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Studies on Ergothioneine. V.[1] Determination by High Performance Liquid Chromatography and Application to Metabolic Research

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The high performance liquid chromatographic procedure was established for the determination of ergothioneine in biological materials. The procedure is also applicable to the purification of ^{3}H-ergothioneine.

The metabolism of ergothioneine given to rats was very slow; the ergothioneine was not disappear even after 1 week fasting. The ergothioneine administrated was mostly concentrated in the liver and the level was 10 times as high as that in blood. All ergothioneine in the blood was found in blood corpuscles 24 hr after the administration.

Keywords——ergothioneine; high performance liquid chromatography; tritiated ergothioneine; thiopropyl-sepharose 6B; affinity chromatography; content in rat tissue; distribution in rat

The authors have been studying ergothioneine (2-mercaptohistidine trimethyl betaine) to elucidate its physiological role as a part of their investigation on histidine derivatives which are physiological constituents in animal organism. Since Melville reviewed ergothioneine,[3] especially regarding its physiological function, in 1959, various investigations of the compound have been carried out. The major physiological role has not been clarified yet. It was reported by the present authors that respiration and survival rate of spermatozoa in guinea pig sperm, where the ergothioneine content is high, are enhanced by the compound.[4] In addition, ergothioneine labeled with tritium was prepared to study its distribution into the whole body and hepatosubcellular fraction of rats 1 or 24 hr after the administration, but we could not avoid using the specific radio activity which was calculated by a simplified method based on colorimetry.[5]

A high performance liquid chromatographic procedure for the separation and the determination of ergothioneine was established and the *in vivo* dynamics was precisely investigated by taking advantage of the chromatographic procedure.

Experimental

Animals——Wistar strain rats used in this study were maintained on commercial food (Nippon Clea Lab.) and water *ad libitum*, in a room with regulated temperature (23°±1°), air (50% fresh air with 60% moisture) and lighting (12 hr light and 12 hr dark).

High Performance Liquid Chromatography (HPLC)——A Hitachi Model 635 or 633 apparatus was used. A column (1/4 inch × 1 ft) was packed with μ-Bondapak CH by Waters' Co. Ltd., which was carefully chosen after examinations, and eluted with a mixture of acetonitril and water (80: 20). The compound was identified by the retention time and the elution pattern in which each peak was checked with a Union Technical Institute SM-303 high speed UV-spectromonitor.

1) Part IV: T. Hama, H. Okumura, N. Tamaki, and T. Konishi, *Yakugaku Zasshi*, **93**, 369 (1973). This work was presented at the 3rd Symposium on Analytical Chemistry of Biological Materials, Tokyo, Nov. 11, 1977.
2) Location: *Arise, Igawadani-cho, Tarumi-ku, Kobe 673, Japan.*
3) D.B. Melville, *Vitamin and Hormone*, **17**, 153 (1959).
4) T. Hama, H. Okumura, N. Tamaki, and T. Konishi, *Yakugaku Zasshi*, **93**, 369 (1973).
5) T. Hama, T. Konishi, N. Tamaki, F. Tsunemori, and H. Okumura, *Vitamin*, **46**, 121 (1972).

labeling ^{3}H-ergothioneine
(Radiochemical Center, Amersham)
↓
Sephadex G-25
↓
rechromatography
↓
Amberlite CG-400
↓
rechromatography
↓
HPL chromatography
(μ-Bondapak CH column)

Fig. 1. Scheme of Purification of Tritiated Ergothioneine

Ergothioneine was labeled by catalytic exchange in tritiated aqueous medium. Explanation is described in the legend to Fig. 2.

Tritium Labeled Ergothioneine——^{3}H-Ergothioneine was prepared by the Radio Isotopes Association by means of T_2O treatment of cold ergothioneine, a product of Sigma. The ^{3}H-ergothioneine after purification had a specific radio activity of 30 mCi/100 mg and the radiochemical purity of 99.99%. The procedure of the purification is shown in Fig. 1 and 2.

