The Oxidation of Thiamine and its Analogues with Potassium Ferricyanide.

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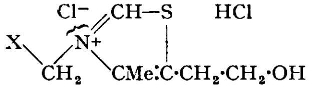
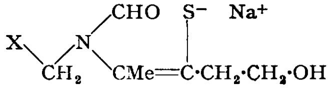
Study of the oxidation of thiamine and its analogues with potassium ferricyanide confirms its free-radical mechanism, by way of dihydrothiochrome (cf. Sykes and Todd, *J.*, 1951, 534).

Sykes and Todd (*J.*, 1951, 534) suggested that oxidation of thiamine chloride hydrochloride (I; R = NH_2) with potassium ferricyanide to thiochrome proceeded through the autoxidisable intermediate, dihydrothiochrome. The initiating reaction was considered to be the one-electron change from the sodium salt of the " open chain " form (II; R = NH_2) to the free thiol radical (III; R = NH_2), which then gave the cyclic radical (IV).

Confirmation for the one-electron oxidation step was sought by investigating the action of potassium ferricyanide on thiamine analogues such as 4-hydroxythiamine (I; R = OH) and *N*-methylthiamine (I; R = NHMe) (Nesbitt and Sykes, *J.*, 1954, 3057). It was found that both these compounds were converted into the corresponding disulphides (V; R = OH and NHMe, respectively).


(I)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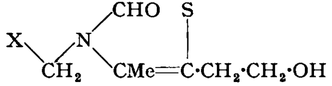
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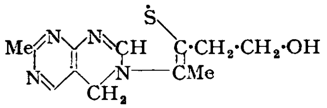
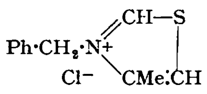
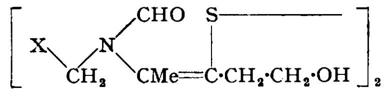
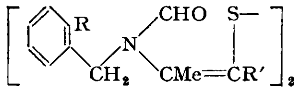
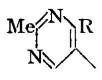



(VI)


(V)


(VII)

X =


Oxidation of the salts (II; R = OH and NHMe, respectively) obtained by the action of alkali on the thiazolium compounds (I) would yield the free thiol radicals (III), as with thiamine itself; but since no cyclisation, as in the formation of (IV), is possible, the free radicals should yield the disulphides (V); this is, in fact, observed. It has now been found that thiamine (I; R = NH_2), itself, with potassium ferricyanide gives analogously a small amount of the disulphide (V; $R = NH_2$) alongside the known major product, thiochrome; the amount of disulphide obtained is more than that produced when a similar alkaline solution of thiamine, containing no ferricyanide, is kept in the air for the same length of time.

The normal oxidation of thiamine to the disulphide (V; $R = NH_2$) by air (very slow), iodine, hydrogen peroxide, etc., is a two-electron process which is initiated by the conversion of RS^- (II; $R = NH_2$) into RS^+, followed by its reaction with more RS^- to form RS·SR. The ease with which thiazolium compounds (I) undergo this oxidation is in the order $R = NH_2 > OH > NHMe$. In the last case, however, part of the explanation probably lies in the fact that ring fission by alkali to form the salt (II; R = NHMe) is slow, whereas in the other two cases it is virtually instantaneous.

The oxidation of simple benzylthiazolium analogues (*e.g.*, VI) with ferricyanide follows the same course as with two-electron oxidising agents, the disulphides (VII; $R' = R'' = H$; $R = NO_2$; $R' = H$; and $R = NO_2$, $R' = CH_2{\cdot}CH_2{\cdot}OH$) being formed, there being no reaction other than dimerisation open to the intermediate free thiol radicals. The action of potassium ferricyanide on (VI) leads to the precipitation of a crystalline thiazolium ferricyanide complex, $R_3Fe(CN)_6$, which is decomposed by alkali to yield the disulphide (VII; R = R′ = H). *N*-Methylthiamine (I; R = NHMe) gives a similar but very much more stable complex.

Confirmation was also sought for formation of the autoxidisable intermediate, dihydrothiochrome, in the ferricyanide oxidation of thiamine. Assays on a standard solution of thiamine (1·75 μg./ml.) were carried out by the usual thiochrome method and, as expected, quantitative results were obtained if two equivalents of ferricyanide were used. If only one equivalent was used, quantitative results were still obtained (although two equivalents are stoicheiometrically required for the conversion of thiamine into thiochrome) provided that free access of air was allowed; if air was excluded, however, the assay dropped to approximately 50%. The formation of an autoxidisable intermediate is thus confirmed.

EXPERIMENTAL

Ferricyanide Oxidation of 4-*Methyl*-3-2′-*nitrobenzylthiazolium Chloride*.—An aqueous solution of the thiazolium chloride (1·35 g.) was made alkaline with N-sodium hydroxide (10 ml., 2 equiv.), and 5% aqueous potassium ferricyanide (33 ml., 1 equiv.) was added dropwise. A sticky solid separated, which crystallised; it had m. p. 115° alone or mixed with di-[2-(*N*-2′-nitrobenzylformamido)prop-1-enyl] disulphide (yield, 71%) (cf. preceding paper).

