STUDIES ON THE ORIGIN OF ERGOTHIONEINE IN ANIMALS*

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(Received for publication, August 5, 1954)

The wide-spread occurrence of ergothioneine in animals (1–3) suggests that the compound is either synthesized by several animal species or is present in common foodstuffs. However, data in the literature on this point are equivocal and contradictory. Eagles and Vars (4) suggested that the corn protein, zein, contains 2-thiolhistidine which serves as a dietary precursor of ergothioneine. Repeated attempts by other workers to identify thiolhistidine as a constituent of corn have failed (5–7). The conclusion of Potter and Franke (8) that corn-containing diets lead to increased blood ergothioneine levels in rats, and the similar conclusion of Eagles and Vars with regard to pigs, could not be confirmed by Hunter (6).

Consideration of the structure of ergothioneine (I) suggests as likely precursors the amino acids histidine and 2-thiolhistidine, the methyl group of methionine, and the sulfur atom of methionine or cystine. In this paper we report studies undertaken to determine whether ergothioneine is synthesized in animal tissues from such precursors, or whether it is of solely dietary origin. Our results strongly indicate that ergothioneine is not synthesized by the rat, chicken, or guinea pig. While this work was in progress, Heath and coworkers (9) published data from which they concluded that the boar is capable of synthesizing ergothioneine.

EXPERIMENTAL

Materials—L-2-Thiolhistidine was prepared from L-histidine by modifications of the method of Ashley and Harington (5). L-Histidine labeled

* This work was aided by grants from the Nutrition Foundation, Inc., and Eli Lilly and Company.
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with C\textsuperscript{14} in the imidazole ring was prepared from NaC\textsuperscript{14}N by a method similar to that used by Borsook and coworkers (10) and was crystallized as the dihydrochloride from acetic acid ([\alpha]_D\textsuperscript{20} +6.68\degree, c = 14 in water; an authentic sample of L-histidine dihydrochloride gave +6.65\degree under similar conditions). L-Methionine labeled in the methyl group with C\textsuperscript{14} was prepared by the method of Melville, Rachele, and Keller (11). L-Methionine labeled with S\textsuperscript{35} was purchased from the Abbott Laboratories, North Chicago, Illinois.

Methods—Blood and liver were examined for ergothioneine by the chromatographic method previously described (3, 7). Briefly, this consists of treatment of the lyzed cells or homogenized tissue with glutathione and dithionite, precipitation of the proteins with trichloroacetic acid, treatment of the filtrate with IRA-410 acetate, and chromatography of the resulting material on alumina. The developing solvent was either 75 per cent aqueous ethanol, 80 per cent aqueous ethanol, or 75 per cent aqueous ethanol containing 1 per cent formic acid (3). The effluent from the column was collected in 1 ml. fractions, and alternate tubes were analyzed for ergothioneine by means of the color reaction with diazotized sulfanilic acid (12). The remaining fractions were tested for radioactivity by evaporation to dryness in a thin film on 25 mm. watch glasses and examination in a windowless flow counter. In most cases the residues weighed 0.2 mg. or less and were spread over an area of approximately 1 cm\textsuperscript{2}.

We have shown previously that the ergothioneine of rat tissues decreases to undetectable levels when the animals are fed purified diets containing casein as the source of protein (3). While this fact in itself suggests that ergothioneine cannot be synthesized by the rat, the possibility remains that purified diets lack a factor which is present in ordinary diets and which is essential for the synthesis of ergothioneine. To determine whether this factor might be L-2-thiolhistidine, two adult Sherman strain rats were placed on the purified diet and each was injected daily with 5 mg. of thiolhistidine for a period of 27 days. At the end of this time neither animal showed any detectable blood ergothioneine by the direct method of determination (12).

Administration of Histidine-2-C\textsuperscript{14} to Rat—A neutralized solution of 181 mg. of L-histidine-2-C\textsuperscript{14} dihydrochloride, possessing approximately 2 \times 10\textsuperscript{7} c.p.m. as determined by direct count on a 7 \gamma aliquot, was injected subcutaneously in equal daily doses for a period of 20 days into a 260 gm. Sherman strain male rat. The animal was maintained on a commercial stock diet (Rockland rat diet) which we have found to be adequate in maintaining blood ergothioneine levels. At the end of the injection period 8 ml. of blood were subjected to the chromatographic analysis procedure. The results of the ergothioneine and radioactivity determinations are shown in Fig. 1.
Administration of $\text{C}^{14}$-Labeled Methionine to Rat—An aqueous solution of 100 mg. of L-methionine labeled in the methyl group with $\text{C}^{14}$ and showing approximately $2.2 \times 10^7$ c.p.m. was injected subcutaneously into a 115 gm. Sherman strain male rat, maintained on the commercial stock diet, in equal daily doses for 20 days. At the end of this time blood from the animal was analyzed chromatographically. It was found that a radioactive peak partly overlapped the ergothioneine peak area. The liver from the same animal was therefore analyzed, but again a radioactive peak overlapped the ascending portion of the ergothioneine curve. The radioactive material was separated effectively from the ergothioneine by combining and rechromatographing the alternate fractions in the ergothioneine area which had been used for radioactivity determinations (Fig. 2).

