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A New Route to the Imidazole-2-thiones from 2-Thiohydantoins

IMPLICATIONS IN THE STUDY OF ERGOTHIONEINE

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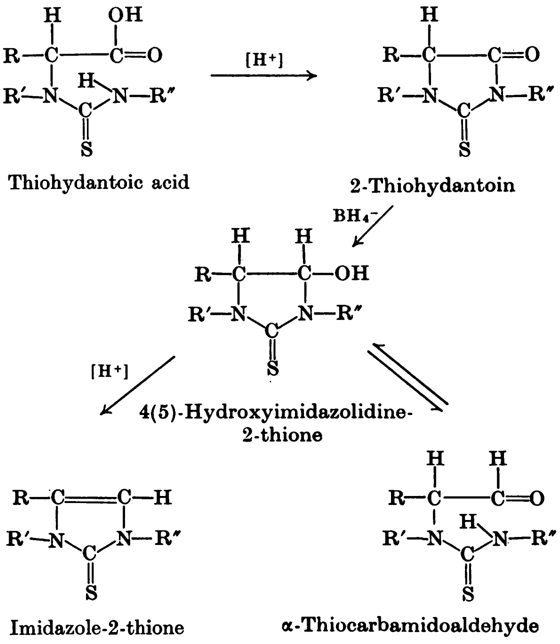
1. 2-Thiohydantoins are reduced by borohydrides to 4(5)-hydroxyimidazolidine-2-thiones, which eliminate water in acid to form imidazole-2-thiones. Both steps take place in mild conditions, in high yield. A number of imidazole-2-thiones have been synthesized by this sequence of steps, with one, two or three substituents in the 1-, 3- and 4(5)-positions. 2. 4(5)-Hydroxyimidazolidine-2-thiones are ammonium pseudo-bases, giving rise to an equilibrium mixture of amino aldehyde, carbinolamine and mesomeric ammonium cationic forms. The elimination of water is suggested to be a property of the mesomeric ammonium cation. 3. The mild conditions in which imidazole-2-thiones are formed from 4(5)-hydroxyimidazolidine-2-thiones are similar to those in which ergothioneine, a naturally occurring imidazole-2-thione of uncertain function, is normally released and measured. It is suggested that the occurrence *in vivo* of a precursor to ergothioneine, in the form of a 4(5)-hydroxyimidazolidine-2-thione, would explain many otherwise conflicting published data.

Imidazole-2-thiones are usually prepared by heating α-thiocarbamido derivatives of acetals and ketones in strong acid (the Wohl–Marckwald reaction). Burtles, Pyman & Roylance (1925) suggested that the acetal alkoxy groups are cleaved before cyclization. The direct route from α-amino aldehydes is not readily available, because of the difficulty of making this reactive type of compound. Bullerwell & Lawson (1951), following the principle of Akabori, prepared α-amino aldehydes *in situ*, by reduction of α-amino acid esters with sodium amalgam in the presence of thiocyanate, but yields of imidazole-2-thiones were sometimes very low. No fixed procedure was discovered, and it is not a general method.

One possible intermediate in the Wohl–Marckwald reaction is a 4(5)-hydroxyimidazolidine-2-thione (Scheme 1), formed by the cyclization of the aldehydothiourea, which could then dehydrate to the imidazole-2-thione. Seldom, if at all, have these intermediates been prepared or isolated, except with 2-amino-2-deoxyaldoses, which react with isothiocyanates to give a 4(5)-hydroxyimidazolidinethione (Scott, 1964; Scheme 1: R = polyhydroxyalkyl, R′ = H, R″ = phenyl etc.). In this context, 2-amino sugars are hemiacetals analogous to those that would be produced in the hydrolysis of acetals according to Burtles *et al.* (1925). The product of phenyl isothiocyanate with glucosamine could alternatively be made by reducing the phenylthiohydantoin of glucosaminic acid with sodium borohydride (Scott, 1964; Scheme 1: R = D-*arabino*-tetrahydroxybutyl, R′ = H, R″ = phenyl). The analogous reduction of the 3-phenyl-2-thiohydantoin of alanine was rapid and quantitative at room temperature.

