I. A NEW TEST FOR ERGOTHIONEINE UPON WHICH IS BASED A METHOD FOR ITS ESTIMATION IN SIMPLE SOLUTION AND IN BLOOD-FILTRATES.

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INTRODUCTION.

TANRET [1909] isolated from ergot of rye a base which he termed ergothioneine, which was shown by Barger and Ewins [1911] to be the betaine of thiolhistidine. There is no further record of the occurrence of ergothioneine in the plant or animal kingdom until the isolation of a substance from blood by Hunter and Eagles [1925, 1927], which has recently been shown by Eagles and Johnson [1927] to be identical with ergothioneine. The isolation of a similar substance from blood was also reported by Benedict, Newton and Behre [1926] and that substance has also been shown to be ergothioneine [Eagles and Johnson, 1927; Newton, Benedict and Dakin, 1927].

Two delicate colour tests for ergothioneine have been discovered in the course of recent work in this laboratory. One of these is that ergothioneine gives a blue colour in alkaline solution with the special phosphotungstic acid reagent used for the estimation of uric acid, but the test is not as specific as might be desired, as both uric acid and reduced glutathione also give a blue colour with this reagent. With suitable fractionation of tissue extracts however this test may yet prove of value for quantitative purposes.

The new test to be described for ergothioneine is a special modification of the well-known diazo-reaction.

STANDARDS AND SOLUTIONS FOR THE TEST.

1. *Ergothioneine standard.* An aqueous solution of ergothioneine saturated with chloroform and containing 1 mg. in 1 cc. remains unchanged for at least one month if stored in an ice-chest. From this, convenient standards may be prepared containing 0·005–0·050 mg. in 2 cc.

2. *Artificial standard of phenolsulphonephthalein.* (*Phenol red*[1].)

(*a*) *Stock.* 0·10 g. of pure vacuum-dried phenol red is dissolved in 5·7 cc. $M/20$ NaOH in a 100 cc. volumetric flask and water added to the mark.

(*b*) *Standard.* 0·40 cc. of the stock solution is measured into a 100 cc. volumetric flask and diluted to the 100 cc. mark with a buffer solution at p_H 8·0, consisting of 50 cc. $M/5$ H_3BO_3 in $M/5$ KCl, and 3·97 cc. $M/5$ NaOH diluted to 200 cc. with water, according to Clark [1920].

The colour of this artificial standard is indistinguishable in daylight in a Duboscq colorimeter from that developed in the test to be described. A test solution (see below) having the same depth of colour as this standard contains 0·015 mg. of ergothioneine.

3. *The diazo-reagent* is made as follows. In a 50 cc. volumetric flask, immersed in running water under 10°, or cooled by ice, are placed 1·5 cc. of a solution containing 9 g. sulphanilic acid and 90 cc. of 37 % hydrochloric acid per litre, followed by 1·5 cc. of 5 % sodium nitrite. The mixture is allowed to stand for 5 minutes, then a further 6 cc. of the nitrite solution is added. At the end of another 5 minutes cold water is added to the mark and the contents are mixed. When kept cold the reagent is good for 2–3 days, but the development of any tinge of colour is an indication that a new reagent should be made.

4. *Sodium carbonate in sodium acetate.* 1 g. anhydrous sodium carbonate and 10 g. anhydrous sodium acetate made up to 100 cc. with water.

5. 10 *N sodium hydroxide,* from which carbonate has been allowed to settle.

THE TEST AS PERFORMED FOR QUALITATIVE AND QUANTITATIVE PURPOSES.

1 cc. of the diazo-reagent is delivered into a test-tube. The time is noted, then 0·5 cc. of the carbonate-acetate solution is added, followed after 15 seconds by 2 cc. of the solution to be tested. The contents of the tube are mixed and kept cold. A clear yellow colour develops at this stage. Thirty seconds after the test portion has been added, 2 cc. of the 10 *N* NaOH are added and the mixture is quickly shaken. A beautiful red colour with a purple tinge rapidly develops and is at a maximum after 15 minutes. The colour is stable for at least 40 minutes but on standing for several hours or overnight a purple-red precipitate forms. Very weak solutions fade after 12–24 hours, often without formation of a precipitate, and even a heavy precipitate dissolves with decomposition on longer standing in the strongly alkaline medium. A perceptible colour is obtained with 0·001 mg. ergothioneine in a volume of 5·5 cc.; the test is therefore sensitive to a dilution of 1 in 5,000,000 of ergothioneine.

