The Distribution of Ergothioneine in Blood as Determined by a New Method of Estimation

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Ergothioneine is usually determined in biological media by measuring the intensity of the red colour which develops when diazotized sulphanilic acid and strong alkali are added to a deproteinized solution (Hunter, 1928). Other methods which have been used lack specificity or fail to give quantitative recoveries of added ergothioneine (e.g. those of Salt, 1931; Sullivan & Hess, 1933; Touster, 1951). Hunter's method, however, is itself unsatisfactory since other compounds present in blood and tissue either inhibit the development of the red colour (e.g. glutathione and cysteine) or mask it by combining with the reagent to produce differently coloured complexes (e.g. phenols, histidine, uric acid). Moreover, the same red colour is given by thiolurocanic acid and other thiolimidazoles (Lawson, Morley & Woolf, 1951).

In consequence, several modifications of Hunter's method have been proposed. These include coprecipitation with various reagents, either of the ergothioneine (Lawson, Morley & Woolf, 1950) or of the interfering substances (Hunter, 1949). Techniques involving a preliminary separation of the ergothioneine by chromatography have been described (Melville & Lubschez, 1953; Heath & Wildy, 1956) but these are too lengthy for general use.

In this paper, a method is presented for estimating ergothioneine which is both easy to perform and apparently specific in biological samples. It is based on the fact (Barger & Ewins, 1911) that when ergothioneine (I) is heated in 50% (w/v) potassium hydroxide solution at 100°, thiolurocanic acid (II) is formed by loss of trimethylamine.

Under suitable conditions this reaction can be made quantitative. The trimethylamine released, which can be aerated into benzene-picric acid and estimated as the picrate, is then a measure of the original concentration of ergothioneine.

MATERIALS AND METHOD

Reagents. Ergothioneine hydrochloride (Burroughs Wellcome Ltd., London. Analytically pure when dried *in vacuo* over phosphorus pentoxide) was standardized by titration with mN-iodine in neutral solution (Woodward & Fry, 1932).

Trimethylamine solution, prepared by collecting the distillate from an alkaline solution of the hydrochloride (British Drug Houses Ltd.), was standardized with acid before dilution.

Benzene, acetic acid, picric acid, sodium sulphate, ammonium sulphate, potassium carbonate, sodium hydroxide and potassium hydroxide were of A.R. quality. The potassium hydroxide solution used contained 100 g. of alkali/100 ml. of solution.

Glutathione and L-cysteine hydrochloride were from British Drug Houses Ltd. The working Nessler solution was made up from 1 vol. of stock solution, 1 vol. of 40% (w/v) sodium hydroxide solution, 4 vol. of a saturated solution of potassium carbonate and 4 vol. of water. The stock solution contained 46 g. of mercuric iodide and 32 g. of potassium iodide/100 ml.

Dialysis. Solutions were dialysed in cellophan against running water for 48 hr.

Packed cells. Oxalated whole blood was centrifuged for 20 min. at 1000 g and the plasma subsequently decanted.

Deproteinized solutions. The proteins were precipitated from plasma, whole blood or packed cells by the heat and acetic acid method (Hunter, 1949), 2 ml. of sample giving 10 ml. of solution. The protein precipitate was separated from the supernatant by centrifuging. In one series of experiments, the proteins were precipitated by tungstic acid (Folin & Wu, 1919) and by zinc hydroxide (Hagedorn & Jensen, 1923). Dialysed plasma-protein solutions from 2 ml. of plasma were diluted to 9 ml. with water and protein precipitation effected by adding 1 ml. of 25% (w/v) trichloroacetic acid.

Albumin and globulin fractions. These were prepared from plasma by half-saturation with ammonium sulphate. The albumin fraction was filtered from the precipitated globulins and the latter dissolved in water. Both solutions were then dialysed.

Spectrophotometry. A Unicam SP. 500 spectrophotometer was used.

