CLXXXIV. THE ERGOTHIONEINE CONTENT OF THE BLOOD IN HEALTH AND DISEASE.

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It is now definitely established that ergothioneine is present in the corpuscles of human blood and of certain animal bloods. Hunter and Eagles [1925] isolated the compound from pig's blood and later [1927] referred to its isolation from children's blood. Benedict, Newton and Behre [1926] also isolated the substance from both pig's and human blood. Hunter [1928] has briefly surveyed the history of ergothioneine and has pointed out the importance of two delicate colour tests to which the substance responds, the development of a blue colour with the uric acid reagents and a new reaction developed by Hunter from the Ehrlich diazo-reaction. Methods for the estimation of ergothioneine based upon the colour reaction with the uric acid reagents were used by Benedict *et al.* [1926, 1929, 1, 2], and by Rockwood, Turner and Piffner [1929]. In the earlier publication of Benedict, the following quantitative data were given:

Normal human blood	14–15 mg. ergothioneine per 100 cc.
Diabetic „ „	20–27 mg. „ „ „
Nephritic „ „	Normal or low values.

However, in Benedict's later publication, the above figures were stated to be too high. A new normal average for human blood was given as 7·5 mg. per 100 cc. whole blood (range 4·2–15·0), and apparently no further study of pathological cases was made.

Rockwood *et al.* [1929] show values for the ergothioneine content of various pathological bloods "in terms of uric acid." The factor of conversion of "ergothioneine in terms of uric acid" to actual ergothioneine was not determined or used by them for the particular method of estimation employed. It thus appeared that more reliable information concerning the ergothioneine content of pathological blood would be desirable. The work fell into two sections: firstly, the development of a reliable technique for the estimation of ergothioneine, which could be also combined with the blood-uric acid estimation, and secondly, the application of this to a series of blood specimens.

Discussion of certain factors in connection with the estimation of ergothioneine.

Benedict *et al.* [1926] found that, in the preparation of protein-free blood filtrates, some tungstic acid reagents caused a loss of all or much of the ergothioneine. This was confirmed by Eagles and Vars [1928]. As a remedy, Benedict

and Newton [1929, 1] adopted a molybdic acid reagent and later [1929, 2] a tungstomolybdic acid reagent for the removal of blood-proteins. Each of these reagents was stated to cause no precipitation of ergothioneine.

In order to choose a suitable blood-protein precipitant, a series of thirty blood samples (mostly pathological) has been used to determine which of the above three methods of protein removal yielded blood-filtrates with the greatest content of ergothioneine. As both uric acid and ergothioneine contribute to the colour developed in the "direct" uric acid estimation, "direct" uric acid values were compared for this purpose. It was found that using reagents prepared from the samples of chemicals stocked in this laboratory, the tungstomolybdic method caused least loss of ergothioneine, and the molybdic method most loss. The tungstic method was found to be nearly as satisfactory in this respect as the tungstomolybdic method (Table I).

Table I.

				"Direct" uric acid, mg. per 100 cc.		
24 samples of human blood				Tungstic	Molybdic	Tungstomolybdic
Maximum	...	...	...	8.1	—	8.6
Minimum	...	...	...	1.6	1.1	1.6
Mean of 24	...	...	...	3.70	—*	3.75
6 samples of pig's blood						
Maximum	...	...	...	1.5	1.8	2.4
Minimum	...	...	...	0.9	0.6	1.2
Mean	...	...	...	1.17	1.03	1.54

* 14 samples only, estimated by the molybdic method; all the figures were definitely low.

This indicated that the use of tungstomolybdic acid as a protein precipitant would be most desirable in blood-ergothioneine determinations. A support for this conclusion was established by the finding that ergothioneine added to blood could be quantitatively "recovered" from tungstomolybdic filtrates. The ergothioneine used was very kindly supplied by Dr G. Hunter.

Folin claimed [1930] that the whole of the ergothioneine of normal blood is retained within the corpuscles when the unclaked blood extract is prepared. That this is also true for pathological bloods has been shown by an examination of twenty-five blood specimens. In the same experiments it was also shown that the centrifugate obtained in the preparation of unclaked extracts, which contains the whole of the blood-ergothioneine, could be used for the estimation of ergothioneine. The actual method, in an improved form, is described later in this paper.