[1] A very pure product was obtained from Messrs Hynson, Westcott and Dunning, Baltimore. It left no residue on ignition but it was not completely soluble in dilute sodium hydroxide. Some was accordingly purified by dissolving in alkali, filtering and reprecipitating with hydrochloric acid. The precipitate was washed free from sodium chloride and dried to constant weight *in vacuo*. The solution made up from the purified material gave a just perceptibly greater colour by the colorimeter than a corresponding solution made from the original product. The necessity for pure dyes for colorimetric purposes has already been emphasised [Hunter, 1925, 1].

Remarks on the test.

The characteristic colour of the test is obtained only by the use of strong alkali; sodium or potassium carbonate, sodium cyanide or ammonium hydroxide fails to give the colour. The red colour is changed to yellow on acidification, but returns again on making alkaline with sodium hydroxide. The purplish red precipitate obtained on standing is insoluble in water and in the common organic solvents. Its colour is changed by acids but returns again on making alkaline. An investigation of the chemical nature of this precipitate is desirable before any suggestion as to the possible mechanism of the test can be made.

The test, however, apparently depends on two main reactions. The first of these is the very rapid interaction, or coupling, of ergothioneine with the sodium salt of diazotised sulphanilic acid, with the production of the substance giving the bright yellow colour to the solution. This reaction product of ergothioneine and the diazonium compound is very unstable in an excess of weak alkali, and the second reaction, the formation of a highly coloured sodium salt in the presence of sodium hydroxide, depends on the addition of the latter under conditions where complete coupling has taken place, and at the same time, where a minimum destruction of the coupled product has occurred. The conditions of the test have thus been made as far as possible to meet those requirements.

Thus, it is assumed that the solution to be tested is neutral or very faintly acid, as the quantity of sodium carbonate recommended for use with 1 cc. of the diazo-reagent is just sufficient to leave the solution faintly alkaline after the test portion has been added. The use of sodium acetate along with the carbonate serves to buffer the slight excess of acid in blood-filtrates and leads to the production of both stronger and purer colours than sodium carbonate alone. As the alkalinity of the coupling medium is increased the instability of the coupled product also increases. Thus, for example, if 2·5 cc. of 1 % sodium carbonate are used with 1 cc. of the diazo-reagent, the ergothioneine solution added, and the mixture allowed to stand for 5 minutes, as is the usual technique when testing for iminazoles, then no red colour is obtained on the subsequent addition of strong alkali. The restriction of the coupling time to 30 seconds has the further advantage that it greatly enhances the specificity of the test for ergothioneine by the elimination of various potentially interfering substances. Among these are the iminazoles, especially carnosine, which occurs in blood and tissue extracts [Hunter, 1925, 2], and other substances such as tyrosine and the "acetone bodies." Aromatic phenols, however, couple much more rapidly and at a lower alkalinity than do the iminazoles and acetone, so that phenolic preservatives should be avoided in material to be tested for ergothioneine. Apart from these potentially interfering substances the test has a very high degree of specificity for ergothioneine. No other substance, although many have been examined, has so far been found to give the test.

In certain blood-filtrates in which the concentration of ergothioneine is very low, or the carnosine content is high, a characteristic colour may not develop. Whether the test is positive or negative may however be decided by allowing the mixture to stand overnight, when the formation of even a very slight purplish precipitate indicates the presence of ergothioneine. This precipitate appears to form at greater dilutions of ergothioneine in blood-filtrates than in corresponding simple solutions of ergothioneine, so that for qualitative purposes this aspect of the test is of importance.

On standing with the strong alkali even very dilute test solutions of ergothioneine or blood-filtrates smell of trimethylamine.

Relationships of the test.

