and Methods

Chemicals

L-Ergothioneine was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. 2,2'-Dipyridyl disulphide, 4,4'-dipyridyl disulphide, 4-thiopyridone and 1-methyl-2-mercaptoimidazole were obtained from Ralph N. Emanuel Ltd., Wembley, U.K. 4,4'-Dipyridyl disulphide was purified by ion-exchange chromatography (T. Stuchbury & K. Brocklehurst, unpublished work) before use. Thiourea, thioaceta-

mid, iodoacetamide and $^2\text{H}_2\text{O}$ were the products of BDH Chemicals Ltd., Poole, Dorset, U.K. Iodoacetamide was recrystallized twice from 50% ethanol to give a product with m.p. 93.5–96.5°C. 2-Mercaptoimidazole was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and 2-thiopyridone from Fluka A.-G. Chemische Fabrich, Buchs SG, Switzerland.

Other chemicals were of the highest commercially available grade.

Millipore filters

Millipore filters (24mm diameter, 0.45 μm pore size and 47mm diameter, 0.22 μm pore size) were obtained from Millipore Filter Corp., Bedford, Mass., U.S.A.

Preparation of stock solutions and determination of concentrations

All stock solutions were made up in deionized water or in $^2\text{H}_2\text{O}$. Stock solutions of 2-Py-S-S-2-Py (approx. 1.5mM) and 4-Py-S-S-4-Py (approx. 3mM) were prepared essentially as described previously (Brocklehurst & Little, 1973), except that the original suspensions were stirred for only 30min before filtration. Concentrations were determined by spectrophotometric analysis at the wavelengths and with the extinction coefficients given in Table 1. The extinction coefficients of thiourea and thioacetamide were determined by using solutions prepared by dissolution of accurately weighed samples on the solids that had been previously dried for 24h over P_2O_5 *in vacuo*.

Buffers

Buffers were made up in 1mM-EDTA and in most cases such that threefold dilution gave $I = 0.1$. The following buffers were used: pH9.0–10.6, NaHCO_3 ;

Table 1. *U.v.-spectral characteristics of the aminothiones and disulphides at pH7*

Compound	$\lambda_{\text{max.}}$ (nm)	$10^{-4} \times \epsilon_{\text{max.}}$ ($\text{M}^{-1} \cdot \text{cm}^{-1}$)	Reference
2-Py-S-S-2-Py*	281	0.97	Grassetti & Murray (1967)
4-Py-S-S-4-Py*	247	1.63	Grassetti & Murray (1967)
Py-2-SH*	343	0.706	Grassetti & Murray (1967)
Py-4-SH*	324	1.98	Grassetti & Murray (1967)
L-Ergothioneine	257	1.40	Carlsson <i>et al.</i> (1974)
2-Mercaptoimidazole†	259	1.36	Stanovik & Tisler (1964)
1-Methyl-2-mercaptoimidazole†	260	1.44	Stanovik & Tisler (1964)
Thiourea	236	1.20	Present work
Thioacetamide	261	1.24	Present work

* The pH-dependences of the u.v.-absorption parameters of these compounds are given in Brocklehurst & Little (1973).

† Solvent, ethanol.

pH 8.4–8.9, sodium borate; pH 7.2–8.4, Tris-HCl; pH 5.6–7.2, potassium phosphate; pH 3.6–5.6, sodium acetate; pH 2.8–3.6, sodium formate; pH 2.4–2.8, glycine-HCl; pH values less than 2, KCl-HCl.

When the reaction of L-ergothioneine with 2-Py-S-S-2-Py was studied in $^2\text{H}_2\text{O}$, 2-Py-S-S-2-Py was dissolved in $^2\text{H}_2\text{O}$ and small volumes of concentrated aqueous buffer and L-ergothioneine were added to give a final reaction mixture in 90% $^2\text{H}_2\text{O}$. Values of pD were calculated from pH values measured with a glass electrode from $\text{pD} = \text{pH} + 0.4$ (Bender & Hamilton, 1962).

Spectrophotometric analysis of the progress of the various thiol-disulphide interchanges

All reactions except the reactions of 2-Py-S-S-2-Py with 4-thiopyridone (Py-4-SH) in the pH range 3–8 and with thioacetamide. The reactions were followed by recording the increase in E_{343} (due to the production of 2-thiopyridone, Py-2-SH), or the increase in E_{324} (due to the production of Py-4-SH) at 25.0°C in a Cary 15 or a Cary 16K spectrophotometer. The concentrations of reactants are given in the various figure legends of the Results and Discussion section. A typical experiment is described.

A reaction volume of 3.6 ml was used. Mixtures of disulphide (x ml), H_2O (y ml) and buffer (1.2 ml) were made up in both reference and sample cells and a base line was recorded. Water (z ml) was then added to the reference cell and the aminothione (z ml) was added to the sample cell to start the reaction. The last addition was made while the recorder was running as the pen crossed a line marked on the chart. The progress curve was recorded until the reaction was judged to be complete and the pH values of the contents of both cells were then measured with Radiometer equipment.

In experiments in which [aminothione] was much greater than [disulphide] the aminothione was present in both reference and sample cells and the reaction was started by addition of the disulphide.

Stoichiometries were calculated from the pH-dependent ϵ_{max} values of Py-2-SH and Py-4-SH (see Brocklehurst & Little, 1973).