Permeability studies. Packed cells, washed once with normal saline, were suspended in an equal volume of 0.9% sodium chloride solution with or without a known amount of added ergothioneine. The mixture was incubated at 37° or 0° and shaken by occasional inversion. If any visual haemolysis had occurred during the incubation, the results were discarded. Samples of the mixture were taken immediately after mixing and at suitable intervals during the incubation. The cells were separated by centrifuging and the concentration of ergothioneine was estimated in the supernatant.

Method

A standard solution of ergothioneine or trimethylamine (5 ml.) or the filtrate from deproteinized human blood, packed cells or plasma (5 ml.) is heated in a 20 ml. glass-stoppered tube in a boiling-water bath. The potassium hydroxide solution similarly preheated (5 ml.) is carefully added, the tube stoppered and the contents are kept in the bath for 1 hr. The stopper is removed and replaced by an all-glass bubbler connected to a tube containing 5 ml. of the Nessler-carbonate solution and 2 drops of octan-2-ol. The outlet from the Nessler tube is connected to a mixture of 5 ml. of benzene and 0.05 ml. of 2% picric acid in benzene, which traps the trimethylamine as the benzene-soluble picrate (Dyer, 1945). By joining the outlet from the benzene tube to the inlet of a second alkali-treated sample, a large number of specimens can be aerated in one operation.

The tubes are kept at room temperature and 2 l. of air, washed by preliminary bubbling through *N*-hydrochloric acid and 2% picric acid in benzene, is drawn through during 1 hr. The picric acid-trimethylamine picrate-benzene solutions are poured into 5 ml. measuring flasks, the tubes washed out with benzene and the volumes (reduced to about 4.5 ml. by evaporation) made up to 5 ml. with benzene. The absorption at 410 $m\mu$ is then measured in a 20 mm. glass cell against a picric acid-benzene blank.

To estimate free trimethylamine in the presence of ergothioneine, the potassium hydroxide solution is replaced by an equal volume of saturated potassium carbonate solution and the mixture aerated directly. To estimate the release of trimethylamine, by means of alkali, from the proteins precipitated by heat and acetic acid from 1 ml. of blood, packed cells or plasma, the precipitate is suspended, after washing with water, in 4 ml. of water and the alkali added as described. After incubation, the aeration is prolonged for 2 hr. and the volume of Nessler solution used is doubled to ensure the complete trapping of ammonia produced from the alkali-treated proteins.

RESULTS

After treating standard solutions of trimethylamine with either alkali or carbonate, the optical density of the trimethylamine picrate-picrate acid-benzene solutions at 410 $m\mu$ was found to be linearly related to the original trimethylamine concentrations up to at least 5 mg./100 ml. (Fig. 1). When ammonia or methylamine (1 ml. of solution containing 150 mg./100 ml.) was previously added to the trimethylamine, there was no interference, the

Fig. 1. Optical density of benzene-picric acid solutions after aeration of trimethylamine from standard solutions.

Table 1. *Determination of trimethylamine released from ergothioneine solution by adding potassium hydroxide solution*

The volume of ergothioneine solution was 5 ml.; 5 ml. of 100% (w/v) KOH solution was used.

Concn. of ergothioneine soln. (mg./100 ml.)	Optical density of benzene-picric acid solution at 410 $m\mu$	Trimethylamine (μ g.)	
		Found	Calc.
0.72	0.07	9	9
1.44	0.11	14	18
2.16	0.18	23	28
2.88	0.28	36	37
3.60	0.31	40	46
4.32	0.40	51	56
5.04	0.49	63	65
5.76	0.51	67	74
6.48	0.65	84	84
7.20	0.69	90	93

two bases being quantitatively removed by the Nessler solution.

A false positive was given by dimethylamine but the colour produced was only a fifth of the intensity for the same weight of trimethylamine (see also Dyer, 1945).

When the method was applied to standard solutions of ergothioneine, the trimethylamine released after treatment with potassium hydroxide was equivalent to the theoretical amount of 1 mole/mole of the thiol (Table 1). This result was also found after first treating the ergothioneine with an equivalent of iodine to convert it into the disulphide form (Woodward & Fry, 1932). On the other hand, no amine was released when ergothioneine was treated with an equal volume of

saturated potassium carbonate solution in which mixture free trimethylamine is insoluble.