Table II.

				Uric acid, or "ergothioneine in terms of uric acid," mg. per 100 cc.		
				Unclaked extract		Centrifugate,
25 human blood specimens				Uric acid	"Ergothioneine"	"Ergothioneine"
Maximum	...	...	...	13.3	< 0.2*	1.0
Minimum	...	...	...	1.3	—	0.2

* By the method used for this series, amounts of less than 0.2 mg. per 100 cc. blood could not be estimated. The actual values probably approached zero.

The method of differential determination of uric acid and ergothioneine.

The following reagents were used:

- (1) Sulphate-tungstate solution, containing 34 g. crystallised sodium sulphate and 6 g. sodium tungstate per litre of solution.
- (2) $N/3$ sulphuric acid.
- (3) Tungstomolybdic reagent of Benedict and Newton [1929, 2].
- (4) 0.62 N sulphuric acid.
- (5) Acid silver lactate reagent prepared according to Folin [1922]. The solution was always filtered immediately before use.
- (6) 10 % NaCl in 0.1 N HCl.
- (7) Cyanide-urea reagent of Folin [1930].
- (8) Folin-Marenzi uric acid reagent [1929].
- (9) Standard uric acid solution of Folin [1930].

4 cc. oxalated non-haemolysed whole blood were added to 32 cc. sulphate-tungstate solution. After standing with occasional gentle rotatory shaking for 15 minutes, 4 cc. $N/3$ sulphuric acid were added slowly and with shaking, and the whole mixture was centrifuged as completely as possible. The supernatant liquid was poured off completely and used for the determination of uric acid by the method of Folin [1930].

The centrifugate obtained was transferred quantitatively with small amounts of water and repeated shaking to a long narrow test-tube graduated at 32 cc. about half-way up. The volume was made up with water practically to this mark and the froth removed by means of a trace of capryl alcohol. The volume was then made up to 32 cc. and the contents of the tube were mixed by closing with a bung and inverting several times, avoiding further frothing. This process was found completely to lysis the corpuscles. 4 cc. tungstomolybdic reagent were then added and the contents of the tube mixed again, this effecting re-solution of much of the precipitated plasma-proteins, and causing a colour change from opaque red-brown to a clear deep red. The whole mixture was then poured completely into a wide-necked flask, adequate time being allowed for drainage. 4 cc. 0.62 N sulphuric acid were then added slowly and with shaking to precipitate the whole of the protein, after which the mixture, occupying a total of 40 cc., was filtered through a 9 cm. No. 40 Whatman paper.

20 cc. filtrate were almost always available and were transferred to a round-bottomed cylindrical centrifuge tube. If 20 cc. were not available, some other aliquot portion, say 18 cc., was used and a correction applied in the calculation. To the filtrate, 7 cc. acid silver lactate solution were then added, and, after standing for a few minutes in the dark, the precipitate was centrifuged. The supernatant liquid was discarded and 1 cc. acid sodium chloride added to the precipitate, which was then thoroughly extracted by triturating with a rounded glass rod. The rod was washed down with about 4 cc. water and the precipitate again centrifuged. The supernatant liquid, containing any uric acid

present, was discarded and the extraction with sodium chloride and water repeated. Finally, 5 cc. cyanide-urea reagent were added to the precipitate which was dissolved by stirring, and the solution was washed quantitatively into a large hard glass test-tube graduated at 14 cc. and water added to this mark.

As a standard, 2 cc. dilute "blood-uric acid" standard solution (containing 0.008 mg. uric acid) were transferred to a similar test-tube, and 5 cc. cyanide-urea reagent and 7 cc. water added.

To both standard and unknown, 1 cc. uric acid reagent was then added and mixed. After allowing the tubes to stand for 4 minutes at room temperature, they were heated in a boiling water-bath for 2 minutes and then rapidly cooled in running water. The colours were compared in the colorimeter, without further dilution of the liquid. The unknown solution was set at 20 mm. and the standard colour matched against it. The calculation was made from a graph plotted for the particular reagents used. This was found necessary, mainly on account of the variable "blank" colours yielded by different samples of sodium cyanide. For the estimations here recorded, the following graph was obtained.