Measurement of Radio Activity——The sample was dissolved in 10 volumes of Packard's cocktail Insta-gel and the radio activity was counted by a Packard Model 3320 liquid scintillation counter. External standard was employed for the calculation of dpm.

Thiopropyl-Sepharose 6B——This reagent was purchased from Pharmacia.

Results

Determination by High Performance Liqiud Chromatography

Preparation of Samples——(1) Blood: A blood sample, 5 ml, taken from rat aorta was mixed with 10 ml of H_2O for hemolysis. The mixture was kept at 95° for 15 min and centrifuged. The supernatant was lyophilized.

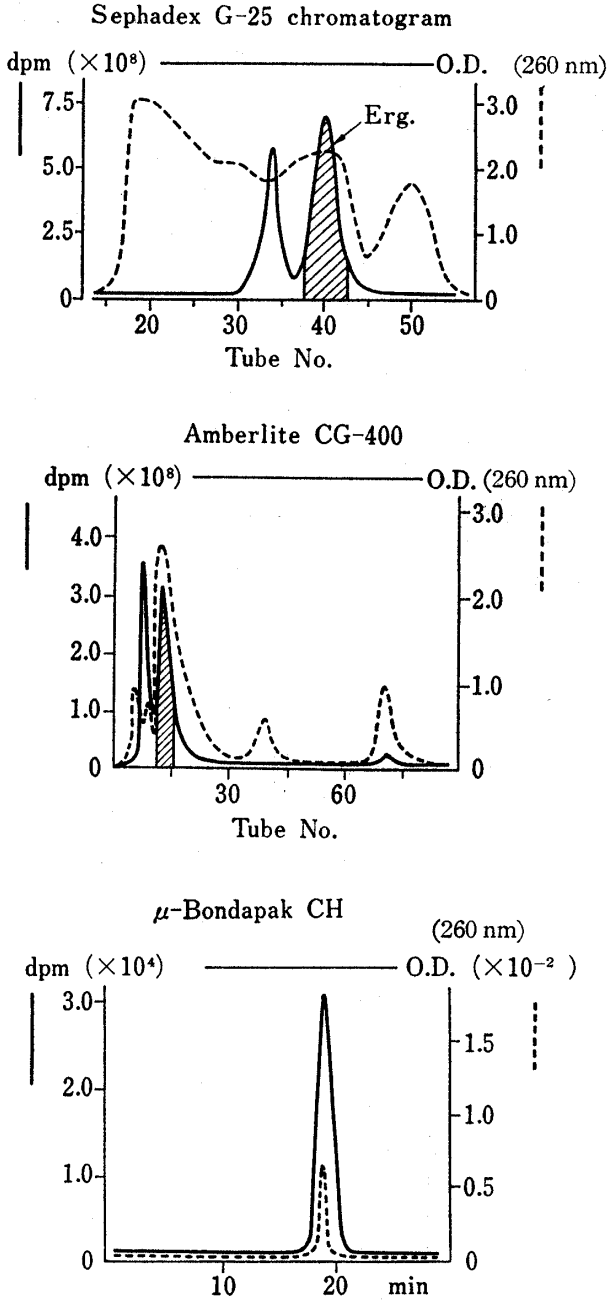

Fig. 2. Purification of Tritiated Ergothioneine by Sephadex G-25, Amberlite CG-400 and μ-Bondapak CH Chromatography

Sephadex G-25 chromatogram: column size; 2.0×50 cm, eluent; 0.015 M NaCl, flow rate; 25 ml/hr, tube; 4 ml. Amberlite CG-400 chromatogram: column size; 1×22 cm, eluent; 0.1 M Gly-buffer (pH 9.7), flow rate; 10 ml/hr, tube; 2 ml. μ-Bondapak CH chromatogram (high performance liquid chromatography): eluent; 20% H_2O in acetonitrile, flow rate; 1 ml/min.

(2) Organs: Taking the liver as an example, the rat was anesthetized with ether and bled. The liver was immediately perfused from the vena portae with cold saline to remove remaining blood. The 1—2 g of the tissue, accurately weighed was homogenized with 2 volumes of water in a glass homogenizer at 10 strokes per min to prepare a 33% homogenate. An

aliquot was kept at 95° for 15 min and centrifuged. The supernatant was lyophilized. Other tissues were treated in the same manner.

