ERGOTHIONEINE IN THE URINE*

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In previous work Sullivan and Hess (1) applied the Rupp-Schied (2) thiocyanate method as improved by Thiel (3) to various urines, normal and pathological, and found that a number of substances actually or potentially present in urine behave like thiocyanate in the Rupp-Schied-Thiel procedure. Among the substances tested was ergothioneine, a blood cell constituent, which, *a priori*, might occur in urine. On making an investigation, detailed presently, ergothioneine-like substances were found in urine and a certain amount of ergothioneine was actually isolated.

Ergothioneine—This compound isolated from blood by Benedict (4), Benedict, Newton, and Behre (5), and Hunter and Eagles (6, 7), was found by Newton, Benedict, and Dakin (8) to be identical with the base ergothioneine isolated from ergot by Tanret (9) and shown by Barger and Ewins (10) to be the betaine of thiohistidine. Previously no one has shown its presence in normal or pathological urines.

Proof of Presence of Ergothioneine-Like Substances in Urine—Benedict, Newton, and Behre (5) showed that ergothioneine is precipitated by silver lactate in the presence of lactic acid but is not freed from the silver compound by treatment with a 10 per cent solution of sodium chloride in 0.1 N hydrochloric acid as used in the Folin-Wu (11) method for uric acid. In this way ergothioneine can be separated from uric acid which is extracted by acid sodium chloride from the silver precipitate. This procedure was applied to urine as follows: To 3 cc. of urine, Folin-Wu's silver lactate-lactic acid mixture was added in slight excess.

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The mixture was centrifuged and the supernatant liquid decanted. The insoluble residue was washed by stirring with acid-sodium chloride (10 per cent sodium chloride in 0.1 N hydrochloric acid) in 50 cc. lots, centrifuging, and decanting until the washings no longer gave a color with the uric acid reagent.

A study was then made of the optimum amount of sodium cyanide needed to bring the silver precipitate into solution and give the maximum development of color with the uric acid reagent. Finally the use of 6 cc. of 5 per cent cyanide was found best for both purposes. The validity of this statement is shown by the following experiments, Experiment A on ergothioneine-like material in urine and Experiment B with blood ergothioneine added to a solution of a number of urinary ingredients.

Experiment A—The contents of four tubes, each containing 3 cc. of the same urine, were precipitated by silver lactate, centrifuged, and washed four times with 40 cc. of the acid-sodium chloride until the washing gave no color with the Folin-Marenzi (12) reagent and alkali. Then the respective solid was treated as follows:

Tube	1	+	0.5	cc.	5	per cent	NaCN	+	5.5	cc. 0.8 N NaOH
"	2	+	1.0	"	5	"	"	"	+ 5.0	" 0.8 " "
"	3	+	3.0	"	5	"	"	"	+ 3.0	" 0.8 " "
"	4	+	6.0	"	5	"	"	"	+ 0.0	" 0.8 " "

To each tube were then added 5 cc. of water and 0.5 cc. of reagent.

Tubes 1 and 2 gave no blue color. Tube 3 gave a good blue but less than Tube 4.

Experiment B—Ergothioneine from blood was added to an artificial mixture containing 1 gm. of NaCl, 2.5 gm. of urea, 0.07 gm. of creatinine, 0.01 gm. of potassium thiocyanate, 0.001 gm. of oxalic acid, and 0.15 gm. of sulfuric acid in 100 cc. of water. Four tubes, each containing 0.25 mg. of ergothioneine in 3 cc. of the mixture were treated as in Experiment A. Similar results were obtained.

Accordingly, the use of 6 cc. of the cyanide was made a routine procedure. To the silver complex dissolved in the sodium cyanide were added 1 cc. of the Folin-Marenzi reagent and 1 cc. of N sodium hydroxide. The standard solution for comparison was 0.5 mg. of ergothioneine dissolved in 6 cc. of 5 per cent sodium cyanide and treated in the same manner as the unknown.

In this way, ergothioneine-like material was estimated in eleven normal urines, thirteen urines from patients with cancer, including cancer of the lung, breast, uterus, prostate, and sarcoma of chin, lower bowels, etc., nine arthritics, and twenty other cases with a wide variety of pathological conditions. The findings are given in Table I.

The values for cancer cases tend to run high. Since, however, three of the cases of malignancy, a cancer of the prostate, a cancer of the lung, and a lymphosarcoma of the lower bowel gave ergothioneine values of 82, 80, and 62 mg. respectively, in the 24 hour urine, the estimation of ergothioneine-like material does not seem to have any diagnostic value for malignancy. This conclusion is corroborated by the fact that high values were obtained occasionally in other conditions, as for example, arthritis.