Reaction of Py-4-SH with 2-Py-S-S-2-Py in the pH range 3–8. In this pH range the extinction coefficients of Py-2-SH and Py-4-SH bear the following relationship to each other in which $\text{p}K_{\text{aII}}^{\text{Py-4-SH}} = 8.6$ and $\text{p}K_{\text{aII}}^{\text{Py-2-SH}} = 9.8$ (Grasseti & Murray, 1967; Brocklehurst & Little, 1973):

$$\begin{aligned}\epsilon_{324}^{\text{Py-4-SH}} &= 1.98 \times 10^4 / (1 + K_{\text{aII}}^{\text{Py-4-SH}} / [\text{H}^+]) > \\ \epsilon_{343}^{\text{Py-4-SH}} &= \text{approx. } 0.8 \times 10^4 / (1 + K_{\text{aII}}^{\text{Py-4-SH}} / [\text{H}^+]) > \\ \epsilon_{343}^{\text{Py-2-SH}} &= 0.706 \times 10^4 / (1 + K_{\text{aII}}^{\text{Py-2-SH}} / [\text{H}^+]) > \\ \epsilon_{324}^{\text{Py-2-SH}} &= \text{approx. } 0.6 \times 10^4 / (1 + K_{\text{aII}}^{\text{Py-2-SH}} / [\text{H}^+])\end{aligned}$$

The reaction could be followed therefore by recording the decrease in E_{324} that accompanies the consumption of Py-4-SH. Reaction mixtures were made up as described above except for the following details. Py-4-SH was introduced into the sample cell; its E_{324} was approx. 1. This E was backed off to give a reading of 0.1 on the 0–0.1 E slide-wire and then 2-Py-S-S-2-Py was added first to the reference cell and then to the sample cell to start the reaction.

At pH values of approx. 2 it becomes more difficult to follow the reaction spectrophotometrically. This is because Py-4-SH becomes protonated in this region of pH ($\text{p}K_{\text{aI}}^{\text{Py-4-SH}} = 1.43$; Albert, 1963) and there is a consequent shift to shorter wavelengths of the absorption band.

Reaction of thioacetamide with 2-Py-S-S-2-Py at pH approx. 1. An aqueous solution (500 ml) of 2-Py-S-S-2-Py (1.4 mm) was mixed with 88 ml of 1 M-HCl. This solution and an aqueous solution of thioacetamide (0.2 M) were passed separately through Millipore filters (0.22 μm pore size). Reaction mixtures were made up by adding to separate 160 ml samples of the 2-Py-S-S-2-Py solution in one case 0.5 ml of the thioacetamide solution and in another 1 ml of the thioacetamide solution. The concentrations of the reactants in these two mixtures were [2-Py-S-S-2-Py], 1.22 mm in both cases; [thioacetamide], 0.62 mm and 1.24 mm.

Both reaction mixtures became cloudy immediately after mixing the reactants. The reactions were allowed to proceed for 2 h and the mixtures were then filtered separately through Millipore filters (0.45 μm pore size) to remove the pale-yellow solid. The u.v. spectra of dilutions (0.2 ml added to 3 ml of 0.3 M-potassium phosphate buffer, pH 6.6) of the clear filtrates were recorded from 400 to 240 nm with a Cary 15 spectrophotometer. Analysis of the two filtrates by using E_{343} showed that they contained 1.2 mm-Py-2-SH in the case when 0.62 mm-thioacetamide was used and 2.2 mm-Py-2-SH in the case when 1.24 mm-thioacetamide was used.

Identification of the products (other than Py-2-SH) of the reaction of thioacetamide with 2-Py-S-S-2-Py at pH approx. 1. The products of these reactions were separated as described above into (i) the pale-yellow solid and (ii) the filtrate. (i) The pale-yellow solid. This was shown by elemental analysis to consist essentially of sulphur ($96 \pm 5\%$). (ii) The filtrate. This was fractionated by ether extraction into an ether-soluble fraction and the residual aqueous fraction. The ether phase was subjected to analysis by conven-

tional g.l.c. (Perkin-Elmer 990 gas chromatograph) by using a column (2.5 m $\times$ 3.2 mm) of 10% Carbowax($\times$ 20M) on 100–120 mesh Chromosorb W, AW-DMCS, and also by mass spectrometry (LKB gas-chromatograph-mass spectrometer Type 9000) with the column described above. In the g.l.c. analysis a peak was observed with the same retention time as that of an authentic sample of acetonitrile and the mass spectrum gave a mol.wt. of 41. Analysis of the ether-soluble fraction and of the solid obtained by rotary evaporation of the aqueous phase by i.r. spectroscopy provided no evidence for the presence of acetamide.

Kinetics of the reaction of L-ergothioneine with iodoacetamide

This reaction was followed by recording the decrease in ϵ_{257} that accompanies alkylation of L-ergothioneine (see the Results and Discussion section). Reaction mixtures were made up essentially as described for the thiol-disulphide interchanges. The reactions were started by addition of iodoacetamide to the sample cell after adding an equivalent quantity to the reference cell. The pH of the sample cell was measured immediately after progress-curve

recording was abandoned by using Radiometer equipment. At pH values less than approx. 9 the reactions are too slow for the progress of the reaction to be conveniently followed to completion and rate constants were computed from initial rates. At higher pH values where faster reactions allow convenient recording of complete progress curves, rate constants were determined both from initial rates and from first-order plots.

Results and Discussion

Ionization characteristics of L-ergothioneine and some other aminothiones

A prerequisite for any study of nucleophilicity characteristics is knowledge of the ionization characteristics of the conjugate acids of the nucleophiles in question. In the discussion that follows, the nucleophilicity characteristics of L-ergothioneine are compared with those of 2-mercaptoimidazole (IV), 1-methyl-2-mercaptoimidazole (V), thiourea (VI), thioacetamide (VII), 2-thiopyridone, Py-2-SH (VIII) and 4-thiopyridone, Py-4-SH (IX).

