# CCCII.—*Colour Reactions of Thiolglyoxalines (Thioliminazoles) with Sodium Diazobenzene-p-sulphonate.*

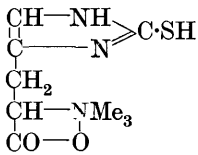
By GEORGE HUNTER.

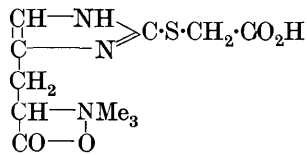
THE author (*Biochem. J.*, 1928, **22**, 4) recently described for ergothioneine a test which depends upon coupling this substance with sodium diazobenzene-*p*-sulphonate in a weakly alkaline aqueous medium and subsequent addition of concentrated alkali. The substance couples very rapidly with production of a lemon-yellow colour which changes gradually in the presence of concentrated alkali to a beautiful red with a purple tinge. If the yellow solution be kept too long before the addition of concentrated alkali, no intensification of colour is obtained, indicating that the product of reaction of ergothioneine and sodium diazobenzene-*p*-sulphonate is very unstable, and that the characteristic colour increase on the addition of concentrated alkali probably depends on a tautomeric change. It is remarkable that under the conditions specified for the performance of the test the change takes place in a quantitative manner, as the colours produced (and measured by a Duboscq colorimeter) are linearly proportional to the amounts of ergothioneine used. The test is also extremely sensitive, as a colour is still perceptible with ergothioneine at a dilution of one in five millions.

Subsequent experience, here reported, with the test shows that it is given not only by ergothioneine but by a number of other thiolglyoxalines (not found in biological material) under certain conditions of substitution. A thiolglyoxaline in order to respond to the test must be capable of coupling with the diazo-reagent, the conditions for which are probably similar to those postulated for glyoxalines by Fargher and Pyman (J., 1919, **115**, 217): ". . . that

glyoxalines, in order to be capable of coupling, must contain a free imino-group and also a hydrogen atom, or some other displaceable group, such as the carboxyl group, in one of the 2-, 4-, or 5-positions." Since the 2-position is occupied in the thiolglyoxalines here examined, the 4- or 5-position (or both) must remain open to permit of the formation of an azo-compound. And if the case is analogous to that of the glyoxalines, only *C*-azo-compounds will be formed. Besides these conditions the glyoxaline ring must contain a free thiol group.

The thiolglyoxalines tested were ergothioneine (I); acetic acid derivative of ergothioneine (II); thiolurocanic acid (III); acetic acid derivative of thiolurocanic acid (IV); 2-thiol-4(or 5)-methylglyoxaline-5(or 4)-carboxylic acid (V); 2-thiol-5(or 4)-carbethoxy-4(or 5)-methylglyoxaline (VI); 2-ethylthiol-5(or 4)-carbethoxy-4(or 5)-methylglyoxaline (VII); and 2-thiol-4(or 5)-aminomethylglyoxaline (VIII).


(I.)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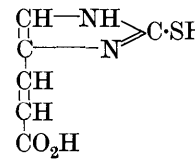
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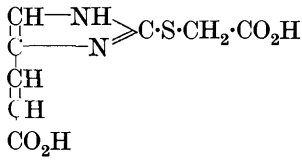
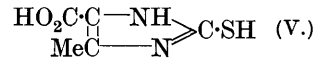
(IV.) 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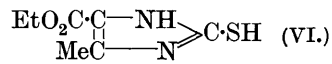
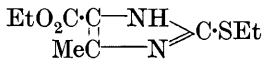
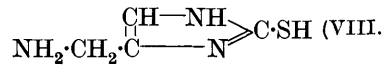

(VI.)

(VII.) 


(VIII.)

## EXPERIMENTAL.

The test was given by ergothioneine (I), thiolurocanic acid (III), 2-thiol-4(or 5)-methylglyoxaline-5(or 4)-carboxylic acid (V), and 2-thiol-4(or 5)-aminomethylglyoxaline (VIII). In all of these there is a free imino-group, and a free hydrogen atom or a displaceable carboxyl group in position 4 or 5. There is also a free thiol group in position 2. When the hydrogen of the thiol group is substituted, as in the acetic acid derivative of ergothioneine (II), in the acetic acid derivative of thiolurocanic acid (IV), and in the 2-ethylthiol compound (VII), the test is negative. Blocking the thiol group is of itself sufficient to render the test negative, as shown by the fact that the acetic acid derivative of ergothioneine (II) and the acetic

