REFERENCES

- Barker, H. Ennor, A. H. & Harcourt, K. (1951). *Biachim. Biophys. Acta* (in the Press).
- Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
- Ennor, A. H. & Stocken, L. A. (1950). *Aust. J. Exp. Biol. Med. Sci.* **28**, 647.
- Ennor, A. H. & Rosenberg, H. (1951). *Nature, Lond.*, **168**, 199.
- Ernster, L., Zetterström, R. & Lindberg, O. (1950). *Acta chem. scand.* **4**, 942.
- Hevesy, G. (1948). *Radioactive Indicators*. New York: Interscience.
- Kalckar, H. M., Dehlinger, J. & Mehler, A. (1944). *J. biol. Chem.* **154**, 275.
- Kamen, M. D. & Spiegelman, S. (1948). *Cold Spr. Harb. Sym. quant. Biol.* **13**, 151.
- Lehninger, A. L. (1949). *J. biol. Chem.* **178**, 625.
- Martin, J. M. & Doty, D. M. (1949). *Anal. Chem.* **21**, 965.
- Oddie, T. H. & Lynn, A. R. (1951). *C.S.I.R.O. Tracer Element Report No. 1. Preparation of Filtered Samples for Radioactive Assay*.
- Rowles, S. L. & Stocken, L. A. (1950). *Biochem. J.* **47**, 489.
- Sacks, J. (1940). *Amer. J. Physiol.* **129**, 227.
- Sacks, J. (1951). *Arch. Biochem.* **30**, 423.
- Veall, N. (1948). *Brit. J. Radiol.* **21**, 347.
- Vogel, A. (1947). *Quantitative Inorganic Analysis*. London: Longmans.

Some Effects of Administering Ergothioneine to Rats

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The presence of ergothioneine in erythrocytes is well established. This has been made possible by the delicate and highly specific Hunter diazo reaction (Hunter, 1928, 1949). Latner (1948) used a modified diazo reaction to determine ergothioneine in blood ultrafiltrates, while Lawson, Morley & Woolf (1950) precipitated ergothioneine with iodobismuthous acid prior to colorimetric determination by the Hunter reaction. Touster (1951) has published a method for the estimation of ergothioneine in which the thiol group is oxidized with bromine water to sulphate, which is precipitated as benzidine sulphate and determined colorimetrically. The reaction of certain mercaptoglyoxalines with 2:6-dichloroquinone-chloroimide described by McAllister (1951*a, b*) might also be applicable to the determination of ergothioneine, but like Touster's method would not have the specificity of the Hunter reaction.

Ergothioneine has been stated to occur in human, rat, pig, ox, cat, guinea pig, dog, fowl and pigeon erythrocytes in amounts up to 30 mg./100 ml. of whole blood. There is no report in the literature of any species in which ergothioneine is entirely absent. It is found exclusively in the red cells and is not present in the plasma (cf. Fraser, 1950). Variations of ergothioneine blood levels with geographical location have been recorded by Hunter (1951) for men, rats and pigs. Significant increases in diabetic patients have been reported by Salt (1931) and by Fraser (1950). Latner & Mowbray (1948) observed

lower ergothioneine levels in some thyrotoxic patients and raised levels in some cases of myxoedema.

Ergothioneine is the only 2-mercaptoglyoxaline known to occur naturally, and has been found only in blood, in ergot and in some other fungi. See, however, Leone & Mann, 1951. The possibility that erythrocyte ergothioneine is derived from dietary ergothioneine seems to be remote, and the report of Eagles & Vars (1928) that a maize diet increased the blood ergothioneine level in pigs has not been confirmed by Hunter (1951). Hunter also showed that when ergothioneine or 2-thiolhistidine was given to young rats (5 mg./animal) for 3 days, there was no increase in blood ergothioneine 2 days later. As will be shown later, however, the erythrocyte concentration of ergothioneine can be greatly increased by feeding rats a diet containing 0.1 % ergothioneine, although the increase is gradual.

The question of the occurrence of ergothioneine in urine has been examined by various workers. Sullivan & Hess (1933) and Work (1949) claimed that it was excreted in appreciable amounts, whilst Woolf (1949) and Lawson *et al.* (1950) failed to detect any by paper chromatography. The last named workers also reported that a single injection of ergothioneine in the rat did not bring about any detectable excretion during the following 24 hr. The use of a new procedure which has likewise given no indication of the presence of ergothioneine in

normal urine has, however, established that a considerable proportion of ergothioneine administered to rats either subcutaneously or in the diet is rapidly excreted.

