Chem. Pharm. Bull. 30(5)1760—1765(1982)

Studies on Ergothioneine. VI. Distribution and Fluctuations of Ergothioneine in Rats

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(Received August 24, 1981)

(1) Ergothioneine is contained as a component of the organella membranes in the rat liver, and is liberated by heat treatment or dithiothreitol treatment. The amount of bound form increases with aging up to 11 weeks of age, and then decreases. All of the ergothioneine measured by the normal determination procedures can be considered to be free form.

(2) Ergothioneine is bound to proteins in the blood plasma, but is present in free form in erythrocytes.

(3) Biosynthesis of ergothioneine from amino acids in rats was not detectable. Ergothioneine is ingested from the diet and accumulated in the body.

(4) When dietary ergothioneine is restricted for 18 weeks, the amount in the liver falls to a threshold value but apparently does not fall further, and no deficiency symptoms are observed.

(5) Experimental ergothioneine-free rats were successfully produced in the second generation (born to parent rats fed on ergothioneine-free diet).

Keywords——ergothioneine; ^{3}H-ergothioneine; ergothioneine-accumulation; ergothioneine biosynthesis; binding form; ergothioneine free diet; ergothioneine deficiency rat

In the previous paper, we reported a method for determining by high performance liquid chromatogrephy (HPLC) the amount of ergothioneine (2-mercaptohistidine trimethyl betaine) (Erg) in biological samples.[1] Erg is known to be formed *via* hercynine from histidine, methionine, and cysteine in wild-type strains of microorganisms.[2–6] In animals, however, its biosynthesis has been considered not to occur, though there has been no report on deficiency symptoms.[2,7]

We examined the possibility of Erg biosynthesis by intestinal flora and found higher contents in animals having no microorganisms than in control animals having intestinal flora. We also considered the formation of a substance having a repressive effect by intestinal flora, but could not reach any conclusion owing to the insensitivity of the method for determination.[8] Though we had found that the intake of Erg from feed apparently accounted for the amount accumulated,[1] in this report we reexamined the possibility of biosynthesis of Erg in rats from RI-labeled, Erg-related amino acids. We also examined the subcellular distribution and the content fluctuation in liver, the presence of Erg-binding proteins, and the effects on these of starvation and Erg-free diet.

Experimental

Animals——Wistar strain rats were maintained in stainless steel cages with a mesh floor at 23±1°C and 50—60% humidity, and with light and dark periods of 12 h each. Rats were given diet pellets (CLEA Japan, Inc.) and water *ad libitum*.

Fasting Tests——The animals were given only water and fasted for 2 weeks in separate cages. During the period, 6 animals in each group were sacrificed by exsanguination under ether anesthesia at prescribed intervals, and the livers were perfused with saline, freed of blood, and washed.

Tests with an Erg-free Diet——Eight groups of 3 rats were fed for 25 weeks on the complete diet, composed of 20% casein, 10% corn oil, 2% vitamin mixture, 5% salt mixture and 63% glucose. Since the commercial casein contained Erg, it was purified by isoelectric point precipitation three times followed by lyophilization

to remove Erg. The complete diet was used after it had been confirmed to contain no detectable Erg, and it was also confirmed that the rats could not indulge in coprophagy in the metabolic cages.

The second generation of rats, produced by crossbreeding male and female rats fed on Erg-free diet, was also examined in the same way.

Examination of Erg-binding Protein——Various tissues were homogenized with cold saline. An aliquot of the homogenate was treated at 100°C for 15 min with 20 mM dithiothreitol (DTT) or 1% sodium lauryl sulfate (SDS), and Erg in the supernatant fraction was measured. The recovery of Erg in this treatment was reproducible and satisfactory (103±4.0%).

Erg-containing Fraction from Tissues——Erg fraction was obtained by chromatography of samples of each tissue on Amberlite CG-400 (1×16 cm), with 0.1 M Gly-buffer pH 7.9 as the eluent (flow rate 0.4 ml/min), followed by rechromatography on LCR-2 (0.8×30 cm) with 0.25 N Li-citrate-buffer pH 2.4 as the eluent (flow rate 0.84 ml/min at 30°C). Determination of Erg was carried out by means of the diazo reaction.[1]

RI-Labeled Compounds——^{35}S-Cys (352 μCi/ml; Spe. Act. 105.6 mCi/mmol), [^{14}C-methyl]Met (2 μCi/ml; Spe. Act. 5.3 mCi/mmol) and ^{14}C-labeled amino acid mixture (48 μCi/ml; Spe. Act. 55 mCi/m atom carbon) were purchased from the Japan Radio Isotope Association. Administration was by intraperitoneal injection in all cases. ^{3}H-Ergothioneine (^{3}H-Erg), labeled with 100Ci T_2O was obtained from the Japan Radio Isotope Association and purified as described in the preceding paper,[1] and a sample having 30 μCi/100 μg specific radioactivity and not less than 99.99% radiochemical purity was used. The radioactivity was measured with a liquid scintillation counter (Packard Co., Ltd.) and dpm values were determined by external source correction.