Fig. 1.

The graph is produced back to cut the ordinate at a point representing 6.0 mm., *i.e.* the equivalent of the "blank" colour. The slope of the line, $\tan \theta = 0.7$.

If c be the unknown concentration of ergothioneine in a test portion,

y be the reading of the standard colour, matched against the unknown set at 20 mm.,

a be the value in mm. represented by the "blank" colour (constant),

then

$$c = \frac{y - a}{\tan \theta}.$$

This actually reduces to

$$c = \frac{y - 6}{200} \text{ mg.}$$

In the estimation of blood-ergothioneine, the test portion contains the ergothioneine of 2 cc. blood, if 20 cc. filtrate are used, hence

$$\text{mg. ergothioneine per 100 cc. whole blood} = \frac{y-6}{200} \times \frac{100}{2} = \frac{y-6}{4}.$$

The standard colour was found to be stable for only about 1 hour, and hence was conveniently prepared for each ergothioneine estimation. The use of uric acid as a standard was found to be quite suitable, while various dyes were unsatisfactory. The use of pure ergothioneine would necessitate a demand for this substance which cannot as yet be met.

Glutathione and cysteine present in blood in normal amounts exert no interfering effect upon the accuracy of ergothioneine determinations by this method. This has been shown by experiments in which ergothioneine was estimated in blood, with and without the addition of these substances. Finally, the method was found to yield satisfactory duplicate figures.

The findings for normal and pathological blood.

As the ergothioneine of the blood is confined to the corpuscles and as the percentage volume of corpuscles varies, particularly in pathological cases, the corpuscle volume was determined for each blood sample. Uric acid and ergothioneine were estimated by the method described above, the figures being obtained as mg. per 100 cc. whole blood. The ergothioneine was then calculated from the corpuscle volume, as mg. per 100 cc. corpuscles. The results of 60 estimations are shown in Table III. For each group, the figures are arranged in order of increasing ergothioneine content. Blood-sugar values are included for the diabetic bloods and urea values for most of the other pathological samples.

The most striking feature about the results is the large range of ergothioneine values in all the human blood groups studied. The normal range (3–12 mg. per 100 cc. corpuscles) agrees well with that given by Hunter [1928], using an entirely different method of estimation. This range is such as to obscure any variation from the normal due to pathological conditions, and to yield a mean figure of little significance. There is no evidence of any constant variation of the blood-ergothioneine content with sex or with age. Eagles and Vars [1928] have shown that the dietary history of pigs determines the blood-ergothioneine content. However, it is considered that normal human subjects, such as were studied, would be receiving a mixed and balanced diet, without any undue amount of maize protein or other material rich in ergothioneine-producing substances.

In the diabetic series the ergothioneine figures certainly tend to be slightly higher than the normal, but again the range of values is so great as to make this variation of questionable significance. There is no correlation between the ergothioneine and sugar values; neither was any variation in the ergothioneine value related to any stage of the disease or to the treatment. However, this point merits more detailed study.

Table III.

(a) *Normal blood.*

Specimen	Age	Sex	% volume of cor- puscles	Uric acid, mg. per 100 cc. blood	Ergo- thioneine, mg. per 100 cc. corpuscles
B.	Adult	F.	47	2.80	3.05
Mixed	"	—	40	2.92	5.60
C.	"	M.	49	2.40	6.70
M.	"	M.	46	3.67	6.85
A.	"	F.	45	2.75	11.60
D.	"	F.	51	3.64	11.60
L.L.	"	F.	45	3.15	12.00
S.S.	11	M.	42	2.93	3.55
B.L.	10	M.	43	2.65	4.05
D.B.	3	M.	37	1.73	6.90
G.H.	4	M.	39	3.32	8.20
Mean of normal samples			...	...	2.85
					7.28