The synthesis of ergothioneine by Heath, Lawson & Rimington (1950, 1951) made possible the preparation of sufficient quantities for feeding experiments, the main objects of which were to determine the fate of ergothioneine after administration, and to show conclusively whether or not ergothioneine has any antithyroid activity.

Lawson & Rimington (1947) reported that administering ergothioneine to rats caused hyperplasia and decreased the total iodine content of the thyroid glands. No such activity was found, however, by Astwood & Stanley (1947), Wilson & McGinty (1949) or by Searle, Lawson & Hemmings (1950), although Pitt-Rivers (1948) found that ergothioneine, like the known antithyroid drugs, inhibited the *in vitro* conversion of acetyldiiodotyrosine into acetylthyroxine. Since even a slight antithyroid activity might have significance in a substance naturally occurring in blood, ergothioneine has now been administered continuously to rats at a higher level than previously reported and in a diet of very low iodine content. Under these conditions even a weak antithyroid activity would be expected to cause appreciable changes in the weight, iodine content and radioactive-iodine uptake of the thyroid gland. No such activity has, in fact, been found.

MATERIALS AND METHODS

Rats. Hooded rats of the Glaxo strain were used in groups of six to seven animals, matched for weight and sex. They were weighed daily, commencing about 4 days before adding ergothioneine to the diet.

Diet. Glaxo powder diet RBSS-9 was used throughout. No iodine was detected in this diet when 0.25 g. samples were analysed as described below. Ergothioneine was incorporated in the diet at a level of 0.1%, calculated as free base.

Ergothioneine. Synthetic ergothioneine was prepared by the method of Heath *et al.* (1951). It was not resolved. Natural ergothioneine hydrochloride was converted to the free base by dissolving in the minimum amount of water, and adjusting to pH 7 with NH_3 ; ergothioneine rapidly crystallized on adding excess methanol.

Iodobismuthous acid. This was prepared and used as described by Lawson *et al.* (1950). After storage for 3 months in the dark at 4° the reagent contains appreciable amounts of free I_2 and its use leads to incomplete precipitation of ergothioneine and bad colour development in the Hunter reaction. Extraction with CHCl_3 after dilution with an equal volume of water restored its original properties.

Hunter diazo reagent. Prepared and used as described by Hunter (1949).

Blood ergothioneine. Blood samples were obtained by heart puncture on the ether-anaesthetized animals immediately before killing. Ergothioneine was then determined as described by Lawson *et al.* (1950).

Urinary ergothioneine. Urinary ergothioneine was detected by the use of ion-exchange resins. A quantity of the total urine output collected over the 14-day period of feeding ergothioneine was diluted ten times with water and passed through a column of Zeo-Karb 215 (The Permutit Co., London, W. 4) which was then washed with water till the washings no longer gave the Folin-Marenzi reaction. The column was fractionally eluted with CO_2 -free 0.1N- NH_3 , those fractions giving a positive Folin-Marenzi reaction being bulked and subsequently adsorbed on a Dowex 2 (Dow Chemical Co., Michigan, U.S.A.) column. After washing, the ergothioneine was obtained by fractional elution with CO_2 -free 0.1N-HCl and was free from all impurities interfering with the Hunter test. Slight but variable ergothioneine losses occurred on the Zeo-Karb, but not on the Dowex columns, and for the determination of ergothioneine in urine the more rapid method described below was preferred.

Determination in urine. A tenfold diluted urine (1 ml.) from rats receiving ergothioneine was heated on the boiling-water bath for 3 min. with 1 ml. water and 1 ml. 5% (w/v) sulphosalicylic acid solution in 0.03N-HCl. After cooling and centrifuging, a sample (2 ml.) of the clear liquid was treated with HBI_4 and the diazo reaction carried out on the precipitate as described by Lawson *et al.* (1950). Addition of choline as co-precipitant was unnecessary. The colour intensities were determined with a Puffrich photometer using filter no. S53. For the blank determination the same procedure was followed using normal rat urine, and a calibration curve was made by substituting 1 ml. of an aqueous solution containing known amounts of ergothioneine for the water.

Determination of ergothioneine in rat tissues. Samples of the liver, kidney, spleen, adrenals and thyroids representative of the group of rats were homogenized with 2.3% (w/v) aqueous sulphosalicylic acid and diluted with this reagent to give a 10% extract. After centrifuging, the ergothioneine in 2 ml. of the supernatant liquid was determined after precipitation with HBI_4 as described above. Separate calibration curves were made for liver, kidney and spleen, using samples of the corresponding tissues from normal animals. This was justifiable since no ergothioneine could be detected in liver homogenates from normal rats using the chromatographic procedure described above for urine.