Results and Discussion

Subcellular Distribution of Erg in Hepatic Cells of Normal Rats

We fractionated hepatic cells by sucrose density gradient centrifugation[9] and measured the distribution of administered ^{3}H-Erg in subcellular fractions as shown in Table I.

Table I. Subcellular Distribution of Radioactivity in Rat Liver after a Single Injection of ^{3}H-Ergothioneine[a]

Fraction	10 min after injection of ^{3}H-Erg			2 weeks after injection of ^{3}H-Erg			
	dpm	%	dpm / mg protein (I)	dpm	%	dpm / mg protein (II)	II/I×100
Nucleus	1404	3.4	968	384	2.6	222	22.9
Mitochondria	7289	17.5	876	3565	24.2	561	64.0
Microsomes	5830	14.0	820	1661	11.3	296	36.0
Supernatant	27103	65.0	5202	8937	60.8	2174	14.8

a) *i.v.* injection (30 μCi/100 μg/0.1 ml).

In hepatic cells, at 10 min after the injection of ^{3}H-Erg, 65% of the total radioactivity was found in the supernatant fraction, about 15% each was distributed in the mitochondria and microsomes, and only a trace amount was found in the nucleus. Two weeks later, there had been a transfer of radioactivity to the mitochondria which probably represents the distribution of ^{3}H or $^{3}H_2O$ formed by degradation, but about 60% of ^{3}H-Erg was still distributed in the supernatant fraction. These findings suggest that Erg is mainly located in the cytoplasmic fraction.

Examination of the Forms of Erg

We then investigated whether Erg was present in the free form or the bound form. As shown in Fig. 1, the amount of Erg found in the lung, heart, spleen and liver increased after DTT treatment. In the liver, it increased by 44% after the DTT treatment but it was not affected by treatment with SDS. Further, as shown in Table II, the largest amount of Erg was recovered after the DTT treatment of the 10000 rpm cold-saline-washed residue.

Moreover, we attempted to recover Erg by 1% SDS treatment of the residue of the cold saline extraction. After the SDS treatment, the sample was dialyzed against SDS-containing medium. Eighty-eight % of the residual Erg was recovered in the outside solution, and another 12% of Erg was recovered in the outside solution after DTT and heat treatments of the solution inside the dialysis sac in the case of a liver sample. It was considered that the Erg extracted by treatment with hot saline was derived from various membrane vesicles in the cells, while the Erg released by DTT treatment may have originated from –S–S– binding to them. In other words, a part of the Erg exists in a bound form and can be freed by these treatments.

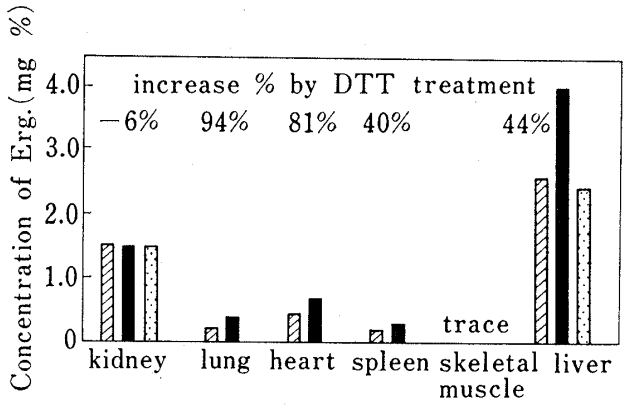

Fig. 1. Treatment of Rat Organs with DTT or SDS for Determination of Ergothioneine

Organs from 6 rats (400 g body weight) were pooled.
■ DTT treatment: Liver was homogenized with saline containing 20 mM DTT and heated at 100°C for 15 min.
▨← SDS treatment: Liver was homogenized with 1% SDS.
▨→ Saline (control).