(b) *Diabetic blood.*

						Sugar, mg. per 100 cc. blood
J.G.	65	F.	46	2.95	6.95	263
E.J.	25	M.	41	2.39	8.15	220
W.	Adult	F.	44	2.38	8.30	180
V.H.	26	F.	45	2.25	8.55	340
C.	Adult	F.	46	1.50	8.80	136
E.B.	23	F.	51	2.52	8.90	250
A.B.	29	F.	45	2.33	9.00	347
A.K.	70	F.	50	3.17	9.30	144
E.H.	61	F.	48	3.39	10.10	208
F.C.	25	M.	53	1.51	10.60	76
W.M. (a)	39	M.	41	3.20	11.10	109
N.S.	15	M.	42	2.92	14.05	260
W.M. (b)	39	M.	33	3.33	15.15	111
P.L.	10	M.	40	2.94	7.90	170
G.H.	8	M.	41	1.86	9.50	98
R.W. (a)	Child	M.	31	5.13	11.95	200
R.W. (b)	"	M.	28	5.16	14.65	266
Mean of diabetic samples			...	...	2.85	10.17

(c) *Nephritic blood.*

						Urea, mg. per 100 cc. blood
M.M. (a)	12	M.	29	1.72	4.85	129
B.R.	8	F.	49	2.13	4.90	81
M.M. (b)	12	M.	44	1.75	5.25	42
B.H.	7	M.	42	2.63	5.70	80
D.C.	6	M.	56	5.06	5.70	65
I.W.	10	F.	36	4.19	5.85	45
B.G.	12	F.	40	2.94	6.00	59
T.H. (a)	12	M.	33	2.92	6.35	54
O.K.	6	F.	29	1.98	7.40	39
T.H. (b)	12	M.	46	2.20	7.80	47
G.T.	6	F.	33	2.29	8.20	22
C.W.	5	F.	42	1.82	8.55	42
K.C.	2	M.	21	3.50	10.00	84
M.R.	10	F.	32	2.67	14.55	35
Mean of nephritic samples			...	...	—	7.78
						—

Table III (*continued*).(d) *Various pathological bloods.*

Specimen	Age	Sex	% volume of cor- puscles	Uric acid, mg. per 100 cc. blood	Ergo- thioneine, mg. per 100 cc. corpuscles	Urea, mg. per 100 cc. blood
Chorea	10	F.	44	0.97	4.00	33
Prostatitis	55	M.	44	2.59	4.00	40
?	37	M.	46	3.64	4.55	40
Chorea	12	M.	37	1.51	5.00	—
?	57	F.	40	3.69	5.75	67
?	47	M.	25	2.40	6.00	—
Coeliac rickets	7	F.	37	3.43	6.75	76
?	Adult	M.	50	6.56	8.00	—
? Renal tumour	11	M.	48	3.08	9.15	—
Pyelitis	9	F.	46	1.78	9.80	48
Rheumatism	8	M.	43	1.65	10.25	40
Pulm. T.B.	10	F.	45	2.32	13.75	28
Hemiplegia	67	F.	50	3.85	14.70	—
Mean of miscellaneous pathological samples ...				—	7.82	—

(e) *Pig's blood.*

1	—	—	43	0.36	15.60	—
2	—	—	50	0.35	20.00	—
3	—	—	45	0.37	21.10	—
4	—	—	36	0.32	22.80	—
5	—	—	55	0.34	24.20	—
Mean ...			46	0.35	20.74	—

In the nephritic series the ergothioneine values are normal and do not vary with the retention of nitrogenous excretory products as evidenced by the uric acid and urea figures. A similar large range of values is shown for thirteen miscellaneous pathological blood samples. On the other hand, four out of five specimens of pig's blood, obtained from the abattoir, yielded ergothioneine values substantially higher than those of human blood.

The uric acid figures are of interest as they were obtained by the new method of Folin [1930], for which normal figures have not yet been fully established. In other experiments with both normal and pathological human blood the author has found that normal figures by this new method are consistently slightly lower than those obtained by the older "direct" technique of Folin [1923] for the same blood.

Summary.

A new method for the differential determination of uric acid and ergothioneine in blood is described. Figures are recorded for the uric acid and ergothioneine contents of normal and certain pathological bloods.

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