TABLE I

Ergothioeine-Like Material in Urine

Condition	Amount in 24 hr. urine			Mean volume	Mean weight
	Minimum	Maximum	Mean		
	mg.	*mg.*	*mg.*	*cc.*	*mg. per l.*
Normal	77.6	154	113.4	1267	89.5
Cancer	62.0	198	135.0	911	147.1
Arthritis	50.5	215	123.7	1261	98.1
Other pathological cases	34.0	225	90.6	1020	88.5

Since Behre and Benedict (13) in their study of blood give evidence for the presence of other material behaving like ergothioneine in their procedure, the material judged to be present in urine is called ergothioneine-like. There is evidence, however, that ergothioneine itself, at least in a small amount, is present in normal urine.

Isolation of Ergothioneine from Urine—The next step was to isolate ergothioneine from urine. This was accomplished by a modified Hunter and Eagles (7) procedure for blood followed in part by Benedict's (5) procedure. 12 liters of normal urine were brought to pH 3.5 by the addition of glacial acetic acid. Basic lead acetate (Goulard's reagent) was then added until the supernatant liquid gave a precipitate with sulfuric acid. 500 cc. of

Goulard's solution were required. The filtrate was freed from lead by hydrogen sulfide. The filtrate from lead sulfide, freed from hydrogen sulfide by passing air through it, was treated with a saturated alcoholic solution of mercuric chloride as long as a precipitate formed. The mercury precipitate was decomposed by hydrogen sulfide and thoroughly washed. The solution, made to 0.5 N acid with sulfuric acid, was precipitated with phosphotungstic acid until precipitation ceased. A pink precipitate formed which was allowed to stand overnight at 5°. Then the phosphotungstic precipitate was ground with an excess of barium hydroxide and filtered. The filtrate freed from barium was concentrated under reduced pressure to 30 cc. Considerable material separated out. The filtrate from this precipitate was treated with silver lactate in slight excess and centrifuged. The silver precipitate was then washed by stirring with a solution of 10 per cent sodium chloride in 0.1 N hydrochloric acid in 50 cc. lots to a total of 600 cc. until the supernatant liquid gave a negative reaction with the Folin-Marenzi uric acid reagent and sodium hydroxide. The silver precipitate was then washed into a beaker containing 30 cc. of hot 0.5 N hydrochloric acid and the mixture was boiled for 5 minutes. The filtrate was then placed in the ice box at 5° overnight. After filtering from a precipitate the solution was concentrated to a few cc. and an equal volume of absolute alcohol was added. On standing a white compound settled out. This was filtered and dried in a desiccator over calcium chloride. The weight was 60 mg.

This material behaves like ergothioneine in that it reacts with the Folin-Marenzi uric acid reagent, gives a red color with Hunter's (14) diazo reaction for ergothioneine, and titrates like ergothioneine in Okuda's iodometric titration as used for cystine.

Analyzed for nitrogen by Kjeldahl and for sulfur by Parr bomb it gave nitrogen 15.26 per cent, sulfur 11.95 per cent. The calculated values for ergothioneine hydrochloride ($C_9H_{15}N_3SO_2HCl$) are N 15.81 per cent, sulfur 12.07 per cent. The free base obtained by the method of Benedict, Newton, and Behre melted at 261° and decomposed between 285–290°.

In previous work (15) ergothioneine from blood was found to give a titer in Okuda's (16) iodometric method for cystine. In the present work, the free base reduced by zinc and hydrochloric

acid, 2.5 mg., gave a titer of 0.52 cc. of 0.001 M KIO_3 or 0.45 cc. of 0.001 M KIO_3 for the hydrochloride. The material isolated from urine, similarly treated gave a titer of 0.45 cc. for 2.5 mg., the theoretical for ergothioneine hydrochloride.

Analysis and reactions indicate the presence of ergothioneine in urine. The amount isolated, 5 mg. per liter, is small but this may be due to the wasteful procedure involved. The procedure is by no means quantitative and was used merely to show the actual presence of ergothioneine. Colorimetrically, there were found about 90 mg. per liter of ergothioneine-like material in normal urine.

5 liters of normal urine were then tested for ergothioneine by the red oxide of copper precipitation method of Williamson and Meldrum (17). Colorimetrically the urine showed 446.5 mg. in the 5 liters. However, in concentrating the urine under reduced pressure to 550 cc. preparatory to treatment with the red oxide a loss of about 34 per cent was encountered in material reacting like ergothioneine in treatment with silver lactate and colorimetric comparison with blood ergothioneine. With the copper oxide procedure, the ergothioneine isolated was again of the order of magnitude as given above, 5 to 6 mg. per liter. These figures are undoubtedly minimal figures. Assuming that the isolation procedures are satisfactory when applied to urine, it would seem that most of the ergothioneine-like material found by us in normal urine is not ergothioneine. Its nature is being further investigated.

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