The pK_a values of the conjugate acids of compounds (III)–(IX) are less than (in most cases very much less

Table 2. Ionization characteristics of L-ergothioneine and some other aminothiones

pK_{aI} probably approximates to the intrinsic pK_a characterizing the formation of neutral thione from the conjugate cationic acid, and for all compounds except L-ergothioneine pK_{aII} probably approximates to the intrinsic pK_a characterizing the formation of the resonance-stabilized thiolate ion (see e.g., Scheme 1). For L-ergothioneine, which contains an extra ionizing group, pK_{aII} probably approximates to the intrinsic pK_a characterizing the ionization of the carboxyl group and pK_{aIII} to the intrinsic pK_a characterizing the formation of the thiolate ion.

Compound	Molecular dissociation constants			Reference
	pK_{aI}	pK_{aII}	pK_{aIII}	
L-Ergothioneine (III)	-2.33	1.3	10.75	Stanovik & Tisler (1964)
2-Mercaptomidazole (IV)	-1.6	11.6	—	Stanovik & Tisler (1964)
1-Methyl-2-mercaptoimidazole (V)	-2.0	11.9	—	Stanovik & Tisler (1964)
Py-2-SH (VIII)	-1.07	9.8	—	Albert (1963); Brocklehurst & Little (1973)
Py-4-SH (IX)	+1.43	8.6	—	Albert (1963); Brocklehurst & Little (1973)
Thiourea	-0.96	?	—	Albert & Serjeant (1962)
Thioacetamide	<-0.5	?	—	*

* Albert & Serjeant (1962) give the following pK_a values: urea, 0.1-0.3; thiourea, -0.96; acetamide, -0.5. Thus it seems reasonable to suppose that pK_{aI} for thioacetamide will be <-0.5.

Scheme 1. Ionizations of the mercaptoimidazole moieties of compounds (III)-(V)

than) 2 and the pK_a values of the compounds (III)-(IX) themselves are greater than (in most cases very much greater than) 8 (see Table 2). The ionizations of the mercaptoimidazole moieties of compounds (III)-(V) are described by Scheme 1 [see Stanovik & Tisler (1964)].

Nucleophilicity characteristics of the aminothiones (III)-(IX)

By analogy with the reactions of aromatic disulphides with simple aliphatic thiols (Brocklehurst & Little, 1972, 1973) only the anionic form of compounds (III)-(IX), e.g. (XIII) (Scheme 1), would be expected to possess appreciable nucleophilic reactivity. There is some evidence in the literature, however, which suggests that aminothiones of the type (III)-(IX) might possess nucleophilic reactivity in their neutral forms. Probably the best known evidence that suggests

this is the commonly used derivative formation of alkyl halides by thiourea (IV) to provide *S*-alkylisothiuronium salts. These reactions proceed readily in essentially neutral media. Pearson *et al.* (1952) have studied the reactions of alkyl halides with a number of nucleophiles, including thiourea, and have suggested that the abnormally high nucleophilicity of thiourea is due to the high polarizability of the sulphur atom.

Dipyridyl disulphides as reagents for the characterization of the nucleophilicity of aminothiones

Our previous work with the dipyridyl disulphides (I) and (II) (Brocklehurst & Little, 1972, 1973; Brocklehurst *et al.*, 1972) has demonstrated that these reagents possess an important property that should allow the detection and characterization of any appreciable nucleophilic reactivity that may reside

in the neutral forms of the aminothiones (III)–(IX). This property is the ability of these reagents to increase their electrophilicity by protonation. This should result in three reactive protonic states in such reaction systems, i.e. the reaction of the thiolate ion with unprotonated disulphide and the reaction of the neutral thiol (or thione) with both unprotonated and protonated forms of the disulphide (see Brocklehurst, 1974). It was considered unlikely that proton partitioning between the thiol (or aminothione) group (pK_a 10.75) and the carboxyl group (pK_a 1.3) (see Table 2) of L-ergothioneine would provide a concentration of the resonance-stabilized thiolate ion at pH values of less than 5 sufficient to give rise to more than two protonic states of appreciable reactivity involving reaction of the thiolate ion. This prediction was supported by the high reactivity in strongly acidic media of the other aminothiones which do not possess an extra ionizing group (see below). Such systems therefore should be characterized by a striking rate maximum in acidic media and probably also by a rate minimum in approximately neutral media (see below). These predictable pH–rate profiles have allowed the detection of nucleophilic reactivity in the thiol group of the cysteine-25–histidine-159–asparagine-175 hydrogen-bonded system of papain (Brocklehurst & Little, 1972, 1973) and the thiol group of the cysteine–histidine hydrogen-bonded system of stem bromelain (Brocklehurst *et al.*, 1972).

The reactions of thiols (RSH) with aromatic disulphides (Ar-S-S-Ar) (thiol–disulphide interchange) may be represented by eqns. (1) and (2).

For the dipyriddy disulphides (I) and (II) the formation of ArSH is readily observed spectrophotometrically (Brocklehurst & Little, 1973; Grassetti & Murray, 1967) at 343 nm in the reactions of compound (I) and at 324 nm in the reactions of compound (II). In the reactions of simple aliphatic thiols with a moderate excess of compound (I) or (II), reactions (1) and (2) are essentially irreversible in acidic and neutral media (Brocklehurst & Little, 1973; Grassetti & Murray, 1967). Analysis of these reactions in terms of a competitive consecutive irreversible second-order model by the Frost–Schwemer (Frost & Schwemer, 1952; Burkhard, 1960) method has established (T. Stuchbury, V. Garner, E. Ager, K. Brocklehurst & H. Suschitzky, unpublished work) that k_{+1} is about 10 times k_{+2} . The reactions of the thiol groups in papain and bromelain with compounds (I) and (II) may be

analysed even more easily because reaction (2), i.e. reaction of the enzyme thiol with the enzyme–pyridyl mixed disulphide to provide an enzyme–enzyme disulphide, does not occur (Brocklehurst & Little, 1970, 1973).