Choline or betaine did not release any detectable amounts of trimethylamine in quantities of up to 1000 μ g. after treatment in solution with an equal volume of 100% (w/v) potassium hydroxide solution at 100° for 1 hr., either alone or in the presence of a known amount of ergothioneine.

Recoveries from plasma, packed cells or blood. When ergothioneine was added to plasma, recoveries by this method amounted to 95% (Fig. 2). The values obtained in typical recovery experiments from plasma, corpuscles and blood are tabulated below (Table 2), from which it will be

Fig. 2. Recoveries of added ergothioneine from human plasma. (The interrupted line represents 100% recovery.)

Table 2. Recoveries of ergothioneine added to blood, packed cells and plasma

Amount of ergothioneine added to 1 ml. of fluid (μ g.)	Recovery of ergothioneine (μ g.) from		
	Whole blood	Plasma	Packed cells
17	22, 17, 17	16, 17, 22	13, 16, 19
34	34, 32, 33	35, 32, 37	31, 40
51	51, 53, 46	52, 46, 53	46, 47
68	71, 66, 68	70, 71, 65	68, 60, 62
85	85, 82, 87	86, 84, 89	83, 85, 79

seen that reasonably consistent recoveries are attainable.

Normal values in human plasma, packed cells and blood. The values for a number of human subjects are set out in Table 3. In some cases an estimation was performed for free trimethylamine by using potassium carbonate in place of the alkali, but the values thus obtained were never significantly above the control levels.

Permeability of packed cells to ergothioneine. After incubation of the cells suspended in ergothioneine-0.9% sodium chloride solution, subsequent measurements of the ergothioneine remaining in the saline solution showed that there was no entry into the cells in up to 21 hr. at either 0° or 37° and with an initial ergothioneine concentration of up to 12 mg./100 ml. On the other hand, after incubation of the cells with saline alone, at these two temperatures and after 21 hr., no ergothioneine could subsequently be detected in the separated saline solution.

Protein-bound ergothioneine. When the proteins precipitated by heat and acetic acid from blood, plasma and packed cells were examined for ergothioneine as described above, considerable amounts of trimethylamine were released. The values for ergothioneine obtained were larger than for the corresponding deproteinized supernatants and less subject to variation as between different samples (Table 3). The amount of ergothioneine held by the blood proteins was not significantly affected by the use of different protein precipitants and, with plasma proteins, it was also unchanged after dialysis of the plasma either alone or after treatment with reducing agents (Table 4).

After separation of the plasma proteins into albumin and globulin fractions, the trimethylamine released by alkali after dialysis of the separated protein solutions was slightly greater for the albumin fraction than for the globulins from the same amount of plasma (Table 4).

DISCUSSION

The results with potassium carbonate show that there is no free trimethylamine, detectable by this method, in normal human blood. Since ammonia and primary amines are trapped by the Nessler

Table 3. Normal values for 'free' ergothioneine in man

Material	No. of estimations	Average value (mg./100 ml.)	Range (mg./100 ml.)	Standard deviation
Whole blood	12	1.7	1.0-2.6	0.5
Packed cells	13	3.3	1.8-5.0	0.9
Plasma	12	0.35	Nil-0.9	0.2
Total blood proteins	5	2.7	2.3-2.9	0.2
Packed cell proteins	4	3.0	2.7-3.4	0.25
Plasma proteins	8	1.3	1.0-1.6	0.2

Table 4. *Properties of 'protein-bound' ergothioneine*

The same batch of blood or plasma was used throughout. The results represent ergothioneine bound to the proteins precipitated from 1 ml. of blood or plasma after the treatment described. Each of the ergothioneine values represents the mean of three estimations.