The present test for ergothioneine was discovered when investigating a test obtained in uraemic sera by Andrewes [1924], who found that when the Van den Bergh test was applied to such sera, a yellow or buff colour developed after standing for 24 hours, when, on addition of strong alkali a red colour developed. From the fact that relatively strong solutions of ergothioneine couple in very faintly acid solutions to give a yellow colour the writer at first suspected that ergothioneine accounted for the test described by Andrewes. Subsequent work, however, with uraemic sera has now convinced him that the substance there giving the diazo-reaction is not ergothioneine.

A reaction of a different type, but also given by relatively strong solutions of ergothioneine, has been recorded by Leimdörfer [1924] as given by a substance which he obtained apparently only in small quantity from the blood. Leimdörfer's substance on micro-analysis gave the relationship C 3·5, N 1, H 7–9 which is not sufficiently near to that of these elements in ergothioneine ($C_9H_{15}O_2N_3S$) to establish an identity, especially as sulphur is also said to be absent from Leimdörfer's substance. Nevertheless, it appears very probable that Leimdörfer's base is ergothioneine, a supposition resting almost entirely on the report that when mixed with Ehrlich's diazo-reagent made alkaline with strong alkali a dark red colour slowly develops on standing at ordinary temperature, and more quickly on warming the solution. On long standing a reddish violet precipitate is formed.

It is of importance to note that the thioliminazole ring of ergothioneine does not behave towards the diazo-reagent as does the unsubstituted iminazole ring in such substances as histidine. With the Pauly method, only a yellow colour is developed from ergothioneine, a colour indeed not more red than that given by phenol. Of course, in the Pauly method the reagents in themselves develop a yellowish orange colour on standing for 5–10 minutes and this fact may account for the statement of Barger and Ewins that ergothioneine gives a red colour with this test. This point was of some significance in identifying ergothioneine prepared from blood with that prepared from ergot, and, through the kindness of Prof. Treat B. Johnson, of Yale University, the

writer has had the opportunity of testing ergothioneine prepared from ergot. The substance behaved exactly as ergothioneine prepared from blood.

It was originally stated by Hunter and Eagles [1925] that the substance gives a yellow colour in extreme dilution with the Koessler and Hanke [1919] method. The presence of an unsubstituted iminazole ring in the substance was thus rendered most improbable. When it was discovered, however, that the substance was ergothioneine, the absence of a positive Pauly or Koessler and Hanke test was easily explainable. When the sulphydryl group was removed by ferric chloride with the formation of trimethylhistidine, or, after treatment with strong alkali, by nitric acid to form urocanic acid, according to the methods of Barger and Ewins [1911], then the sulphur-free products in both cases showed the undoubted presence of the iminazole ring by both the Pauly and Koessler and Hanke tests.

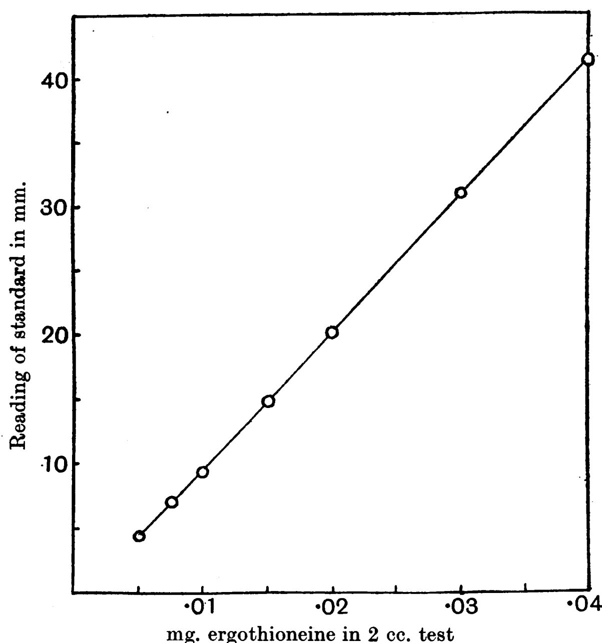

Fig. 1.

QUANTITATIVE APPLICATIONS OF THE TEST.

(*a*) *In simple solutions of ergothioneine.* For quantitative purposes the test is performed as has been described. The colours obtained in test solutions are conveniently matched against standards made from known amounts of ergothioneine, or they may be matched, preferably in daylight, against the phenol red standard described.