If 4(5)-hydroxyimidazolidine-2-thiones are intermediates in the Wohl–Marckwald synthesis, the reduced products of 2-thiohydantoins ought to be converted into imidazole-2-thiones in acid conditions, and this was found to occur. Conversion was rapid and quantitative, and could be followed easily by spectrophotometry. The imidazole-2-thione from the 3-phenyl-2-thiohydantoin of alanine crystallized spontaneously at room temperature in pure form in 86% yield from the acidic aqueous solution. This is compared with a 28% yield from the acetal after 30min. reflux in 5N-hydrochloric acid (Burtles *et al.* 1925) or 55% (Bullerwell & Lawson, 1951) from the α-amino acid ester.

The ease and convenience of this preparation stimulated attempts to prepare other imidazole-2-thiones from 2-thiohydantoins, substituted in all possible positions. The results show that mono-, di- and tri-substituted imidazole-2-thiones are conveniently prepared in high yields by this route. Because of the ease of desulphurization of imidazole-2-thiones, a new route is opened to imidazoles in general. The mild conditions used throughout



Scheme 1.

should ensure that labile groups in the molecule remain intact.

A preliminary account of some of this work has been published (Scott, 1968).

EXPERIMENTAL

Preparation of 2-thiohydantoins. 2-Thiohydantoins without substituents at N-3 were made by the general method of Johnson & Nicolet (1913), from the α-amino acid, in high yield and with great convenience. With the exception of 1,3-dimethyl-2-thiohydantoin, which was prepared by the method of Cook & Cox (1949), 2-thiohydantoins with methyl or phenyl substituents at N-3 were prepared from methyl or phenyl isothiocyanate and the α-amino acid: 1 mole of amino acid in aq. 67% (v/v) pyridine, 1·1 mole of triethylamine and 1·1 mole of isothiocyanate were incubated for 4hr. at 37° with stirring. In some cases the amino acid was not completely soluble at the outset, but as the reaction continued solution of the amino acid became complete. The solution was extracted three times with 3vol. of benzene. Conc. HCl (2 moles) was added to the aqueous layer, which was heated on a steam bath for 30min. and then cooled to 4°. In many cases heating was not required: conversion into the 2-thiohydantoins occurred at room temperature on leaving overnight. The solid that separated was recrystallized. Melting points, recrystallizing solvents and other relevant data are given in Table 1 for those 2-thiohydantoins that were used as starting materials for the preparation of the imidazole-2-thiones shown in Table 2.

Preparation of imidazole-2-thiones. All the available

Table 1. *2-Thiohydantoins*

	Melting point	Literature melting point	Literature reference	Crystallizing solvent	Preparative method	Yield (%)	
3-Methyl	163–164°	162°	Jeffreys (1954)	Water	This paper	50	
3-Phenyl	244–246	245–248	Edman (1950)	Aq. 25% (v/v) acetic acid	This paper	65	
5-Isobutyl	175–176	174–176	Jackman, Klenk, Fishman, Tullar & Archer (1948)	Aq. 30% (v/v) acetic acid	Johnson & Nicolet (1913)	68	
1,3-Dimethyl	92–92·5	93	Cook & Cox (1949)	Water	Cook & Cox (1949)	50	
3,5-Dimethyl	170–173	168	Horner, Kimmig & Schreiner (1952)	Aq. 25% (v/v) acetic acid	This paper	75	
3-Methyl-5-phenyl	159–160			Aq. 50% (v/v) ethanol	This paper	70	(Found: C, 58·3; H, 4·9; N, 13·6. Calc.: C, 58·4; H, 4·7; N, 13·4%)
5-Methyl-3-phenyl	185–186	185	Edman (1950)	Aq. 67% (v/v) acetic acid	This paper	70	
5-Carboxypropionyl-3-phenyl	165–166	166–167	Edman (1950)	Aq. 75% (v/v) acetic acid	This paper	70	Monohydrate
5,5-Dimethyl-3-phenyl	174–176			Aq. 50% (v/v) acetic acid	This paper	50	(Found: C, 59·9; H, 5·2; N, 13·0; S, 14·6; $C_{11}H_{12}N_2OS$ requires: C, 60·0; H, 5·5; N, 12·7; S, 14·6%)