Similar oxidation of 5-2′-hydroxyethyl-4-methyl-3-2′-nitrobenzylthiazolium chloride (Livermore and Sealock, *J. Biol. Chem.*, 1947, **167**, 699) yielded the corresponding disulphide, m. p. 110° (decomp.). Oxidation of 3-benzyl-4-methylthiazolium chloride also yields the disulphide, but, if ferricyanide without alkali is added, a crystalline *complex* separates as colourless plates, m. p. 56° [Found: C, 51·0; H, 5·5; N, 13·6. $(C_{11}H_{12}NS)_3Fe(CN)_6,8H_2O$ requires C, 50·6; H, 5·6; N, 13·6%]. Addition of alkali causes this to redissolve, with precipitation of the disulphide, m. p. 98° (Sykes and Todd, *J.*, 1951, 534).

Ferricyanide Oxidation of N-*Methylthiamine*.—Sodium hydroxide solution (10%; 10 ml.) followed by N-potassium ferricyanide solution (15 ml.), was added to aqueous *N*-methylthiamine chloride hydrochloride (Nesbitt and Sykes, *J.*, 1954, 3057) (0·99 g. in 10 ml.). The solution was extracted with butanol (25 ml.) after 5 min. and subsequently at 10-min. intervals for 2 hr. Evaporation of the extracts followed by recrystallisation from methanol–acetone yielded *N*-methylthiamine disulphide (*loc. cit.*), m. p. and mixed m. p. 156° (0·21 g., 25%). The aqueous solution was acidified with 10% hydrochloric acid and freeze-dried, the residue was extracted with boiling dry methanol, and the solution filtered to remove sodium chloride. The methanol extract was evaporated, and the resultant residue dissolved in water, brought to pH 5 (Na_2CO_3), and again freeze-dried. Extraction of the residue with butanol, followed by recrystallisation from methanol–ether yielded *N*-methylthiamine chloride hydrochloride (0·4 g., 40%). Paper chromatography of the alkaline oxidation solution with saturated aqueous butanol as solvent showed initially only one spot (on the starting line; ferricyanide complex) which absorbed ultra-violet light.

Ferricyanide Oxidation of 4-*Hydroxythiamine*.—Similar oxidation of 4-hydroxythiamine chloride hydrochloride with ferricyanide (2·2 equiv.) in alkali (3 equivs.) yielded the disulphide (*loc. cit.*) rapidly (extraction after 15 min.) and in high yield ($>75\%$). Paper chromatography of the oxidation solution after 1 hr., with saturated aqueous butanol as solvent and examination in ultra-violet light, disclosed the disulphide (V; R = OH) (R_F 0·22) as sole absorbent spot (except for the ferrocyanide–ferricyanide spot at the base line). Addition of hydrochloric acid (3 equivs.) before chromatography led to the disulphide hydrochloride (R_F 0·18) as sole moving absorbent spot. A similar experiment but with the ferricyanide omitted gave the sodium salt (II; R = OH) (R_F 0·14) of the thiol as sole absorbent spot, or after addition of hydrochloric acid gave only hydroxythiamine chloride hydrochloride (I; R = OH) (R_F 0·07). Control experiments confirmed the R_F quoted.

Ferricyanide Oxidation of Thiamine.—Oxidation of thiamine with subsequent chromatography, the whole procedure being as in the previous experiments, gave absorbent spots for ferrocyanide–ferricyanide (at base line), the sodium salt of the thiol (R_F 0·14), thiochrome (R_F 0·37), and the disulphide (V; R = NH_2) (R_F 0·48). After addition of hydrochloric acid the spots for the bases were replaced by those for the disulphide hydrochloride (R_F 0·22) and thiochrome hydrochloride (R_F 0·28). If the ferricyanide was omitted from the experiments, spots were obtained for the thiol sodium salt and disulphide, or, after addition of acid, for the disulphide hydrochloride and thiamine chloride hydrochloride (R_F 0·085). Control experiments confirmed the R_F quoted.

Elution of the thiochrome spot in the original experiment with *iso*butanol, followed by dilution and fluorimetric estimation of the solution, indicated *ca.* 90% yield of thiochrome.

Oxidations of thiamine solutions (1·75 µg./ml.), and subsequent fluorimetric estimations of the thiochrome produced, were carried out, all in 5 replicates with varying amounts of ferricyanide. The apparent thiamine contents of the resultant solutions (µg./ml.) derived from the amounts of thiochrome produced were as follows for the amounts of ferricyanide stated: excess, 1·74—1·76; 2 equiv., 1·74—1·77; 1 equiv. (air excluded), 0·89—0·94; 1 equiv. (free access of air), 1·74—1·77.

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