(3) Lyophilized Samples: The samples were dissolved in 1.5 to 2.0 ml of 50% ethanol and the solution was centrifuged. The supernatant, 10 μl, was subjected to further study.

(4) Standard: Ergothioneine·$2H_2O$ was dissolved in 50% ethanol to prepare a 2 μmol/ml stock solution. The stock solution was diluted to prepare solution containing 100, 250, 500, 1000 and 2000 pmoles of ergothioneine/10 μl. The peak height measured by absorbance at 254 nm of wavelength was linearly related to the concentration in the range studied. An inner standard was practically unnecessary.

(5) Pretreatment for Food, Urine and Matters as required: A fractionation was performed by the thiopropyl-sepharose 6B column chromatography according to demand. In case that there were some difficulties during the procedure to prepare an estimation sample, because of the large volume of starting material or lyophilized residue, affinity column chromatographic separation was performed. Analysis for 24 hr urine is shown in Fig. 3. Ten to twenty ml of urine was adjusted to pH 2.0 with 1.0 N HCl and was applied to a column (5 mm × 10—14 cm) of thiopropyl-sepharose 6B (bed vol. 4 ml) previously equilibrated with 0.01 N HCl containing 1 mM ethylene diaminetetraacetate (EDTA). The column was washed with the same solution after sampling. And then the ergothioneine was replaced and eluted with

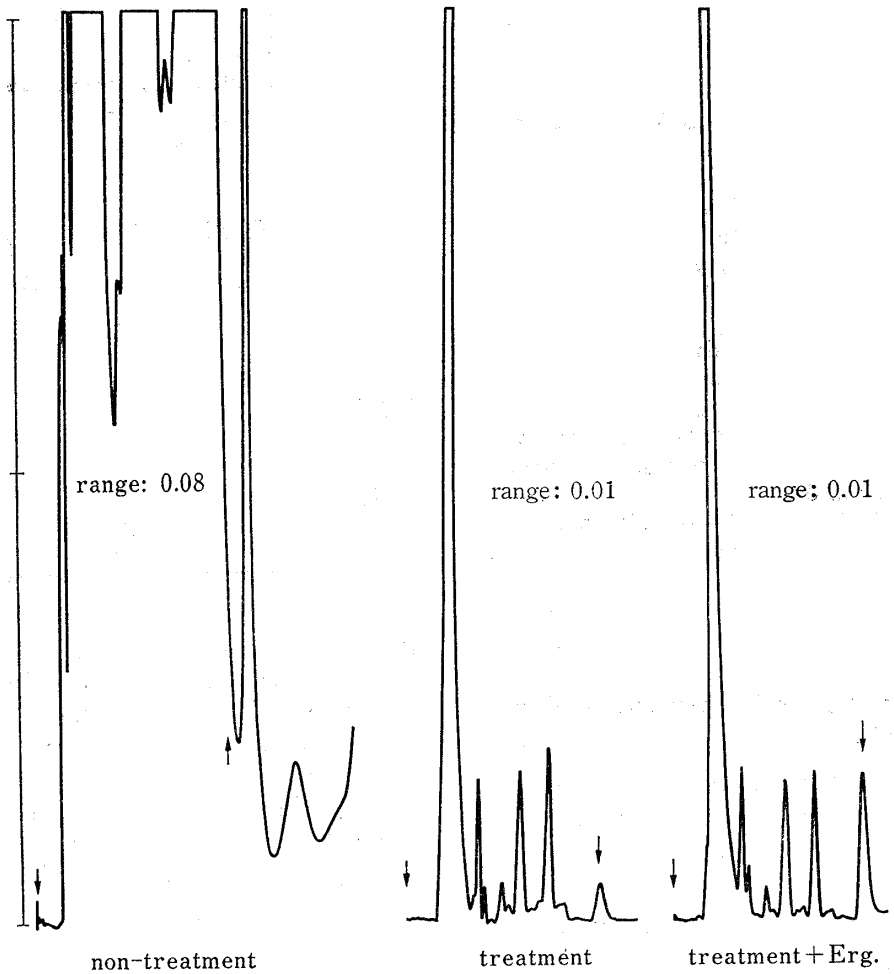

Fig. 3. Removal of Interfering Contaminant to HPLC by Thiopropyl-Sepharose 6B Chromatograpy