Table 2. *Imidazole-2-thiones*

	Melting point	Literature melting point	Crystallizing solvent	Preparative method	Yield (%)	Literature reference	
1-Methyl	140–143°	143–144°	Diethyl ether	$LiBH_4$–dioxan		Easson & Pyman (1932)	
1-Phenyl	178–181	181–182	Diethyl ether	$LiBH_4$–dioxan	30	Burtles *et al.* (1925)	
4(5)-Isobutyl	186–187	188–189	Water	$LiBH_4$–dioxan	70	Jackman *et al.* (1948)	(Found: C, 46·2; H, 6·2; N, 22·2; S, 25·0. Calc.: C, 46·9; H, 6·3; N, 21·9; S, 25·0%)
1,3-Dimethyl	185–186		Water	$NaBH_4$–water	30		
1,4-Dimethyl	209–210	211–212	Water	$NaBH_4$–aq. 25% (v/v) diglyme	63	Burtles *et al.* (1925)	
1-Methyl-4-phenyl	210–212	220–221	Aq. 33% (v/v) acetic acid	$LiBH_4$–dioxan	72	Dodson & Ross (1950)	(Found: C, 62·5; H, 5·3; N, 14·5; S, 16·4. Calc.: C, 63·2; H, 5·3; N, 14·7; S, 16·8%)
4-Methyl-1-phenyl	191–192	191–192	Aq. 25% (v/v) diglyme	$NaBH_4$–water	80	Burtles *et al.* (1925)	
4-Carboxypropyl-1-phenyl	190		Aq. 25% (v/v) acetic acid	$NaBH_4$–aq. 25% (v/v) diglyme	65		(Found: C, 58·2; H, 4·3; N, 11·5. Calc.: C, 58·0; H, 4·8; N, 11·3%; neutralization equivalent=247)

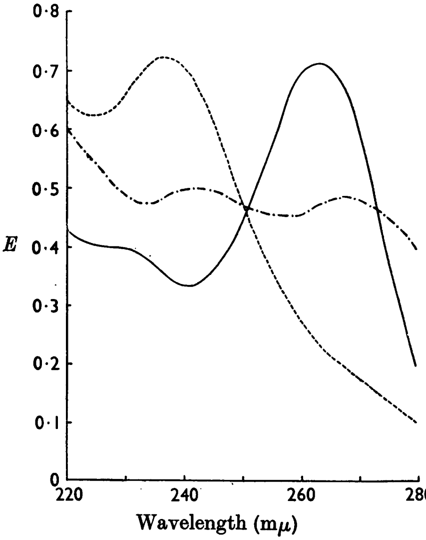


Fig. 1. Spectra (in water) of 5-methyl-2-phenyl-2-thiohydantoin (——) ($\epsilon_{max.}$ 16000), 5-hydroxy-4-methyl-1-phenylimidazolidine-2-thione (-------) ($\epsilon_{max.}$ 17500) and 4-methyl-1-phenylimidazole-2-thione (–•–•–) (ϵ_{243} 7900).

2-thiohydantoins could be reduced to the 4(5)-hydroxyimidazolidine-2-thiones by $LiBH_4$ in dioxan.

The 10% (w/v) thiohydantoin in dioxan was added in portions to a fresh 5% (w/v) suspension of $LiBH_4$ in dioxan at 37° with stirring. About 1g. of $LiBH_4$ was used/g. of thiohydantoin. Vigorous effervescence occurred. After 30–60min. aq. 10% (v/v) acetic acid was added, in the ratio 2 moles of acetic acid/mole of $LiBH_4$. The solution was boiled for 5–10min. and then, while gently simmering, was over-neutralized to approx. pH 10 with solid Na_2CO_3. The solution was cooled to 4°. In many cases crude imidazole-2-thione separated and could be further purified. Some imidazole-2-thiones (e.g. imidazole-2-thione, 1-methylimidazole-2-thione and 1-phenylimidazole-2-thione) were more efficiently recovered by extraction with diethyl ether.