The test solution, after the colour has fully developed, is transferred to the left cup of the Duboscq colorimeter and its plunger is set at 15 mm. The right cup is partly filled with the standard and the plunger in this cup is

moved until the colour of the standard matches that given by the test. The readings obtained with the phenol red standard when the test was set constantly at 15 mm., from different concentrations of ergothioneine, in the range of 0·005–0·040 mg., are given in Fig. 1.

It is seen from Fig. 1 that the depth of colour produced in the test solution is a straight line function of the amount of ergothioneine in it. The slope of this line is 1·06 and the number of mg. of ergothioneine in the test solution can be calculated from the equation

$$\text{ergothioneine in mg.} = \frac{\text{reading of standard in mm.} + 0{\cdot}9}{1060}.$$

(*b*) *In blood-filtrates.* Protein-free blood-filtrates made according to the tungstic acid method of Folin and Wu [1919] have been found to be suitable for the test in all types of blood so far examined. By this method whole blood is precipitated at 1 in 10 dilution and corpuscles generally at 1 in 20 dilution. The 2 cc. portion used for the test thus represents 0·2 cc. of whole blood and 0·1 cc. of corpuscles. In many bloods this is a sufficiently large test-portion from which to obtain colours suitable for reading, and indeed in some pig bloods a fifth of those amounts may be sufficient. In many other bloods, however, the 2 cc. test-portion is insufficient to permit of satisfactory readings on account of their low concentration of ergothioneine. By decreasing the dilution in the precipitation of the protein, this difficulty may be overcome.

Estimations of ergothioneine of a preliminary nature have been carried out on a number of bloods from various animals. The values reported in Table I are given as mg. of ergothioneine in 100 cc. of corpuscles as the substance is confined almost entirely to the corpuscles. The highest accuracy is not yet claimed for the quantitative application of the test, especially where the amount of ergothioneine in the blood is less than 5 mg. per 100 cc. But there is no doubt of the substantial correctness of the values given in Table I as may be seen from the following considerations. In many pig blood-filtrates the proportionality of colour obtained in the test is as good as that obtained in simple solutions of ergothioneine. Ergothioneine added under these circumstances is quantitatively determinable by the method. Further evidence will be given on this point in a forthcoming paper. The addition of ergothioneine representing as little as 2 mg. per 100 cc. of blood is qualitatively recognisable by the test, so that the absence of an appreciable colour in ox blood-filtrates is due to the very small amount of ergothioneine in ox bloods and not to the presence of substances inhibiting the development of colour in the test. The amount of aromatic phenols in blood is negligible when the ergothioneine is more than 5 mg. per 100 cc., but below this value the colour given by the ergothioneine is not quite pure, owing probably to the combined interference of small amounts of phenols and iminazoles.

Of the bloods reported in Table I that from pig has an almost constantly high content, those from ox and sheep a constantly low content of ergothioneine. The amount of ergothioneine in blood even of the same species,

however, varies over a wide range. The amount in human corpuscles varies from about 2–10 mg. per 100 cc. No blood so far examined has been found to be entirely free from ergothioneine.

Table I. *Ergothioneine in blood to nearest mg. per* 100 *cc. corpuscles.*

Normal men	(9)	2·5; 3; 5; 8; 3; 7; 5; 3; 4
Pig	(12)	28; 21; 20; 20; 30; 26; 5; 43; 31; 4; 53; 47
Ox	(10)	All < 2 mg.
Rabbit	(4)	5·5; 7; 9; 2
Guinea-pig	(5)	2; 6; 4·5; 8; 3
Fowl	(12)	3 to 8. Two over 20
Cat	(2)	< 1
Dog	(1)	5·5

Few normal tissue constituents are subject to the variations here indicated for ergothioneine, but it is interesting to note that a similar variation has been recorded for carnosine [Hunter, 1922] which contains, in common with ergothioneine, the iminazole ring.

SUMMARY.

1. A new and apparently highly specific colour test, depending on the use of diazotised sulphanilic acid, has been described, by which it is possible to detect ergothioneine in a dilution of more than one in five millions.

2. A method for the quantitative estimation of ergothioneine in simple solution and in protein-free blood-filtrates has been based upon the test.

3. The ergothioneine contents of a number of human and animal bloods are recorded.

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