By contrast, the reactions of compounds (III)–(IX) with compounds (I) and (II) are not so readily amenable to kinetic analysis. Spectrophotometric study of the reactions with a fixed concentration of compounds (III)–(IX) and different concentrations of compound (I) or (II), or vice versa, has established that the reactions proceed to an equilibrium position (see below). That stoichiometries approaching closely to two for the release of ArSH are observed in at least some of the interchanges when RSH is in large (e.g. 60-fold) excess demonstrates that both of the reversible reactions (1) and (2) are occurring. Very accurate data ($<\pm 1\%$) and substantial computation are required to determine even the two equilibrium constants of reactions of types (1) and (2), let alone their constituent rate constants (Gorin *et al.*, 1967). Quantification of the reactions in these terms has not been attempted in the present work. Nevertheless, interesting conclusions can be drawn about the nature of the reactivity of compounds (III)–(IX) towards compounds (I) and (II) from the spectrophotometric study reported below. The conclusions drawn from this study about the nucleophilic reactivity of the neutral forms of aminothiones are supported by the kinetic analysis of the alkylation of L-ergothioneine by iodoacetamide.

Reaction of L-ergothioneine with 2-Py-S-S-2-Py

The progress curves for the formation of Py-2-SH in the L-ergothioneine–2-Py-S-S-2-Py system (reaction of L-ergothioneine with 2-Py-S-S-2-Py and with L-ergothioneine–Py-2-SH mixed disulphide) were determined in the pH range 5.4–8.3 at 25.0°C and $I = 0.1$ with [L-ergothioneine] = 62.5 μM and [2-Py-S-S-2-Py] = 195 μM . In this pH region the equilibrium position appears to be independent of pH (see Fig. 1 and subsequent discussion) and the reactions all reach the same infinity reading. The reactions are slowest at pH values 6.95 and 7.65 ($v_1 = 4.1 \times 10^{-8} \text{ M} \cdot \text{s}^{-1}$ in both cases) and become faster as the pH is either increased or decreased (pH 5.4, $v_1 > 4.7 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$; pH 5.8, $v_1 = 2.7 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$; pH 8.3, $v_1 = 1.1 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$). The increase in rate as the pH is decreased to 5.4 is particularly striking. The reactions become even faster as the pH is lowered still further but the picture is confused by a concomitant shift to more favourable equilibrium positions (see Fig. 1). Below pH 5, the approach to equilibrium is essentially complete within the 15 s required to mix the reactants.

The proposal that the very rapid increase in E_{343} that accompanies admixture of L-ergothioneine with

Fig. 1. Effect of pH on the equilibrium position of the L-ergothioneine-2-Py-S-S-2-Py system at 25.0°C and $I = 0.1$

The buffers are given in the Materials and Methods section; the points are experimental and the lines are drawn by eye. O, L-Ergothioneine 2.75 mM, 2-Py-S-S-2-Py 19.5 μ M; □, L-ergothioneine, 7.1 μ M, 2-Py-S-S-2-Py 1.04 mM; Δ, L-ergothioneine 62.5 μ M, 2-Py-S-S-2-Py 195 μ M; ●, L-ergothioneine 34 mM, 2-Py-S-S-2-Py 19.5 μ M.

2-Py-S-S-2-Py at pH 1 results from the formation of Py-2-SH is supported by the close resemblance of the u.v. spectrum of the product (difference spectrum versus that of 2-Py-S-S-2-Py) above 320 nm to that of an authentic sample of Py-2-SH.

The effect of pH on the equilibrium position for the L-ergothioneine-2-Py-S-S-2-Py system is shown in Fig. 1. At all the concentrations of the initial reactants studied, the overall equilibrium position is independent of pH in the region 8–5.5 and then changes to a more favourable position (for the formation of Py-2-SH) as the pH is decreased to approx. 1. When relatively high L-ergothioneine concentrations (2.75 mM) are used, the reactions proceed to completion at pH 1. Use of extremely high concentrations of L-ergothioneine (34 mM) demonstrates the approach to completion of the reactions even at pH approx. 6 (see Fig. 1).

It is not surprising that the pH-dependent changes in the equilibrium position of the L-ergothioneine-2-Py-S-S-2-Py system occur in the pH region 2–5 because pK_{aIII} of 2-Py-S-S-2-PyH⁺ = 2.45 (Brocklehurst & Little, 1973) and the pK_a values of the imidazole moieties of the L-ergothioneine-Py-2-SH mixed disulphide and of L-ergothioneine might be expected to be approx. 4–5 (cf. Richardson, 1933).

That the reactions of L-ergothioneine with 2-Py-S-S-2-Py are not complicated by side reactions such as aerial oxidation of L-ergothioneine is strongly

suggested by (i) the stoichiometric release of Py-2-SH when high concentrations of 2-Py-S-S-2-Py are used, (ii) the known stability of L-ergothioneine towards oxidation (Heath & Toennies, 1958; Carlsson *et al.*, 1974) and (iii) the closely similar initial rates of formation of Py-2-SH in both aerobic and anaerobic solutions.

The results presented above demonstrate that L-ergothioneine and 2-Py-S-S-2-Py undergo reversible thiol-disulphide interchange to provide Py-2-SH, the L-ergothioneine-Py-2-SH mixed disulphide and L-ergothioneine disulphide (L-ergothioneine) (i.e. eqns. 1 and 2).