An alternative procedure, which could not be used on 2-thiohydantoins in which N-3 was unsubstituted, utilized $NaBH_4$ in aqueous diglyme. Aq. 2% (w/v) $NaBH_4$ was added to a diglyme solution of the thiohydantoin at room temperature with stirring. About 2–3 equiv. of $NaBH_4$ was used/mole of thiohydantoin. The concentration of diglyme was adjusted to suit the solubility of the thiohydantoin. A considerable amount of insoluble thiohydantoin was permissible at the outset, since the intermediates were very soluble. The reaction was slower in higher concentrations of diglyme. Gas was evolved, and after 30–120min. the solution was adjusted to pH 0·5 with conc. HCl. Imidazole-2-thione frequently began to separate in 2–3hr., especially if the diglyme concentration was low. The conversion into imidazole-2-thione could be accelerated by warming, and at 100° was usually complete in 1min. or less.

Both procedures could be monitored spectrophotometrically with considerable benefit. The characteristic 2-thiohydantoin peak at about 265mμ moves on reduction to an equally clear peak characteristic of 4(5)-hydroxyimidazolidine-2-thiones at about 238mμ (see the examples in Fig. 1). A 0·1ml. reaction mixture was taken into 100ml. of aq. 2mM-boric acid and spectra were drawn, with appropriate controls. Dioxan, diglyme, boric acid, acetic acid and HCl do not absorb light in the regions characteristic of 2-thiohydantoins and imidazole-2-thiones (220–320mμ).

Preparation of 5-hydroxy-4-methyl-1-phenylimidazolidine-2-thione. DL-Alanine 3-phenyl-2-thiohydantoin (4g.) and 1g. of $NaBH_4$ were dissolved in 20ml. of diglyme and 80ml. of water respectively and mixed together. A white precipitate appeared, which slowly dissolved with considerable evolution of gas on stirring for 2hr. at room temperature. The solution was extracted three times with an equal volume of ethyl acetate, and the pooled extracts were evaporated to dryness *in vacuo* at 50°. The clear viscous liquid was extracted with 50ml. of diethyl ether, leaving a small amount of undissolved crystalline material (phenylthiocarbamoylalaninol, m.p. 136–137° on recrystallizing from aq. 50% ethanol; $\lambda_{max.}$ 243mμ; a specimen prepared from DL-alaninol and phenyl isothiocyanate melted at 138–139°; a mixed melting point showed no depression). [A crystalline solid, presumably phenylthiocarbamoylprolinol, was isolated from an $LiBH_4$ reduction of 1,5-trimethylene-3-phenyl-2-thiohydantoin; crystallization from aq. 50% acetic acid gave a product with m.p. 128° (Found: C, 61·2; H, 6·8; N, 11·7; S, 14·0; $C_{12}H_{16}N_2OS$ requires: C, 61·0; H, 6·8; N, 11·9; S, 13·6%). The spectrum in water showed a single peak at 243mμ (ε 17250).] The ethereal solution was concentrated to a clear viscous oil, which was further extracted with 25ml. of dibutyl ether–diethyl ether (1:1, v/v) and left in a stoppered flask at room temperature. After a few days white crystalline deposits appeared in the oil and grew. After several weeks the solid was removed and dried *in vacuo*. The yield was 30% (Found: C, 57·7; H, 5·8; N, 13·5; S, 15·7; $C_{10}H_{12}N_2OS$ requires: C, 57·7; H, 5·7; N, 13·5; S, 15·4%).

The material softened, congealed and apparently re-formed crystals between 112° and 119°. At 188° a second sharp melting point occurred, with charring. It seems likely that conversion into 4-methyl-1-phenylimidazole-2-thione had occurred (m.p. 190–191° quoted by Burtles *et al.* 1925) with loss of water.