acid derivative of thiolurocanic acid (IV) respond negatively to the test although the conditions required by Fargher and Pyman for coupling are fulfilled. These substances indeed give a characteristic glyoxaline test by the Pauly method (*Z. physiol. Chem.*, 1904, **42**, 508), or by the Koessler and Hanke method (*J. Biol. Chem.*, 1919, **39**, 497), which gives results in parallel with the Pauly test but allows of the production of purer colours. Glyoxalines of course partly couple under the conditions of the author's test, but their speed of coupling is apparently much less than in the case of the thiolglyoxalines. For this reason little colour develops in the short time allowed in the weakly alkaline medium and the addition of concentrated alkali practically completely inhibits further coupling.

As is to be expected, there is no coupling in the case of 2-ethylthiol-5(or 4)-carbethoxy-4(or 5)-methylglyoxaline (VII), and no colour is produced in the Pauly or in the author's test. In the case of 2-thiol-5(or 4)-carbethoxy-4(or 5)-methylglyoxaline (VI), in relatively large amount (about 1 mg.), there is a slight yellow colour obtained by the glyoxaline tests, probably through an effect similar to that produced by aliphatic thiol groups on diazo-compounds.

Pyman and his associates in numerous papers in this Journal, and various other workers, have established that, when glyoxalines couple with sodium diazobenzene-*p*-sulphonate in sodium carbonate solution, they give rise to colours predominantly red. The colour hue, varying from orange to purplish-red, is dependent on the character of the side chain(s) on the glyoxaline nucleus (Koessler and Hanke). In no instance, among the substances with a free thiol group here examined, is a red colour obtained by the Pauly or the Koessler and Hanke test. The thiolglyoxalines giving the author's test all yield clear yellow colours by the glyoxaline tests. It thus follows that the glyoxaline nucleus, if it has a free thiol group in the 2-position, is not detectable by the Pauly test, whether the glyoxaline is capable of coupling or not. Only when the hydrogen of the thiol group is replaced by an alkyl, or presumably by an aryl group, will the coupled product yield a red colour indicative of the presence of the glyoxaline nucleus.

As with the glyoxaline test, the author's test for thiolglyoxalines gives colours predominantly red but varying with the character of the side chain in the thiolglyoxaline nucleus. Ergothioneine (I) and thiolurocanic acid (III) give practically identical colours—matching a solution of phenolsulphonephthalein at $p_H$ 8·0. Indeed it is probable that in the course of the test thiolurocanic acid is formed from ergothioneine by the action of the concentrated alkali, as trimethylamine is always perceptible by smell (see also Barger and Ewins, J., 1911, **99**, 2336). The colours obtained from

2-thiol-4(or 5)-methylglyoxaline-5(or 4)-carboxylic acid (V) and from 2-thiol-4(or 5)-aminomethylglyoxaline (VIII) are also very similar to each other, of a pinkish-orange hue resembling that of a dilute solution of sodium alizarinsulphonate at the neutral point. These colours are much less blue than that from ergothioneine.

*Summary.*

The test previously described by the author for ergothioneine is a general one for the thiolglyoxaline ring provided it contains a free thiol group in the 2-position and has at least one of the 4- and 5-positions open or containing a displaceable carboxyl group. A free iminic hydrogen may also be necessary.

It is also shown that the presence of a thiol group in the 2-position alters the reaction of the glyoxaline ring with diazo-compounds in such a way that its presence in solution can no longer be detected by the glyoxaline diazo-tests. That the thiol hydrogen plays a part in this interference, and is responsible for the course of the reaction in the author's test, is made evident.

I am much indebted to Dr. Blythe A. Eagles for small samples of thiolurocanic acid, its acetic acid derivative, and that of ergothioneine, which substances were prepared by him in Professor Treat B. Johnson's laboratory at Yale; to Dr. Harold King of London for specimens of 2-thiol-4(or 5)-methylglyoxaline-5(or 4)-carboxylic acid, 2-thiol-5(or 4)-carbethoxy-4(or 5)-methylglyoxaline, and 2-ethylthiol-5(or 4)-carbethoxy-4(or 5)-methylglyoxaline; and to Professor F. L. Pyman for 2-thiol-4(or 5)-aminomethylglyoxaline.

Dr. Eagles has also informed me that he has applied my test to thiohydantoin, thiotyrosine, and thiophenol, with negative results. This is valuable contributory evidence of the high specificity for the thiolglyoxaline ring which the test appears to possess.

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