The rate minimum in approximately neutral media suggested by the pH-dependence of v_1 demonstrates that by contrast with simple aliphatic thiols L-ergothioneine possesses considerable nucleophilic character in its neutral thiol or thione form. This is exhibited most dramatically at low pH in the reactions with the highly electrophilic 2-Py-S-S-2-Py cation and/or the highly electrophilic L-ergothioneine-Py-2-SH cation. It is important to point out that it is necessary to observe or infer the existence of three reactive protonic states in these reactions, to demonstrate nucleophilic reactivity in the un-ionized aminothione moiety (see Brocklehurst, 1974).

pH-rate profiles are most effectively discussed in terms of various protonic states designated X, XH, XH₂ etc. to indicate stoichiometries (or relative stoichiometries) with respect to protons (see Brocklehurst & Little, 1972). The X state of a reaction of a thiol or thione with 2-Py-S-S-2-Py should be reaction of the thiolate ion with unprotonated disulphide. If an XH₂ state of reactivity higher than that of XH state is observed, reactivity in the XH state cannot result solely from reaction of the thiolate ion with protonated disulphide. This is because the intrinsic nucleophilicity of an un-ionized thiol (or thione) would reasonably be expected to be lower than that of the corresponding thiolate ion and addition of the second equivalent of protons would abolish any thiolate ion remaining in the XH state. The necessary validity of this argument depends of course, on the absence of significant proton partitioning between the thiol group and some other atom in the system (see Brocklehurst, 1974). The absence of such partitioning is strongly suggested by the results of the kinetic study of the alkylation of L-ergothioneine by iodoacetamide (see below).

Reaction of 2-Py-S-S-2-Py with 2-mercaptoimidazole and with 1-methyl-2-mercaptoimidazole

2-Mercaptoimidazole (67 μ M) reacts with 2-Py-S-S-2-Py (195 μ M) very rapidly at pH 5.49 ($v_1 > 2.6 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$); the rate falls as the pH is raised, becomes pH-independent in the pH range 7.06–8.3 ($v_1 = 2.1 \times 10^{-8} \text{ M} \cdot \text{s}^{-1}$) and then increases

Fig. 2. Effect of pH on the equilibrium positions of the 2-mercaptimidazole-2-Py-S-S-2-Py and 1-methyl-2-mercaptimidazole-2-Py-S-S-2-Py systems at 25.0°C and $I = 0.1$

The buffers are as given in the Materials and Methods section and all reaction mixtures contained 2% (v/v) ethanol: the points are experimental and the lines drawn by eye. ○, 2-Mercaptoimidazole, 58 μM , 2-Py-S-S-2-Py, 1.02 mM; □, 1-methyl-2-mercaptoimidazole, 71 μM , 2-Py-S-S-2-Py, 1.02 mM; ▲, 1-methyl-2-mercaptoimidazole, 71 μM , 2-Py-S-S-2-Py, 10.2 mM. In these systems only situations in which $[2\text{-Py-S-S-2-Py}] > [\text{mercaptimidazole}]$ were studied.

Fig. 3. Effect of pH on the equilibrium position of the thiourea-2-Py-S-S-2-Py system at 25.0°C and $I = 0.1$

The buffers are as given in the Materials and Methods section, the points are experimental and the lines drawn by eye. ○, Thiourea, 19.2 mM, 2-Py-S-S-2-Py, 15.4 μM ; ●, thiourea, 8.5 μM , 2-Py-S-S-2-Py, 0.79 mM.

again at higher pH. The pH-dependence of the equilibrium position of these reactions is shown in Fig. 2. The equilibrium position at pH values greater than 5 is less favourable (for the formation of Py-2-SH) than in the corresponding L-ergothioneine system.

These results are qualitatively very similar to those presented above for the analogous reactions of L-ergothioneine and demonstrate that the high reactivity of L-ergothioneine towards 2-Py-S-S-2-Py and in particular towards 2-Py-S-S-2-Py⁺H is a property of the 2-mercaptimidazole moiety of this molecule.

This conclusion is further supported by the observation of the characteristically rapid reaction of 2-Py-S-S-2-Py with 1-methyl-2-mercaptimidazole at low pH. The equilibrium position for the reactions in this system is even more unfavourable and at the concentrations necessary to observe measurable extinction changes the reactions were essentially complete within 15s at pH values between 1.0 and 8.0. The pH-dependence of the equilibrium position for this system is presented in Fig. 2.

Reaction of 2-Py-S-S-2-Py with thiourea and with thioacetamide

That the complete 2-mercaptimidazole moiety is not necessary to provide high reactivity towards 2-Py-S-S-2-Py is shown by the high reactivity of both thiourea and thioacetamide towards this compound at low pH. The pH-dependence of the equilibrium position of the thiourea-2-Py-S-S-2-Py system is shown in Fig. 3. Under the conditions of concentration necessary to provide measurable extinction changes (see e.g., the legend to Fig. 3) the approach to equilibrium was complete within 15s. The adverse equilibrium position of this system probably accounts for the failure to observe any reaction under the conditions of concentration used when $[2\text{-Py-S-S-2-Py}]$ is greater than $[\text{thiourea}]$ at pH values greater than 5. The reaction of thioacetamide with 2-Py-S-S-2-Py is complicated by subsequent reaction. The important observation for the purposes of this discussion is that, as with L-ergothioneine and the other aminothiones discussed above, admixture of thioacetamide and 2-Py-S-S-2-Py at pH values less than 5 results in very rapid release of 2-thiopyridone.