The spectrum (in water), with $\lambda_{max.}$ 237mμ (ε 17500), was different from those of the phenylthiohydantoin ($\lambda_{max.}$ 263mμ), phenylthiocarbamoylalaninol ($\lambda_{max.}$ 243mμ) and phenylthiocarbamoylalanine ($\lambda_{max.}$ 244mμ).

Conversion of 5-hydroxy-4-methyl-1-phenylimidazolidine-2-thione into 4-methyl-1-phenylimidazole-2-thione. A 420mg. sample of the hydroxyimidazolidine-2-thione was dissolved with warming in 25ml. of water, and 25ml. of cold N-HCl was added. After 72hr. at room temperature, a mass of leafy crystals filled the solution. They were filtered, washed briefly with water and dried, m.p. 192°. 4-Methyl-1-phenylimidazole-2-thione melts at 190–191° (Burtles *et al.* 1925). The yield was 325mg. (86%) (Found: C, 62·7; H, 5·6; N, 14·7; $C_{10}H_{10}N_2S$ requires: C, 63·1; H, 5·3; N, 14·7%). The spectrum (in water) had $\lambda_{max.}$ at 242mμ and 265–266mμ, of similar intensity (Fig. 1).

5-Hydroxy-4,4′-dimethyl-1-phenylimidazolidine-2-thione. 5,5′-Dimethyl-3-phenyl-2-thiohydantoin (1g.) was dissolved in 10ml. of diglyme, and 10ml. of fresh aq. 4% (w/v) $NaBH_4$ solution was added. A white precipitate appeared, and effervescence began. After several hours at 37°, 30ml. of 5% (v/v) acetic acid was added, and the precipitated boric acid was filtered off. The solution (the pH of which was 4·7) was boiled down to about 15ml. On cooling, a mass of white crystals appeared (m.p. 180–182°). On recrystallizing from aq. 20% (v/v) diglyme, adjusted to pH10 with a little Na_2CO_3 solution, the melting point was unchanged. The yield was 400mg. (>40%), $\lambda_{max.}$ 238mμ (ε 14800). The material did not migrate at pH7·0 on electrophoresis in 0·05M-sodium acetate (Found: C, 59·7; H, 6·2; N, 12·6; S, 14·2; $C_{11}H_{12}N_2OS$ requires: C, 59·4; H, 6·3; N, 12·6; S, 14·4%).

Other preparations. Tables 1 and 2 list the thiohydantoins reduced and the imidazole-2-thiones formed in experiments from which pure and crystalline products were obtained. Other experiments were performed with pure thiohydantoins from which no attempt was made to isolate crystalline products, but which yielded spectrophotometric, electrophoretic and chromatographic evidence that reduction to a 4(5)-hydroxyimidazolidine-2-thione and subsequent elimination of water to an imidazole-2-thione had occurred. These thiohydantoins were: the 3-phenyl-2-thiohydantoins of tyrosine, histidine, threonine and proline; the 3-methyl-2-thiohydantoin of proline; 5-methyl-2-thiohydantoin; 2-thiohydantoin itself.

Yields in Tables 1 and 2 are of the pure material, recrystallized to constant melting point. Melting points are uncorrected. All analyses were by Weiler and Strauss, Oxford.