Example for 24 hr urine of rat. Sampling: correspond to 10 μl of five-fold dilution urine. Treatment procedure is described in the text. The column consists of 4 ml bed vol. and contains approx. 80 μmole 2-thiopyridyl.

0.1 M Tris buffer (pH 8.0) or H_2O, containing 1 mM EDTA and 20 mM dithiothreitol (DTT). Five ml fractions were collected at a flow rate of 1 ml/min. The ergothioneine containing fractions were checked by ultraviolet (UV)-spectromonitor and combined. The recovery rate was 95—104% and reproducible. This pooled fraction was lyophilized.

Glutathione-2-pyridyl disulfide sepharose (activated thiol sepharose 4B) was not suitable because it is impertinent to affinity chromatography of ergothioneine.

Measurement——The column was packed with μ-Bondapak CH in preference to Hitachi Gel No. 3010, No. 3011 or No. SLX–10DS. After the trials with water, 0.025 M KH_2PO_4, CH_3CN: 0.025 M KH_2PO_4(20: 80), H_2O: CH_3CN (20: 80), H_2O: CH_3CN (30: 70), H_2O: CH_3CN (40: 60) and H_2O: CH_3CN (50: 50), the best separation was obtained when the column was eluted with a mixture of CH_3CN and H_2O (80: 20) as shown in Fig. 4. The recovery rate of ergothioneine added to biological materials was exactly 100%. A calibration curve prepared in terms of peak height on the HPL chromatogram (Fig. 4). The wavelength employed was 254 nm and the molecular extinction coefficient, E^{1M}_{1cm}, was 1.40×10^{-4}.

When the column was eluted at the flow rate of 1 ml/min with the eluate containing 79 to 81% of CH_3CN at room temperature, the retention time of ergothioneine was 18±1 min. The elevation of column pressure was eliminated by pre-washing with 50% CH_3CN for 20 min. The column was then equilibrated with 80% CH_3CN. Cysteine, cystine, glutathione, DTT and mercaptoethanol did not disturb the determination.

Examination of the Substances in the Eluate——Authentic ergothioneine charged on the column was eluted showing a single peak with retention time of about 18 min, which was established as a quantitative method. In order to examine the possible chemical change of ergothionine during the chromatography and the applicability of the procedure to the separation and purification of ^{3}H-ergothioneine administrated in the body, the ^{3}H-ergothioneine purified as shown in Fig. 2 was added to the specimen and collected using a fraction collector and the radioactivity and UV absorption were measured. The results were shown in Fig. 5 (HPL chromatograms) and Fig. 6 (records by a high-speed spectromonitor). The examination of the eluate peak No. 9 from HPLC as shown in Fig. 6 did not show any chemical transformation during the analysis. The peak No. 9 fraction in Fig. 5 was submitted to rechromatography by HPLC under the same condition and checked its UV spectrum and radioactivity. These results agreed with those obtained on HPLC in Fig. 2. It was considered