By contrast with the other interchanges discussed above incubation of equivalent quantities of thioacetamide and 2-Py-S-S-2-Py at pH 1 results in the rapid release of 2equiv. of Py-2-SH, i.e. all of the 2-Py-S-S-2-Py is converted into Py-2-SH. The reaction is accompanied by formation of colloidal sulphur and acetonitrile (see the Materials and Methods section). These findings can be accounted for by a thiol-disulphide interchange to produce 1 equiv. of

(i) Thiol–disulphide interchange

(ii) Spontaneous fragmentation

(iii) Hydrolysis followed by elimination of S and H₂O

Scheme 2. Postulated mechanisms of the reaction of thioacetamide with 2-Py-S-S-2-Py

Py-2-SH and a mixed disulphide that subsequently undergoes fragmentation to provide the other equivalent of Py-2-SH, sulphur and acetonitrile (Scheme 2). This reaction is an example of the known instability of disulphides with adjacent double bonds such as *S*-(guanylylthio)-L-cysteine in neutral and alkaline media (McGowan, 1886; Werner, 1912; Fromm, 1906; Toennies, 1937). Toennies (1937) suggested that such reactions proceed via the hydrolysis mechanism of Scheme 2 (iii) to provide a sulphenic acid which then successively loses S and H₂O. This mechanism seems unlikely in this case because the reaction would be expected to produce acetamide rather than acetonitrile.

Origin of the high nucleophilicity of L-ergothioneine and the other aminothiones

The structural feature common to L-ergothioneine, 2-mercaptoimidazole, 1-methyl-2-mercaptoimidazole, thiourea and thioacetamide is the HN-C=S moiety.

Nucleophilic reactivity in simple un-ionized aliphatic thiols (RSH) cannot be detected in their reactions with aromatic disulphides that cannot increase their electrophilicity by protonation, e.g. 5,5'-dithiobis-(2-nitrobenzoate) dianion (Little & Brocklehurst, 1972). This is inferred from the essentially zero reactivity of the XH state of these reactions.

The only published examples of definitive evidence for nucleophilic reactivity in undissociated thiol groups relate to those in the active centres of the proteolytic enzymes papain and bromelain (Brocklehurst & Little, 1970, 1972, 1973; Brocklehurst *et al.*, 1972; see also Polgar, 1973). In these cases the activation of the thiol group is achieved through space by its proximity to a histidine imidazole group (see formula XIV), and the lack of a deuterium isotope effect in the XH state of both the alkylation of papain (Polgar, 1973) and in its reaction with 2-Py-S-S-2-Py (M. Shipton & K. Brocklehurst, unpublished work) indicates that the reactions may involve pre-transition-state proton transfer.

The high nucleophilic reactivity of L-ergothioneine and the other aminothiones could arise in one of four ways. It could be provided by general base catalysis either by the pyridine nitrogen atom of 2-Py-S-S-2-Py (Scheme 3a) or by one of the sulphur atoms of this molecule in a fully concerted cyclic transition state (Scheme 3b) or by a third molecule (B) such as solvent (Scheme 3c). Alternatively it could arise by unassisted mesomeric release of the electrons of the pyridine nitrogen atom with post-transition-state proton transfer (Scheme 3d).

That general base catalysis by a sterically aligned nitrogen atom of 2-Py-S-S-2-Py (Scheme 3a) is not a necessary requirement is demonstrated by the high reactivity of L-ergothioneine (62.5 μM) towards 4,4'-dipyridyl disulphide (4-Py-S-S-4-Py) (214 μM) which results in the production of Py-4-SH (v_i at pH 5.45 $> v_i$ at pH 5.86 $> v_i$ at pH 7.04).

The pH-dependence of the equilibrium position for the L-ergothioneine-4-Py-S-S-4-Py system is shown in Fig. 4. In marked contrast with any of the other interchanges reported above the equilibrium position for this system becomes less favourable for the derivative formation of L-ergothioneine as the pH is decreased below approx. 6. This presumably reflects the effect of protonation of the 4-pyridyl moieties in the various compounds of the system on the equilibrium position (e.g. for 4-Py-S-S-4-Py $\text{p}K_{a_1} = 4.0$ and $\text{p}K_{a_{II}} = 5.1$; Brocklehurst & Little, 1973).

The other possible type of assistance from the 2-Py-S-S-2-Py molecule (Scheme 3b) is eliminated as a necessary requirement for high reactivity in the aminothiones by the observation of similar high

reactivity in the reaction of Py-4-SH with 2-Py-S-S-2-Py. In this system it was possible to observe the decrease in rate at low pH that would be predicted when the pyridine nitrogen atom of Py-4-SH becomes protonated ($\text{p}K_{a_1} = 1.43$; Albert, 1963). High reactivity is observed also in the reaction of Py-2-SH with 4-Py-S-S-4-Py.

The initial rates of the reactions in the L-ergothioneine (74 μM)-2-Py-S-S-2-Py (159 μM) system at pH (pD) approx. 6-7 (pH 6.26, $v_i = 1.6 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$; pD 6.36, $v_i = 3.7 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$; pD 6.77, $v_i = 2.0 \times 10^{-7} \text{ M} \cdot \text{s}^{-1}$) suggest that the reactions are characterized by a positive deuterium isotope effect. This provides no evidence for general base catalysis (Schemes 3a-3c) but is consistent (see Bruice & Benkovic, 1966) with reaction of the neutral thione with post-transition-state proton transfer (Scheme 3d) assisted by specific acid catalysis (i.e. reaction with 2-Py-S-S-2-PyH⁺).

A mechanism for the reaction at sulphur involving nucleophilic attack by the un-ionized aminothione with post-transition-state proton transfer is in accord with the similar mechanism for the reaction of L-ergothioneine at saturated carbon inferred by the kinetic analysis of its alkylation by iodoacetamide presented below.

Reaction of L-ergothioneine with iodoacetamide

The reactions of L-ergothioneine and the other aminothiones with 2-Py-S-S-2-Py consist of two reversible consecutive thiol-disulphide interchanges proceeding to pH-dependent equilibrium positions. The complexity and rapidity of these reactions precluded quantitative kinetic analysis, at least with conventional spectrophotometry, and it was considered desirable therefore to demonstrate quantitatively the existence of nucleophilic reactivity in the neutral aminothione form of L-ergothioneine in a reaction that could be subjected to kinetic analysis. Such a reaction is alkylation by iodoacetamide.