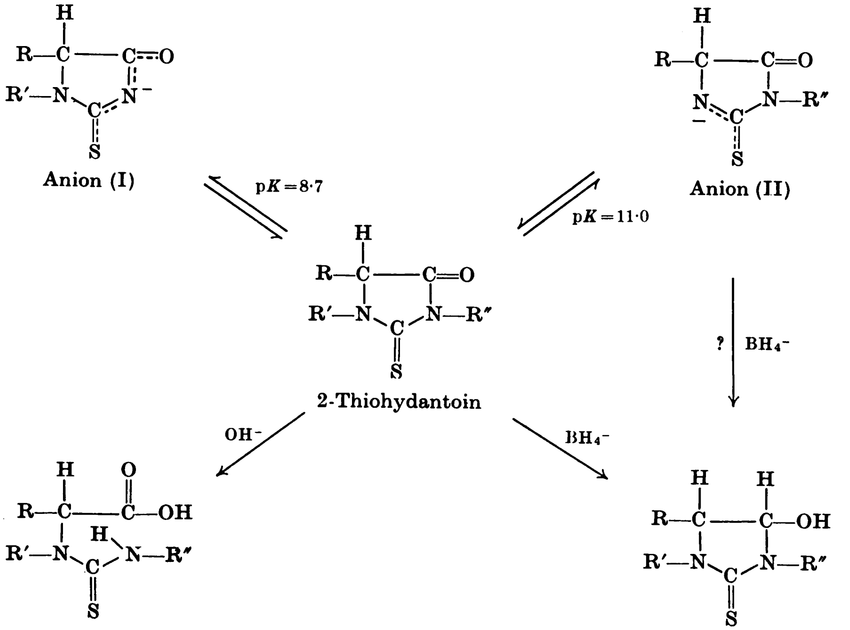
Ultraviolet spectra (in water). The ultraviolet spectra of thiohydantoins have been discussed (see, e.g., Edward & Nielsen, 1957*a*,*b*). There is less information on the ultraviolet spectra of imidazole-2-thiones, with the exception of those monosubstituted at C-4(5), to which class ergothioneine belongs. The spectrum of 5-isobutylimidazole-2-thione is similar to that of ergothioneine (Heath & Toennies, 1958). The spectra of imidazole-2-thiones substituted with 1-methyl, 1,3-dimethyl, 1,4-dimethyl and 1-methyl-3,4-trimethylene (not obtained crystalline) groups are similar in shape to the 4(5)-monosubstituted imidazole-2-thiones with single well-marked peaks in the region 251–258mμ (ε approx. 14000). 1-Methyl-4-phenylimidazole-2-thione has an extra band at 285–290mμ (ε 19000) in addition to that at 265mμ (shoulder) (ε 15200).

The spectra of 1-phenyl- and 4-alkyl-1-phenylimidazole-2-thiones (4-carboxypropyl and 4-methyl) are qualitatively identical, being relatively flat in the region 230–270mμ with ε about 10000. The spectrum of 4-D-*arabino*-tetrahydroxybutyl-1-tolylimidazole-2-thione (Hüber, Schier & Druey, 1960) (a gift of Dr G. Hüber) is of this type.

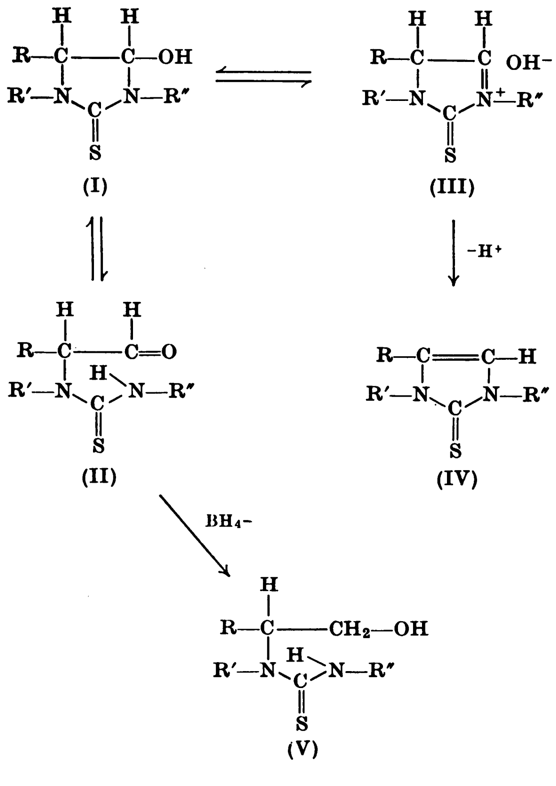
DISCUSSION

Reduction of 2-thiohydantoin by borohydride. Two side reactions are possible: (*a*) hydrolysis of the 2-thiohydantoin to thiohydantoic acid by OH^- (Scheme 2); (*b*) over-reduction to the thiocarbamoyl alcohol (V) (Scheme 3).