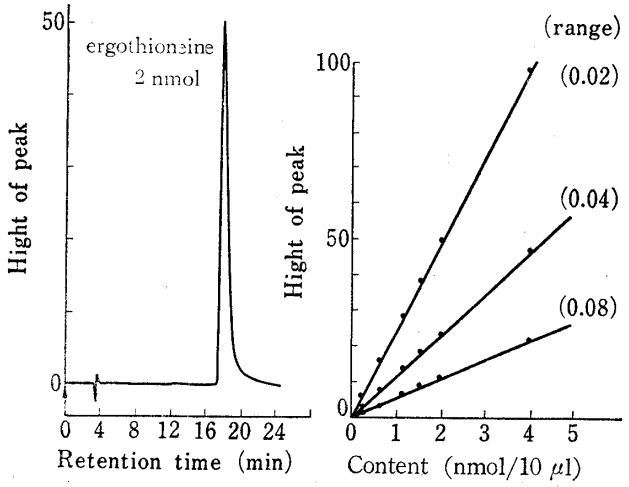

Fig. 4. HPL Chromatogram of Ergothioneine and Calibration Curves

Column: μ-Bondapak CH, φ 40 m/m×30 cm. Eluate: CH_3CN: H_2O (80: 20). Flow rate: 1 ml per min. Pressure: 40 kg per cm^2. Temp.: room temp. Chart speed: 2.5 mm per min.

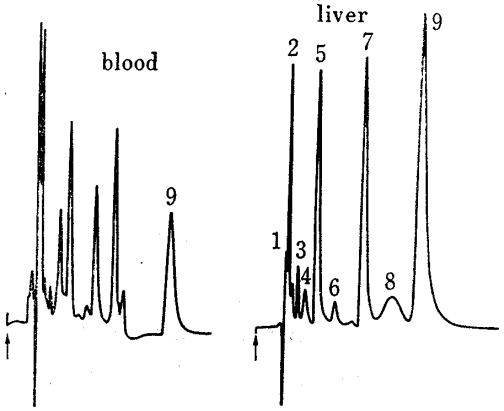

Fig. 5. Determination of Ergothioneine in Blood and Liver of Rat

HPLC: Conditions are described in the legend to Fig. 4. Range: 0.005 to blood, 0.04 to liver.

that tritium labeling was stable through the analytical procedure. So the peak No. 9 in Fig. 5 was confirmed as ergothioneine.

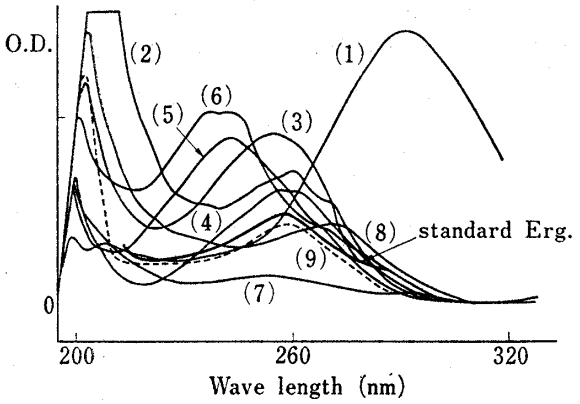

Fig. 6. UV-Scannings of Each Peak from Liver Chromatogram by HPL Chromatography

UV-absorption spectra are measured with high-speed spectromonitor. Numberings correspond with the peak numbers shown in Fig. 5.

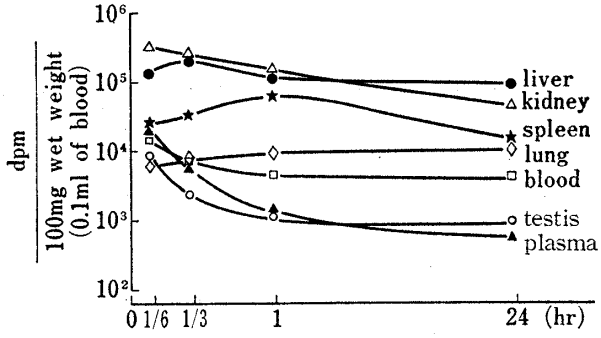

Fig. 7. Retention of Radioactivity in the Ergothioneine Fraction by HPL-Chromatography from Various Tissues of Rat administrated Tritiated Ergothioneine

Single intravenous injection of 100 μg of ^{3}H-ergothioneine (30 μCi) in 0.1 ml saline is performed at 0 time.