U.v. spectra of L-ergothioneine and the product of its reaction with iodoacetamide: the basis of the rate assay

Heath & Toennies (1958) reported that in the pH range 2-9 the u.v. spectrum of L-ergothioneine consists of an absorption band at 258nm, $\epsilon_{\text{max}} = 14500 \text{ M}^{-1} \cdot \text{cm}^{-1}$, and that as the pH is increased above 9, the absorption maximum gradually shifts to shorter wavelengths and becomes broader. The absorption band at 258nm probably arises from electronic transitions in the thione form of the 2-mercaptoimidazole moiety of L-ergothioneine which is destroyed by proton loss from nitrogen ($\text{p}K_a = 10.8$). This suggests that alkylation of the sulphur atom of

(XIV)

Scheme 3. Possible origins of the nucleophilicity of the neutral forms of the aminothiones (III)–(IX)

The geometrical restrictions of mechanisms (a) and (b) make them applicable only to aminothiones in which the two functional groups are linked directly.

L-ergothioneine, e.g. by iodoacetamide, which also destroys the thione character of the molecule (see Scheme 4), should be accompanied by substantial decrease in extinction around 258 nm.

The absorption parameters of L-ergothioneine determined in the present study at pH values 2–8 (λ_{max} , 257 nm, ϵ_{max} , $1.4 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$) are in

reasonable agreement with those reported by Heath & Toennies (1958) and with those of 2-mercaptoimidazole reported by Stanovik & Tisler (1964) (λ_{max} , 259; ϵ_{max} , $1.3 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$; solvent, ethanol). The spectrum of the products of the reaction consists of the absorption band of alkylated L-ergothioneine (λ_{max} , approx. 250 nm) and that of iodide ion

($\lambda_{\max}$, 227 nm). The difference extinction coefficient at 257 nm that corresponds to the reaction in Scheme 4 is $\Delta \epsilon_{257} = 1.0 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in the pH range 2–8. The fall in $\Delta \epsilon_{257}$ at higher pH values which results from the lower values of ϵ_{257} for L-ergothioneine at

Fig. 4. Effect of pH on the equilibrium position of the L-ergothioneine-4-Py-S-S-4-Py system at 25.0°C and $I = 0.1$.

The buffers are given in the Materials and Methods section; the points are experimental and the lines drawn by eye. ●, L-Ergothioneine, 2.7 mM, 4-Py-S-S-4-Py, 15.6 μM; ○, L-ergothioneine, 0.38 μM, 4-Py-S-S-4-Py, 0.32 mM.

higher pH values is described by $\Delta \epsilon_{257} = \{8 \times 10^3 / (1 + K_a/[H^+])\} + 2 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$, in which $pK_a = 10.8$.

pH-dependence of the rate of alkylation of L-ergothioneine

The pH- (and pD-) dependence of the second-order rate constant for the reaction of L-ergothioneine with iodoacetamide (Scheme 4) is shown as a log plot in Fig. 5.

Fig. 5. pH-dependence of the second-order rate constant for the reaction of L-ergothioneine with iodoacetamide at 25°C and $I = 0.05$.

The buffers are as given above. At pH values less than 9 the values of k_2 were calculated from initial rates and the reactant concentrations. At higher pH values the values of k_2 were obtained usually by dividing the first-order rate constants by the iodoacetamide concentration. In some cases values of k_2 at pH values greater than 9 were obtained also from initial-rate data and concordant results were obtained. The points are experimental; the lines of zero slope correspond to the X ($k_2^X = 4.0 \text{ M}^{-1} \cdot \text{s}^{-1}$) and XH ($k_2^{XH} = 0.03 \text{ M}^{-1} \cdot \text{s}^{-1}$) states respectively; the line of slope +1 intersects the X-state line of zero slope at $pK_a = 10.45$; this value and that of the k_2^X were obtained by regression of $1/(k_2 - 0.03)$ on $[H^+]$.

Scheme 4. Reaction of L-ergothioneine with iodoacetamide

That the reaction does conform to a second-order rate law, first order in both L-ergothioneine and iodoacetamide, was demonstrated by the linear dependence of the initial rate on both [L-ergothioneine] and [iodoacetamide] at pH values 4 and 9.

The pH-rate profile in Fig. 5 demonstrates that the alkylation of L-ergothioneine by iodoacetamide (Scheme 4) is characterized by at least two reactive protonic states designated X and XH to indicate their relative stoichiometries with respect to protons (see Brocklehurst & Little, 1972). The X state is reasonably interpreted as a reaction of neutral iodoacetamide with the ionized mercaptoimidazole moiety of the L-ergothioneine dianion (Scheme 5a). The three most chemically reasonable interpretations of the reactive XH state of the reaction are presented in Scheme 5 (b)–(d).