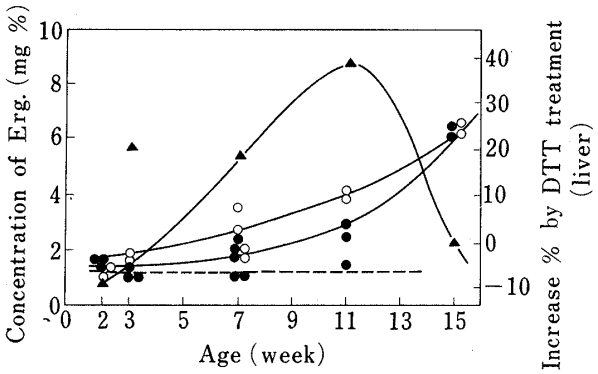

Fig. 2. Concentration of Ergothioneine in the Liver and Kidney of Rats of Different Ages

Concentration of ergothioneine: —●— by saline extraction, —○— by DTT treatment, —— in the liver, ------in the kidney.
Increase % in the liver after DTT treatment: —▲—

TABLE II. Comparison of Ergothioneine Content obtained with Different Extracting Procedures

Treatment	Saline[a)]	DTT[b)]	SDS[c)]	SDS+DTT[d)]
Exp. 1 (μg)	336.0	479.3	271.0	439.0
Exp. 2 (μg)	301.8	413.4	283.6	435.3

Rat liver was homogenized with saline in an ice bath and centrifuged at 10000 rpm for 1 h at 0°C. The residue was washed twice with saline. An aliquot of the washed residue was *a*) heated at 100°C for 15 min in saline, *b*) heated in saline containing 20 mM dithiothreitol (DTT) under the same conditions as for saline extraction, *c*) treated with 1% sodium lauryl sulfate (SDS) in saline, and *d*) treated with 1% SDS saline, followed by 20 mM DTT treatment.

Then the changes in the amount of bound form were investigated in relation to the age of rats (Fig. 2). The bound form could not be found in the kidney. In the liver, the bound form increased with age, reaching the maximum at 11 weeks of age, and then decreased.

In order to investigate the presence of high molecular substances which bind Erg, 0.1 ml of saline solution of ^{3}H-Erg was administered to rats and the 105000×***g*** supernatant fractions of homogenates of liver and kidney were subjected to gel-filtration (Fig. 3). ^{3}H-Erg was all recovered in the low molecular fraction coinciding with Erg and no radioactivity could be found in macromolecular fractions.

Erg in the blood is known to be transferred from the plasma to erythrocytes.[1)] At 1 h after administration of ^{3}H-Erg, the plasma was collected by heparinization followed by gel-filtration (Fig. 4). In contrast to the cases of the liver and kidney (Fig. 3), ^{3}H-Erg was recovered in the macromolecular fraction. When this plasma was stored at 0°C for 8 h before

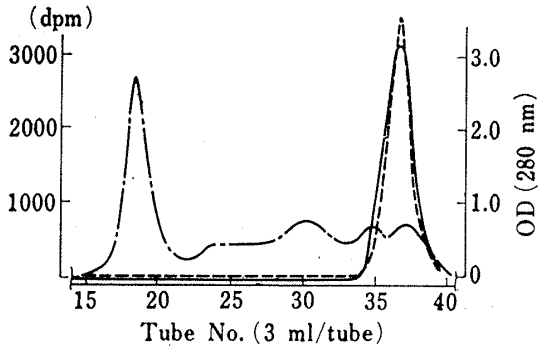

Fig. 3. Gel-Filtration of Liver and Kidney Cytosols after Injection of ^{3}H-Ergothioneine

Rats were sacrificed at 1 h after a single *i.v.* injection of 100 μg of ^{3}H-ergothioneine (30 μCi) in 0.1 ml saline. Cytosols of tissues were applied to a Sephadex-G-25 column (2.2 × 31 cm) and eluted with saline (flow rate 35 ml/h).
------, Liver;——, kidney; ———, OD at 280 nm.

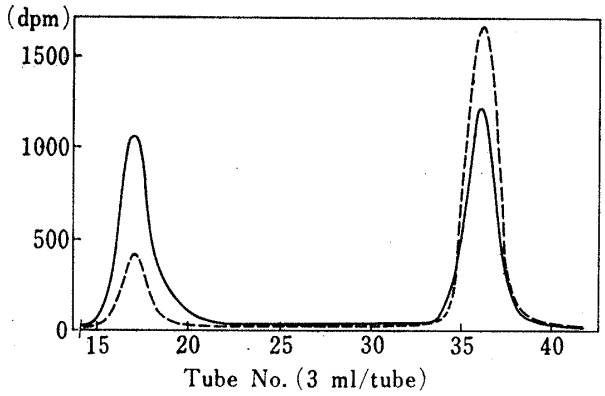

Fig. 4. Gel–Filtration on Sephadex–G-25 of Rat Plasma 1 h after a Single *i.v.* Injection of ^{3}H-Ergothioneine

The animal treatment and conditions of gel-filtration were the same as in Fig. 3.
------, Fresh plasma;——, plasma incubated at 0°C for 8 h.