Table I. Ergothioneine Content in Rat Organs, Urine, Feces and Diet, and Daily Balance

	Ergothioneine mg/wet tissue (100 g)		
	Present method[a]	Diazo method[b]	PCMB method[c]
Liver	4.83±0.45	13.3	4.74
Kidney	1.87±0.07	4.3	6.73
Heart	0.70±0.05	1.5	2.52
Spleen	1.47±0.05	1.1	4.82
Lung	0.69±0.06	1.5	—
Brain	0.21±0.02	—	—
Testis	0.24±0.01	—	—
Sem. Ve. (with fluid)	1.06±0.09	—	—
Blood	0.28±0.01	4.1[d]	1.60[d]
Whole body	*ca.* 928—1000 μg		
24 hr urine	0.94±0.08 μg		
24 hr feces	1.0 μg		
24 hr diet (12—17 g)	16.4—23.3 μg (mean 19.9)		
[Assessed balance]	intake−(feces+urine)≒uptake uptake=deposit+degradation 19.9−(1+0.94)≒18 μg/day $\frac{\text{whole body content}}{\text{uptake/day}} = \frac{1000}{18} = 56$ (day) 220 g body weight ≒7—8 week age after weaning		

mean± standard error.
a) Male rat, body weight 220 g.
b) By Melville *et al.* 6).
c) By Hama *et al.* 5).
d) Rabbit.

Application of HPLC Determination

Determination of Ergothioneine in Rat Organs——Ergothioneine contents in various tissues in rats were determined by this HPLC procedure. The results thus obtained were compared with those determined by conventional methods (Table I).

6) D.B. Melville, W.H. Horner, and R. Lubschez, *J. Biol. Chem.*, **206**, 221 (1954).

No significant difference was observed between the those values. The micro amount (pmol order) of ergothioneine in biological materials can be determined by this method in a short time without any interference with other physiological components in the tissue. The recovery test of authentic ergothioneine added to estimating samples was about 100% as rate. These observations lead us to conclude that this method is an excellent procedure for the determination of ergothioneine.

As shown in Table I, an ergothioneine balance of intake and accumulation was impossible to calculate exactly the value, but reasonably good approximation could be made. From assessed balance, it should seem that consumed ergothioneine was accumulated without any biodegradation or biosynthesis in the body.

Distribution of Ergothioneine in Rats——*In vivo* dynamics of ergothioneine was studied in rats given the ^{3}H-ergothioneine by the HPLC coupled with the radiochemical analysis. The distribution of radioactivity in various organs was studied various times after the injection of 0.1 ml of ^{3}H-ergothioneine (30 μCi/100 μg) solution into femoral vein. The results are shown in terms of dpm (Fig. 7). The decreases of the radioactivity in plasma were faster than in whole blood. The plasma concentration 24 hr after the injection was 4% of the concentration 10 min after the injection, while the concentration in whole blood after 24 hr was 31% of the concentration after 10 min. The half life of ergothioneine in whole blood was about 18 min.

The metabolic turn over rate in various organs appeared to be low. When 1.6 mg/100 g B.W. of ergothioneine was daily given to rats for 1 week, the ergothioneine content in blood and liver was multiplied by 12 and 7, respectively (Fig. 8). No decrease in the level of ergothioneine was observed in blood and liver after fasting for 1 week. Moreover, the ergothioneine concentration in liver increased significantly after fasting for 1 week because of the decrease in liver weight. Ergothioneine, anyhow, is well retained in the tissues.

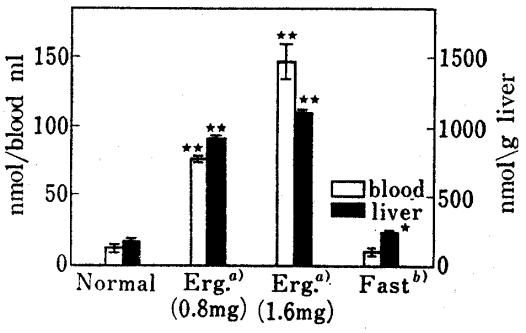

Fig. 8. Ergothioneine Content in Liver and Blood of Rat after Fasting or Ergothioneine Administration

a) 0.8 or 1.6 mg per 100 g body weight of ergothioneine was daily administrated by intraperitoneal injection for a week.

b) Rats were maintained on water only *ad libitum* for a week. Autopsy carried out at 24 hr after the last injection.