Reaction (*a*) takes place in alkaline aqueous solution and is most rapid when R″ and R′ are not hydrogen. If R″ or R′ is hydrogen, the thiohydantoin can ionize, the anion being resistant to attack by OH^- (Edward & Nielsen, 1957*a*,*b*). By



Scheme 2.



Scheme 3.

analogy, it would be expected to diminish interaction with the BH_4^- ion.

Reduction of the mesomeric anion (I) (Scheme 2) should be hindered because of the consequent loss of the resonance energy of the anion.

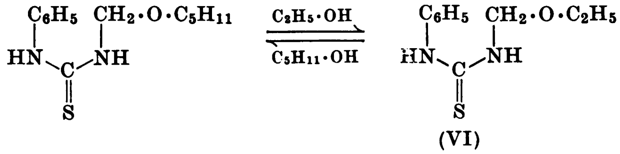
Thiohydantoins giving rise to anion (I) (Scheme 2) are able to do so in the alkaline aqueous solutions of sodium borohydride because of the low pK_a of the N-3-hydrogen, and are therefore not reduced. Anion (II) (Scheme 2) is less readily formed than anion (I) (Edward & Nielsen, 1957*a*,*b*) and, since the $>$C=O group is not involved in the mesomeric anion, reduction of thiohydantoins giving rise to anion (II) by borohydride is possible in unbuffered aqueous solution.

Hydrolysis by OH^- and the formation of anions (I) and (II) may both be decreased or eliminated by carrying out the reduction in non-aqueous solvents, in this case dioxan or diglyme. The rate of reduction by sodium borohydride is much less in diglyme or dioxan than in water, but the more strongly reducing lithium borohydride may be used with advantage instead.

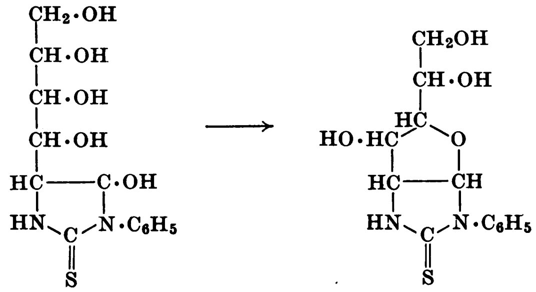
Over-reduction takes place in the presence of a large excess of borohydride. Phenylthiocarbamoylalaninol and phenythiocarbamoylprolinol have been isolated in up to 25% yield by the over-reduction of the appropriate 3-phenyl-2-thiohydantoin. Over-reduction can be avoided by following the reaction spectrophotometrically. It is slow in comparison with the reduction of the thiohydantoin, presumably

Reaction (1) $R_2N{\cdot}CH_2{\cdot}OH + R'{\cdot}OH \longrightarrow R_2N{\cdot}CH_2{\cdot}O{\cdot}R' + H_2O$

Reaction (2)



Reaction (3)



reflecting the small proportion of 4(5)-hydroxyimidazolidine-2-thione in the form of the tautomeric α-thiocarbamoylaldehyde.

The reduction of the carbonyl group of 2-thiohydantoins by sodium borohydride is noteworthy, since amides, imides and esters are not usually reduced, though lactones are (Gaylord, 1956, p. 101). Several thioureas showed no reaction (spectrophotometrically) when treated with sodium borohydride.

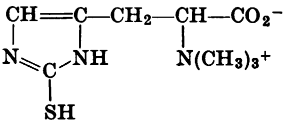
The ready reduction of 2-thiohydantoins reopens the question of the reducibility of hydantoins, which are said to be resistant to sodium borohydride (Gaylord, 1956, p. 634). Presumably, the correct choice of reducing agent and non-aqueous solvent, in accord with the preceding discussion, would permit reduction to take place. The presence of substituents on N-1 and N-3 would be important to the ease of reduction, because of their influence on the formation of anions by the hydantoins.

Pseudo-basic properties of 4(5)*-hydroxyimidazolidine*-2*-thiones*. As defined by Hantzsch (see Béke, 1963), pseudo-bases are carbinols giving salts with acids, with the elimination of water and a change of constitution. Gadamer (quoted by Béke, 1963) postulated that pseudo-ammonium bases are a tautomeric system of amino aldehyde (II), carbinolamine (I) and quaternary ammonium hydroxide (III) (Scheme 3). 4(5)-Hydroxyimidazoline-2-thiones appear to satisfy all these requirements.