Radioactive iodine. Radioactive iodine (^{131}I) was obtained from Atomic Energy Research Establishment, Harwell. 1 $\mu\text{c.}$, free of added carrier, was injected intraperitoneally in 0.5 ml. of 0.9% NaCl solution into each animal 24 hr. before it was killed with CHCl_3 . The uptakes of ^{131}I by the thyroids were then determined as described by Searle *et al.* (1950).

Iodine analyses. Materials for analysis were ashed with $\text{KMnO}_4\text{-H}_2\text{SO}_4$, and the iodine was distilled with oxalic acid (Talbot, Butler, Saltzman & Rodriguez, 1944), and determined colorimetrically by the $\text{As}_2\text{O}_3\text{-Ce(SO}_4)_2$ procedure (Taurog & Chaikoff, 1946). Natural and synthetic ergothioneine (25 mg. samples) and the normal and supplemented diets (0.25 g. samples) have been analysed for iodine in this way.

RESULTS AND DISCUSSION

Blood ergothioneine

The ergothioneine blood level of all the rats used was less than 1 mg./100 ml. whole blood at the start of these experiments. The level slowly increased

Table 1. *Effect of 0.1 % ergothioneine in the diet of rats, for varying periods, on thyroid weight and ^{131}I uptake and on ergothioneine blood levels*

(The diet supplemented with synthetic ergothioneine contained additional I (0.6 $\mu\text{g./g.}$). No ergothioneine was found in the blood of control rats.)

Ergothioneine	Time fed (days)	Change in thyroid weight (%)	Change in ^{131}I uptake of thyroids (%)	Blood ergothioneine of dosed rats (mg./100 ml.)
Natural	2	-13.3	+0.8	<1
Synthetic	2	-14.2	-41.8*	<1
Synthetic	4	+0.8	-35.6*	2.0 $\pm$ 0.1
Natural	11	-14.6	-16.8	7.2 $\pm$ 0.8
Synthetic	11	-19.5*	-68.9*	6.15 $\pm$ 0.7
Synthetic	14	-2.1	-45.5*	10.2 $\pm$ 1.0

* Statistically significant ($P < 0.05$).

when the diet was supplemented with either natural or synthetic ergothioneine, reaching 2 mg./100 ml. after 4 days and 10 mg./100 ml. after 14 days (Table 1). No ergothioneine was found in the plasma. The slow rate of incorporation of ergothioneine into the blood does not conflict with the preliminary observation of Hunter (1951) that a single dose of ergothioneine given orally for 3 days does not increase the blood level.

An experiment was also performed with oxalated human blood, incubated at 37°, to ascertain whether or not ergothioneine could enter the erythrocytes from the plasma. Portions of blood (2 ml.) were used for a preliminary determination of the ergothioneine levels in plasma and cells, then to the remaining 10 ml. of blood was added 1 mg. of ergothioneine dissolved in 0.1 ml. of normal saline and the mixture was incubated with gentle agitation. Samples (2 ml.) were withdrawn for analysis immediately after the addition and at hourly intervals up to 4 hr. The cells were spun down, washed three times with normal saline, and plasma and cells analysed for ergothioneine. No transference of ergothioneine from plasma to cells could be detected. This finding, together with the gradual increase in erythrocyte ergothioneine which occurred during the feeding experiments, would suggest that ergothioneine normally present in blood is incorporated during erythropoiesis.

Urinary ergothioneine

There was no detectable difference in the feeding experiments between the behaviour of natural and synthetic ergothioneine. In both cases some ergothioneine was excreted in the urine within 24 hr. after a single subcutaneous injection (Table 2), although at this time no detectable quantity was present in either the erythrocytes or the plasma, suggesting that ergothioneine has a very low renal threshold in the rat.

Tissue ergothioneine

The ergothioneine found in the spleen (Table 2) after 14 days on the ergothioneine diet could be due to the blood content of this organ, but this is not the case with the liver and kidney, which undoubtedly have the power of accumulating ergothioneine. In another series of experiments, to be reported later, it was found that the ergothioneine content of rat liver 48 hr. after discontinuing the administration of the substance was 60 mg./100 g. liver and levels as high as 100 mg./100 g. have been reached in certain cases. No ergothioneine could be detected in the adrenals or thyroid gland, and no differences in the ergothioneine distribution among the different tissues were observed with different routes of administration of the ergothioneine or between natural and synthetic ergothioneine.