Significant differences from normal:
★) $p<0.05$, ★★) $p<0.001$.

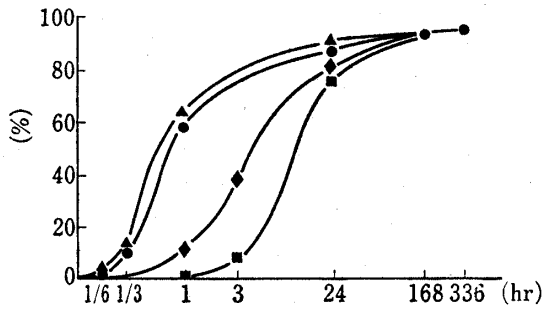

Fig. 9. Transport of Tritiated Ergothioneine into Blood Cell from Plasma

Transport rate is shown as % $\left(\frac{\text{radioactivity in blood cell}}{\text{radioactivity in whole blood}} \times 100\right)$.

Treatment of the experimental groups was as follows;
—●—: normal,
—◆—: Erg.; 0.8 mg/100 g B.W./day,
—■—: Erg.; 1.6 mg/100 g B.W./day,
—▲—: fast (1 week).

Treatments are described in the legend to Fig. 8.
Method of ^{3}H-ergothioneine administration was the same as that in Fig. 7.

The concentration curve of ergothioneine in blood was shown with the time in Fig. 9. The blood ergothioneine was maintained in blood corpuscles in the course of time. Both in fed and fasted rats, the rate of ergothioneine in the blood corpuscles to those in whole blood are 11% and 90%, 20 min and 24 hr after the injection, respectively. All the ^{3}H-ergothioneine in blood was incorporated into blood corpuscles till 168 hr regardless of the level of ergo-

thioneine pre-treatments, even though more time was required for the disappearance from plasma.

Discussion

Diazotization has been utilized for the determination of ergothioneine since Hunter first reported the method.[7] UV-spectrophotometry is also applicable once the compound has been identified as ergothioneine. Ergothioneine does not react with N-ethylmaleimide and reacts with *p*-chloromercuric benzoate. The present authors utilized this property to determine ergothioneine in biological materials.[5] The methods, however, were unsatisfactory because of complex procedures and poor sensitivity. The most excellent procedure for the specific determination of ergothioneine is established by employment of the HPLC. This method can detect the amount of pmol levels of ergothioneine without chemical transformation of the compound during the process. This chromatographic procedure is applicable not only to the purification of ergothioneine, but also to micro preparative chromatography when coupled with the labeled compound for *in vivo* studies. As a determination of ergothioneine in biological materials the method is very excellent in its accuracy, reproducibility, simplicity and rapidity.

The content value in rat organs obtained by the present method nearly agreed with the lower value as previously reported value, as an example, Melville reported 1.5—4.2 mg/100 ml of blood.

After the administration of ^{3}H-ergothioneine to rat, the *in vivo* distribution was studied by the method presented above. The method has been proved to be very useful for the study. Ergothioneine given to rats was first concentrated in the liver; the concentration in liver was 10 times as high as that in blood. Ergothioneine in blood was maintained and accumulated in blood corpuscles, and all the ergothioneine was found in the corpuscles 24 hr after the administration practically. The turn over rate of the ergothioneine *in vivo* was low. Such evidence become clear for the first time by the utilization of HPLC. Although high ergothioneine concentration in blood corpuscles has been known, it is first reported in this presentation that the liver concentration is 10 times high as blood concentration when ergothioneine is administrated. This HPLC will be employed for the future study on the interaction between endogenous ergothioneine and that administrated, the possibility of biosynthesis and the active form or binding form of ergothioneine in organs.

It is considered that the physiological behavior of the so-called *in vivo* thiol compounds can be clarified by a distinguishable analysis about ergothioneine.

7) G. Hunter, *Biochem. J.*, **22**, 4 (1928).