Table III. ^{3}H-Ergothioneine Replacement on Addition of Cold Ergothioneine, Histidine or Crysteine to the ^{3}H-Ergothioneine Bound Fraction in Rat Plasma

Addition	Incubation		Substitution rate (%)[c]
	before[a] (dpm)	after[b] (dpm)	
None	2900	2900	0
Ergothioneine	2750	670	75.5
Histidine	2800	2560	8.6
Cysteine	2700	1930	28.5

Two ml of ^{3}H-ergothioneine bound fraction obtained by Sephadex G-25 column chromatography was incubated with 1 μmol of each compound at 0°C for 4 h.
a) Total dpm in ^{3}H-ergothioneine bound fraction.
b) Residual dpm in ^{3}H-ergothioneine bound fraction after incubation.
c) Liberation of ^{3}H-ergothioneine from bound fraction.

gel-filtration, ^{3}H-Erg in the macromolecular fraction was greatly increased. In order to further confirm the binding properties, ^{3}H-Erg was added to 2 ml of the plasma *in vitro*, allowed to stand at 0°C for 8 h, and then subjected to gel-filtration. Radioactivity due to ^{3}H-Erg was present in the macromolecular fraction. When this fraction bound *in vitro* with ^{3}H-Erg was treated with a large excess of cold Erg (1 μmol) at 0°C for 4 h, 75.5% of ^{3}H-Erg was released. When the bound ^{3}H-Erg was treated with a large excess of His or Cys, only 8.6% or 28.5% of ^{3}H-Erg was liberated, respectively (Table III). These results suggest the presence of a binding substance specific for Erg in the plasma.

On the other hand, in the erythrocyte hemolysate, ^{3}H-Erg was found only in its free form. Generally, Erg can be considered to be contained in the liver and kidney essentially wholly in its free form. The physiological significance of these results remains to be resolved by further investigations.

Retention of Erg in the Body

The level of ^{3}H-Erg decreased rapidly with time in the plasma, and was transferred to the erythrocytes. Its half-life in the blood was approximately 18 min. However, a small amount appeared to remain for a long time. Further, the turnover rate of ^{3}H-Erg was very slow after it had been distributed to the organs, and it tended to accumulate.[1] In this paper, we carried

Table IV. Effect of Diet on Ergothioneine Levels in Rat Liver and Blood

		Normal	Rats fasted for 14 d	Rats refed for 3 d[a)]
Ht. value (%)		39±1	56±0.04[c)]	45±1[b)]
Serum protein (mg/ml),		70±1	75±3	71±0.04
Erg	Liver (mg/100 g)	3.7±0.04	2.8±0.1[b)]	3.2±0.1
	Blood (μg/100 ml)	466±12	298±14[b)]	371±27

a) Ergothioneine-free diet.
b) $<p=0.005$.
c) $<p=0.001$: Significant differences from normal value.
Rats were fasted for 14 d and refed on amino acid mixture for 3 d. Values are means ±S.E. of 3 rats.

out a drastic fasting test in which only water was given over a period of 14 days to examine whether Erg was derived from the feed. When an Erg-free diet was given for 3 days after 14 days of starvation, the hematocrit value, which had been raised by starvation returned to normal (Table IV), and the Erg level, which had decreased significantly to 25% and 37% of the control level in the liver and blood, respectively, showed a slight recovery.

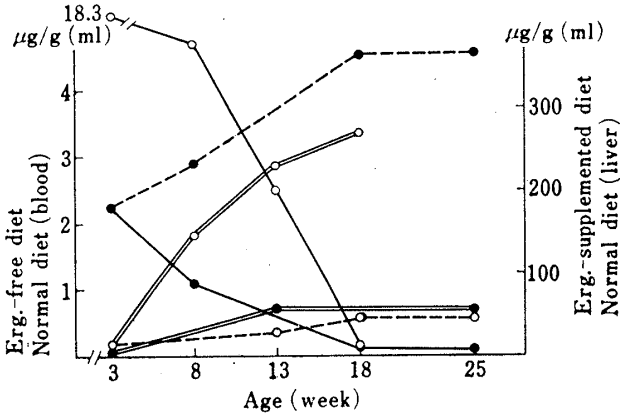

Fig. 5. Ergothioneine Content in the Liver and Blood under Various Diet Conditions
——, Ergothioneine-free diet;═══, ergothioneine-supplemented diet;------, normal diet;○, liver;●, blood.