Table 2. *Distribution of ergothioneine 24 hr. after a single subcutaneous injection of 15 mg. synthetic ergothioneine to a 150 g. rat and at the end of a 14-day period on a diet containing 0.1 % synthetic ergothioneine*

	Ergothioneine concentration (mg./100 g.*)		
	Control diet	After single subcutaneous injection	After 14 days 0.1 % dietary ergothioneine
Urine	<1	74	83
Blood	<1	2.8	10
Kidney	<1	21	40
Liver	<1	27.5	36.5
Spleen	<1	—	5
Adrenals	<1	—	<1
Thyroids	<1	—	<1

* Except for urine and blood, where concentrations are in mg./100 ml.

Effect on thyroid activity

Synthetic and natural ergothioneine have been administered as described for periods up to 23 days. In no experiment has any change in growth rate,

or any significant increase in thyroid weight indicative of antithyroid activity been observed. The results are shown in Table 1.

After 23 days on the synthetic ergothioneine diet, the effects observed were only those attributable to the presence of a small amount of iodine (0.6 $\mu\text{g./g.}$ of diet) present as an impurity in the ergothioneine, namely, a significantly decreased thyroid weight (19.9% below control weight) and a significantly greater thyroid iodine concentration (41.5% above control values). Under these conditions even a weak antithyroid activity would have reversed such effects. As a result of this slightly increased iodine content of the synthetic ergothioneine diet, the ^{131}I -uptake method gave a false impression of activity when this diet was fed owing to dilution of the ^{131}I with non-radioactive iodide. Such dilution effects are an ever-present potential source of misleading results when diets of low iodine content facilitating the detection of weak antithyroid activity are used. With the diet supplemented with natural ergothioneine no significant depression of the 24 hr. uptake of ^{131}I was found after 2 or 11 days.

There seems little doubt, therefore, that the antithyroid activity of ergothioneine is negligible.

SUMMARY

1. Methods for the determination of ergothioneine in tissues and urine are described.

2. Contrary to previous reports it is found that when ergothioneine is administered to rats, either orally or subcutaneously, some is rapidly excreted in the urine.

3. Ergothioneine administered in the diet is rapidly accumulated in the liver and more slowly in the erythrocytes. No ergothioneine has been detected in the plasma at any time during these experiments.

4. Ergothioneine does not exert any antithyroid action in the rat even when tested under conditions which would reveal any slight activity.

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REFERENCES

- Astwood, E. B. & Stanley, M. M. (1947). *Lancet*, **2**, 905.
Eagles, B. A. & Vars, H. M. (1928). *J. biol. Chem.* **80**, 615.
Fraser, R. (1950). *J. Lab. Clin. Med.* **35**, 960.
Heath, H., Lawson, A. & Rimington, C. (1950). *Nature, Lond.*, **166**, 106.
Heath, H., Lawson, A. & Rimington, C. (1951). *J. chem. Soc.* p. 2215.
Hunter, G. (1928). *Biochem. J.* **22**, 4.
Hunter, G. (1949). *Canad. J. Res. E*, **27**, 230.
Hunter, G. (1951). *Biochem. J.* **48**, 265.
Latner, A. L. (1948). *Biochem. J.* **42**, xxxv.
Latner, A. L. & Mowbray, R. (1948). *Biochem. J.* **42**, xxxv.
Lawson, A., Morley, H. V. & Woolf, L. I. (1950). *Biochem. J.* **47**, 513.
Lawson, A. & Rimington, C. (1947). *Lancet*, **2**, 906.
Leone, E. & Mann, T. (1951). *Nature, Lond.*, **168**, 205.
McAllister, R. A. (1951a). *Nature, Lond.*, **167**, 863.
McAllister, R. A. (1951b). *J. Pharm. Pharmacol.* **3**, 506.
Pitt-Rivers, R. (1948). *Biochim. Biophys. Acta*, **2**, 311.
Salt, H. B. (1931). *Biochem. J.* **25**, 1712.
Searle, C. E., Lawson, A. & Hemmings, A. W. (1950). *Biochem. J.* **47**, 77.
Sullivan, M. X. & Hess, W. C. (1933). *J. biol. Chem.* **102**, 69.
Talbot, N. B., Butler, A. M., Saltzman, A. H. & Rodriguez, P. M. (1944). *J. biol. Chem.* **153**, 479.
Taurog, A. & Chaikoff, I. L. (1946). *J. biol. Chem.* **163**, 313.
Touster, O. (1951). *J. biol. Chem.* **188**, 371.
Wilson, M. L. & McGinty, D. A. (1949). *Amer. J. Physiol.* **156**, 377.
Woolf, L. I. (1949). *Lancet*, **1**, 757.
Work, E. (1949). *Lancet*, **1**, 652.