Protein source	Treatment	Protein precipitant	Ergothioneine value ($\mu\text{g./ml.}$)
Whole blood	Untreated	Heat and acetic acid	27
		Tungstic acid	21
		Zinc hydroxide	24
Plasma	Untreated	Heat and acetic acid	17.5
		Trichloroacetic acid	16
		Trichloroacetic acid	17
		Trichloroacetic acid	17
	Dialysed alone		
	Dialysed after adding glutathione (100 mg./ml.)	Trichloroacetic acid	15.5
	Dialysed after adding cysteine hydrochloride (100 mg./ml.)		
	Dialysed after adding sodium sulphite (100 mg./ml.)	Trichloroacetic acid	14
	Dialysed albumin fraction	Trichloroacetic acid	8.5
	Dialysed globulin fraction	Trichloroacetic acid	6

Table 5. *Ergothioneine levels in human blood*

Ergothioneine range (mg./100 ml.)	Authors
5.4-5.7	Hunter (1949)
2.0-3.1	Lawson, Morley & Woolf (1950)
1.9-5.5	Latner & Mowbray (1949)
1.8-4.2	Melville & Lubschez (1953)
1.0-2.6	Jocelyn (present paper)

reagent used, the validity of the method for estimating ergothioneine depends on the absence from blood of other substances which, in 50% (w/v) potassium hydroxide solution, release volatile secondary and tertiary amines. Of substances known to occur in blood and containing a trimethylamine residue, neither choline nor betaine itself liberates the amine under these conditions. The only other betaine known to be present in significant amounts is carnitine (β -hydroxy- γ -butyrobetaine) with a range in human blood of 0.7-1.4 mg./100 ml. (Fraenkel, 1953). This, however, releases trimethylamine only after prolonged treatment with solid alkali at 150° (Krimberg, 1906). Substances containing a triethylamine, a diethylamine or a dimethylamine residue have not been reported to occur in blood.

In any case, from the nature of the new method, values obtained for ergothioneine represent maximum values, yet the range found for normal human subjects is lower than ranges reported by the use of various modifications of the Hunter method (Table 5). Hence the new method gives a truer estimate of ergothioneine levels and the Hunter method probably estimates some other compound in addition to ergothioneine. This could be thiolhistidine or thiolourocanic acid, both of which give a positive Hunter reaction. Neither

acid has, however, been proved to occur in mammalian tissues.

It has been reported (Heath & Toennies, 1958) that ergothioneine disulphide gives a value corresponding to only 65-70% of the equivalent amount of ergothioneine by the Hunter reaction. It is not known whether this disulphide occurs in animal tissues but, if it does, the results show that it would be estimated quantitatively as ergothioneine by the present method.

Until now it has not been possible to detect ergothioneine in the plasma. The Hunter method gives values equivalent to a concentration of about 1 mg./100 ml., but this has been attributed to the presence of interfering substances (Hunter, 1949; Fraser, 1950). Thus Melville & Horner (1954), using a chromatographic technique before the Hunter reaction, concluded that no ergothioneine was present in the blood plasma of rats.

In the present work, small amounts of ergothioneine have consistently been found in human plasma. This ergothioneine probably does not originate directly from the supply in the cells, since no diffusion from cells to saline was detectable at 37°. Diffusion also does not occur in the reverse direction, even with unphysiologically high saline-ergothioneine concentrations or, as already reported by Heath, Rimington, Searle & Lawson (1952), when ergothioneine is added to the plasma itself. This impermeability of the cell membranes to ergothioneine is apparently not maintained by an enzymic mechanism, for there is still no equilibration at 0° in 21 hr.

Since ergothioneine in mammals seems to be of exogenous origin, the plasma level probably depends on, and fluctuates with, the ergothioneine content of the diet at the time; because of the cell impermeability it is therefore unlikely that the

plasma concentration bears any relation to the concentration in the cells.