Possibility of Biosynthesis of Erg in the Rat

Concerning the biosynthesis of Erg in animals, no incorporation of radioactivity into Erg was observed in rat experiments previously conducted using [^{14}C-methyl]Met and [^{14}C-imidazol] His.[7)] We reexamined this result by intraperitoneally administering 12 μCi/0.1 ml/rat of a mixture prepared by adding [^{14}C-methyl]Met and ^{35}S-Cys to a uniformly labeled ^{14}C-amino acid mixture to rats at 3 and 5 weeks of age. The radioactivity was measured in Erg fractions purified by column chromatography (Table V). The possibliity of biosynthesis of Erg seemed

Table V. Incorporation of Radioactivity into Ergothioneine Fraction as determined by Column Chromatography

	Age (weeks) 3[a)]	5[b)]
1 h after injection		
Liver (dpm/10 g)	1924±283	1418±377
Blood (dpm/10 ml)	283±8	202±11
after treatment for 4 d		
Liver (dpm/10 g)	534±143	152±7
Blood (dpm/10 ml)	432±37	126±7[c)]

a) Body weight (g): 35±1.
b) Body weight (g): 91±2.
c) $<p=0.005$: Significant differences from 1 h value.
Labeled amino acid mixture (12 μCi) was administered at 0.1 ml a day.

negligible in view of the results in the table and the very slow turnover rate as described above. The apparent counts were around $10^{-3}\%$ or lower ($7\times10^{-3}\%$ at the most) relative to administered total counts.

Attempts to experimentally induce Erg-deficiency Diseases

Rats (3 weeks of age) were divided into 3 groups and maintained up to 25 weeks of age on 3 kinds of diets; normal (pellets by CLEA Japan: Erg content, 13.7 μg/10 g), Erg-free and Erg-supplemented (5 mg/100 g). Changes in Erg levels in the liver and blood are shown in Fig. 5. In the group given an Erg-supplemented diet, Erg accumulated with time, and the blood levels reached 50—85 μg/ml in 10 weeks. Erg accumulated up to 270 μg/g in the liver. In the group given a deficient diet, the Erg content of the liver decreased more rapidly than in the group given a normal diet, and the content reached the minimum value 15 weeks later, thereafter maintaining a very low level without apparently reaching zero. In this period no abnormalities in the rats were observed. The fact that the amount of Erg, under these conditions, did not fall below a threshold value, suggests its physiological significance.

Mating was carried out between male and female rats fed on an Erg-free diet for 100 days, and rats of the second generation were examined for Erg content 12 weeks after birth. No abnormalities were observed in the reproduction, delivery, or growth of rats. No measurable amount of Erg in the liver or blood, and no Erg deficiency symptoms were detected in rats born to parent rats which had been fed on an Erg-free diet.

References and Notes

1) T. Mayumi, H. Kawano, Y. Sakamoto, E. Suehisa, Y. Kawai, and T. Hama, *Chem. Pharm. Bull.*, **26**, 3772 (1978).
2) D.B. Melville, S. Eich, and M.L. Ludwig, *J. Biol. Chem.*, **224**, 871 (1957).
3) D.B. Melville, M.L. Ludwig, E. Inamine, and J.R. Rachele, *J. Biol. Chem.*, **234**, 1195 (1959).
4) A. Achari and D.B. Melville, *J. Biol. Chem.*, **237**, 1615 (1962).
5) D.S. Genghof and Olga V. Damme, *J. Bacteriol.*, **95**, 340 (1968).
6) Y. Ishikawa, S.E. Israel, and D.B. Melville, J. *Biol. Chem.*, **249**, 4420 (1974).
7) D.B. Melville, *J. Biol. Chem.*, **213**, 61 (1955).
8) T. Konishi, N. Tamaki, F. Tsunemori, N. Masumitsu, H. Okumura, and T. Hama, *Vitamins*, **46**, 127 (1972).
9) G.H. Hogeboon, *Methods in Enzymology*, **1**, 16 (1955).