Schemes 5(b) and 5(c) depict the reaction of the X state assisted by specific acid catalysis; i.e. attack of the L-ergothioneine dianion on iodoacetamide protonated on iodine which would make it a better leaving group or on oxygen which would decrease the

electron density of the electrophilic centre. Since protonation of iodoacetamide would be expected to occur in the H_0 region of the acidity scale, the reactions of Schemes 5(b) and 5(c) would have to be characterized by rate constants approaching the diffusion-controlled limit to account for the results given in Fig. 5. Interpretation of the XH state in terms of specific acid catalysis of the reaction of the L-ergothioneine dianion is therefore considered less likely than the remaining interpretation (Scheme 5d), involving reaction of the neutral mercaptoimidazole moiety of the L-ergothioneine monoanion with neutral iodoacetamide. The low pK_a which would be predicted for the conjugate acid of iodoacetamide precludes the observation of the third reactive protonic state (reaction of the L-ergothioneine monoanion with the iodoacetamide monocation) which would provide definitive evidence for reactivity in the neutral mercaptoimidazole moiety (see Brocklehurst & Little, 1972; Brocklehurst, 1974). Without knowing the pK_a of the conjugate acid of iodoacetamide it is not possible to consider whether the mechanisms of Schemes 5(b) and 5(c) might be rigorously excluded by making use of the concept of the diffusion-controlled limit. It is conventional to reject mechanisms that would have rate constants greater than that of the reaction of a hydroxide ion with a proton ($1.4 \times 10^{11} \text{ M}^{-1} \cdot \text{s}^{-1}$; see Bender, 1972). Calculation shows that since the relevant pK_a of L-ergothioneine is approx. 11 and the experimentally observed rate constant for the XH state is $0.03 \text{ M}^{-1} \cdot \text{s}^{-1}$ the pK_a of the iodoacetamide monocation would have to be equal to or less than -2 if mechanisms involving specific acid catalysis are to be rejected by the criterion of the diffusion-controlled limit. The pK_a values of the conjugate acids of simple amides are approx. 0 (Bender, 1972) and that of the iodoace-

(a)
X state

(b)
XH states

Scheme 5. Possible interpretations of the reactive protonic states of the reaction of L-ergothioneine with iodoacetamide.

tamide monocation might be expected to be even lower owing to the presence of the electronegative iodine atom.

The probable interpretation of the reactive XH state shown in Fig. 5 in terms of nucleophilic reactivity in the un-ionized mercaptoimidazole moiety of L-ergothioneine in reaction at saturated carbon (Scheme 5d) lends support to the evidence presented above for nucleophilic reactivity in neutral aminothiones including L-ergothioneine in reaction at electrophilic sulphur. The absence of a deuterium isotope effect (see Fig. 5) provides no evidence either for specific acid catalysis or for general base catalysis.

It is almost certain that the reactive XH state of Fig. 5 is not provided by proton partitioning in L-ergothioneine between the imidazole nitrogen atom and the carboxylate ion. This interpretation of the reactive XH state may be eliminated if the reasonable assumption is made that the reactivity of the resonance-stabilized thiolate ion of L-ergothioneine ($4\text{M}^{-1}\cdot\text{s}^{-1}$) is essentially unchanged by protonation of the carboxylate ion of this molecule. The $\text{p}K_a$ values of 1.3 and 10.8 (see above) should be good approximations to the intrinsic $\text{p}K_a$ values of the carboxyl and aminothione moieties respectively. Thus, at pH 3 only 5×10^{-10} of the L-ergothioneine will be present such that the carboxylate ion is protonated and the aminothione moiety is ionized. Accordingly, the second-order-rate constant for the reaction with neutral iodoacetamide at pH 3 would be expected to be $4 \times 5 \times 10^{-10} = 2 \times 10^{-9}\text{M}^{-1}\cdot\text{s}^{-1}$, whereas Fig. 5 shows it to be $3 \times 10^{-2}\text{M}^{-1}\cdot\text{s}^{-1}$, some seven orders of magnitude higher.

The results presented above demonstrate that the nucleophilicity of the sulphur atom of the aminothiones is probably best considered to arise from mesomeric release of the lone pair of electrons on the nitrogen atom of the amino group (Scheme 3d). To what extent this is assisted by the approach of the electrophilic centre of the disulphide reagent remains to be established.

It is interesting to compare the high nucleophilicity of these aminothiones with that frequency displayed by compounds containing an electronegative atom

with a lone electron pair adjacent to the nucleophilic atom. Compounds of this sort that display unusually high reactivity include hydroxylamine, hydrazine, and the anions of hypochlorite, hydroxamic acids, oximes, hydroperoxide, nitrite and sulphite, and the effect has been called the α effect. (Jencks & Carriuolo, 1960; Green *et al.*, 1958; Edwards & Pearson, 1962). The α effect has been reviewed by Jencks (1969) and discussed by Hudson (1973) in terms of his perturbation treatment of chemical reactivity, proposed as an alternative to the transition-state approach.

The role most frequently proposed for the electron pair on the electronegative atom adjacent to the nucleophilic atom is that it increases the effective electron density at the reaction centre. In this respect the effect resembles that found with aminothiones. Increase of electron density at the nucleophilic atom in the aminothiones is readily understood in terms of mesomeric electron release. Understanding of the α effect in similar terms is at first sight more difficult because it appears to require an involvement of the nucleophilic atom with more bonds and electrons than is normally possible without expansion of its valence shell. That this difficulty is more apparent than real has been pointed out by Jencks (1969): the number of electrons in an α -effect system is not very large compared with the total number of available atomic orbitals and it should be possible to obtain a stable system by hybridization of the p orbitals of each of the constituent atoms to molecular orbitals involving the total system. This is the same type of hybridization that is used to describe the covalent character of the hydrogen bond, the transition state of a simple $\text{S}_\text{N}2$ reaction at saturated carbon, and the structure of polyhalide compounds such as I_3^- (Rundle, 1962; Pimentel & McClellan, 1960).

Another way to describe the nucleophilic reactivity of these aminothiones would be in terms of the zwitterionic canonical forms (see Scheme 6). In these terms high reactivity in the XH_2 states would be ascribed to reaction of 2-Py-S-S-2-Py ^+H with the thiolate ions protonated on nitrogen, i.e. a special case of the proton-partitioning effect referred to above.

Scheme 6. Canonical forms of conjugated aminothiones

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