The finding that trimethylamine is released by alkali from the washed proteins of plasma and whole blood is of some interest. In the absence of other known trimethylamine-liberating substances, this must be assumed to originate from 'protein-bound' ergothioneine. The main part of the total blood ergothioneine (about 60%) is present in this bound form. Moreover, the values found are unaffected by different ways of precipitating the proteins or by dialysis, so that the binding cannot be due to a loose association complex or to adsorption of ergothioneine on the proteins during their precipitation. It was suggested by Melville & Lubschez (1953) that the existence of a protein-ergothioneine-disulphide complex would account for their observation that, when rat blood was treated with reducing agents before precipitation of protein with trichloroacetic acid, greatly increased values for blood ergothioneine resulted. With human blood, however, the failure to diminish the level of the 'protein-bound' ergothioneine by treatment with cysteine, glutathione or sulphite is evidence against the existence of such a disulphide linkage.

The slightly greater values found for albumin than for globulin binding are consistent with the greater concentration of the former in human plasma and render it unlikely that a specific protein is associated with the ergothioneine.

SUMMARY

1. A new method of estimating ergothioneine in biological fluids is described which depends on liberating trimethylamine from the thiol in

alkaline solution and its determination as the picrate by spectrophotometry.

2. Values are given and discussed for the concentration ranges of ergothioneine found in human blood, plasma and packed cells.

3. Ergothioneine does not diffuse either out of or into human blood cells.

4. Evidence is given for the existence in blood of 'protein-bound' ergothioneine.

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REFERENCES

- Barger, G. & Ewins, A. J. (1911). *J. chem. Soc.* **99**, 2336.
 Dyer, W. J. (1945). *J. Fish. Res. Bd Can.* **10**, 351.
 Folin, O. & Wu, H. (1919). *J. biol. Chem.* **38**, 81.
 Fraenkel, G. (1953). *Biol. Bull., Woods Hole*, **104**, 359.
 Fraser, R. (1950). *J. Lab. clin. Med.* **35**, 960.
 Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
 Heath, H., Rimington, C., Searle, C. E. & Lawson, A. (1952). *Biochem. J.* **50**, 530.
 Heath, H. & Toennies, G. (1958). *Biochem. J.* **68**, 204.
 Heath, H. & Wildy, J. (1956). *Biochem. J.* **64**, 612.
 Hunter, G. (1928). *Biochem. J.* **22**, 4.
 Hunter, G. (1949). *Canad. J. Res. E*, **27**, 230.
 Krimsberg, R. (1906). *Hoppe-Seyl Z.* **49**, 91.
 Latner, A. L. & Mowbray, R. (1949). *Proc. 1st Int. Congr. Biochem., Cambridge*, p. 396.
 Lawson, A., Morley, H. V. & Woolf, L. I. (1950). *Biochem. J.* **47**, 513.
 Lawson, A., Morley, H. V. & Woolf, L. I. (1951). *Nature, Lond.*, **167**, 82.
 Melville, D. B. & Horner, W. H. (1954). *J. biol. Chem.* **206**, 221.
 Melville, D. B. & Lubschez, R. (1953). *J. biol. Chem.* **200**, 275.
 Salt, H. B. (1931). *Biochem. J.* **25**, 1712.
 Sullivan, M. X. & Hess, W. C. (1933). *J. biol. Chem.* **102**, 69.
 Touster, O. (1951). *J. biol. Chem.* **188**, 371.
 Woodward, G. E. & Fry, E. G. (1932). *J. biol. Chem.* **97**, 474.

Toxopyrimidine Phosphate as an Inhibitor of Bacterial Enzyme Systems that Require Pyridoxal Phosphate

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Elucidation of the structure of thiamine was followed by attempts to determine the roles of the two components of the molecule in the nutrition of higher animals and micro-organisms (Abderhalden, 1939). Surprisingly, the pyrimidine unit (4-amino-5-hydroxymethyl-2-methylpyrimidine; toxopyrimidine; pyramin) proved markedly toxic to rats. Makino, Kinoshita, Sasaki & Shioi (1954) showed

that the animals could be protected by simultaneous administration of vitamin B₆. They noted the structural similarity between toxopyrimidine (I) and pyridoxamine (II), and suggested that the former was converted *in vivo* into toxopyrimidine phosphate (4-amino-5-hydroxymethyl-2-methylpyrimidine O-phosphate), which might then competitively displace pyridoxal phosphate from some