The reduction of 5-hydroxy-4-methyl-1-phenylimidazolidine-2-thione by sodium borohydride to phenylthiocarbamoylalaninol (V) is evidence of the existence of the amino aldehyde (II), and the electrophoretic behaviour and spectrophotometric properties of the sugar derivatives (Scott, 1964) are best explicable on the basis of the carbinolamine form (I). The elimination of water in acid can be regarded as a further manifestation of pseudo-basic properties, presumably via the quaternary hydroxide (III). The mesomeric cation (III) by the loss of a proton and a redistribution of electrons could then be converted into the imidazole-2-thione (Scheme 3). With 5-hydroxy-4,4′-dimethyl-1-phenylimidazolidine-2-thione, the quaternary ammonium ion cannot lose a proton to form a neutral stable molecule, and the hydroxyimidazolidinethione is therefore stable in the mildly acid conditions in which imidazole-2-thiones are formed from other compounds of this type.

Ether-formation and ether-exchange reactions, in which alkoxy groups are interchanged freely and easily with alcohols, are characteristic of pseudobases (reaction 1). McLeod & Robinson (1921) demonstrated similar exchanges by thiol compounds. Johnson & Guest (1910) demonstrated the remarkable ease of exchange of alkoxy groups in substituted thioureas that occurred on dissolution at room temperature in the appropriate solvent alcohol (reaction 2). Compounds of type (VI) are formally very closely similar to the 4(5)-hydroxyimidazoline-2-thiones, which undergo a similar reaction (reaction 3; J. E. Scott, unpublished work) in equally mild conditions.

Ergothioneine. For several decades ergothioneine was a subject of outstanding interest. It occurs in many tissues, in plants and in animals (Melville, 1959). Despite considerable research, no clear function has been demonstrated for it, though various activities have been ascribed to it. In view of the remarkable ease with which 4(5)-hydroxyimidazolidine-2-thiones are converted into imidazole-2-thiones in acid conditions similar to those in which ergothioneine has usually been isolated from biological materials, one may ask



Ergothioneine

whether ergothioneine might not be an artifact. A precursor 4(5)-hydroxyimidazolidine-2-thione would be reactive chemically, because of its pseudobasic properties, and might occur bound through the 4(5)-position.

Some consequences of this possibility deserve discussion. In the form of the 4(5)-hydroxyimidazolidine-2-thione, whether free or bound, an ergothioneine precursor would probably not give the typical Hunter (1928) reaction, which takes place in alkaline medium and which depends on the presence of an imidazole-2-thione. However, an assay based on the elimination of trimethylamine in alkaline conditions (Jocelyn, 1958) would be a valid method of estimating both ergothioneine and its hypothetical precursor. It is thus of interest that Jocelyn (1958) found about 60% of blood ergothioneine to be 'bound' to the plasma proteins by using this method, in contrast with other workers (see Melville, 1959), who found ergothioneine only in the erythrocytes. These findings, however, were based on the Hunter reaction. The existence of a precursor 4(5)-hydroxyimidazolidine-2-thione could explain the discrepancy between the results, and could also suggest a mechanism for the binding to protein. Melville (1959) expressed reservations about the specificity of Jocelyn's (1958) method, but in the light of the above discussion the results should be further investigated. Observations in the literature suggest that ergothioneine is often quite strongly bound to tissue elements. In the standard method of Melville & Lubschez (1953), ergothioneine is not measurable in blood-cell supernatants deproteinized with trichloroacetic acid unless dithionite and glutathione are added before protein precipitation. Pace (1964) isolated ergothioneine from interstitial cells of foetal gonads of horses after prolonged acid hydrolysis. It is suggested that the existence of a covalent bond (possibly via the 4-position of the hypothetical imidazolidine-2-thione precursor) is a reasonable alternative to the purely physical binding of ergothioneine implicit in the usual discussions.

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