A small amount of residual radioactivity was present in the fractions containing the ergothioneine. To determine whether this activity was present in the methyl groups of the ergothioneine, use was made of the fact that the methyl groups can be removed readily as trimethylamine by treatment of ergothioneine with strong alkali. The residue from Fraction 66, which contained more than 8 $\gamma$ of ergothioneine and showed an activity of 10 c.p.m., was heated in one arm of a small, sealed U-tube with a few drops of concentrated KOH solution at 60° for 1 hour. The second arm of the apparatus contained a few drops of 6 N HCl to trap the trimethylamine. After a diffusion period of 3 hours the HCl solution was evaporated to dryness on a watch glass and the residue was examined in the flow counter. No detectable radioactivity was present.

Administration of $\text{C}^{14}$-Labeled Methionine to Chicken—A 70 gm., week-old "broad breast" chicken was maintained on a commercial ration (Ralston...
Purina Company, Growena) and injected subcutaneously with the L-methionine labeled in the methyl group with C^{14}. The dosage and injection schedule were the same as those employed with the rat. At the end of the injection period the blood was analyzed. In this case, several of the fractions in the ergothioneine range showed no detectable radioactivity above the background count (Fig. 2).

**Administration of S^{35}-Labeled Methionine to Rat**—A 322 gm. Wistar strain male rat was maintained on the stock diet and was injected subcutaneously with a total of 45.5 mg. of S^{35}-labeled L-methionine over a 20 day period in equal daily doses. The total radioactivity administered was approximately 2 $\times$ $10^8$ c.p.m. as determined by direct count on a small aliquot. The results of the analysis of blood obtained from the animal at the end of the injection period are shown in Fig. 3.

**Administration of S^{35}-Labeled Methionine to Guinea Pig**—A 245 gm. guinea pig was maintained on commercial rabbit pellets (Rockland) supplemented with cabbage and was injected with the S^{35}-methionine at the same dosage and radioactivity levels used with the rat. The analytical results are shown in Fig. 3.

In addition to the foregoing experiments, analyses were carried out on blood samples which became available from work not connected with the ergothioneine problem, but in which isotope-labeled compounds had been administered.

**Analysis of Blood from Human Given S^{35}-Cystine**—A sample of red cells...
was obtained from a 46 year-old male patient with lymphocytic lymphoma, who had been given 0.4 mc. (18.2 mg.) of S$^{35}$-labeled L-cystine by mouth. The blood sample had been collected 21 hours after administration of the cystine. Chromatographic analysis yielded a column effluent containing a well-defined ergothioneine peak with a maximum of 22 $\gamma$ of ergothioneine in one of the fractions. This fraction showed no detectable radioactivity in the flow counter.

**Analysis of Blood from Rat Given C$^{14}$-Glycine**—Blood was obtained from a 370 gm. Sherman strain male rat, maintained on a commercial stock diet, which had undergone partial hepatectomy 31 days earlier and which had been injected intraperitoneally for the first 2 postoperative days with a total of approximately 0.01 mc. (0.54 mg.) of glycine-2-C$^{14}$. After analysis, the effluent fraction containing the largest amount of ergothioneine (11 $\gamma$) was examined, but no radioactivity could be detected.

**Analysis of Blood from Rat Given C$^{14}$-Formaldehyde**—Blood was obtained from a 330 gm. Sherman strain male rat, maintained on a commercial stock diet, which had been injected subcutaneously three times daily for 10 days with a total of 5.7 mmoles of formaldehyde-C$^{14}$,D$_2$ (a mixture of C$^{14}$H$_2$O and CD$_2$O) having a total activity of approximately $5.8 \times 10^4$ c.p.m. The animal was killed 21 hours after the last injection and the
blood was analyzed. The effluent fraction containing the largest amount of ergothioneine (17 γ) was found to contain no detectable radioactivity.

**DISCUSSION**

Our finding of the inability of injected 2-thiolhistidine to support blood ergothioneine levels is in conformity with the work of Heath (13).

The experiments described have all given uniformly negative results in our attempts to demonstrate the synthesis of ergothioneine in the rat, the chicken, the guinea pig, and the human. In the cases of the rat, chicken, and guinea pig, the radioactive compounds were injected over long periods of time to minimize the possibility of inability to detect synthesis because of the slow rate of incorporation of ergothioneine into the red cell (14). It is estimated that, in the experiment with C¹⁴-histidine, significant levels of radioactivity would have appeared in the ergothioneine if as little as 2 per cent of the blood ergothioneine had arisen from the radioactive histidine; in the S³⁵-methionine experiment labeling would have been readily detected if as little as 1 part in 1000 of the sulfur of the blood ergothioneine had been derived from the administered methionine.

The negative results obtained with the rat and the chicken after the administration of methyl-labeled methionine (Fig. 2) show that the methyl groups of ergothioneine are not derived from transmethylation reactions with compounds containing labile methyl groups. Whether ergothioneine can act as a methyl group donor has not been determined. The strongly radioactive peak evident in Fig. 2 in the chromatograms for both the rat and the chicken is due to a ninhydrin-negative substance which is undoubtedly choline, inasmuch as C¹⁴-labeled choline was found to behave identically as the unknown substance on the chromatograph columns. These results are therefore an excellent demonstration of the difference in availability of the methyl group of methionine for transmethylation to choline and to ergothioneine.

The negative results obtained with ring-labeled histidine and methyl-labeled methionine did not exclude the possibility that hercynine, the naturally occurring betaine of histidine, might serve as a dietary precursor of ergothioneine. However, the negative findings with S³⁵-methionine in the rat and the guinea pig (Fig. 3) rule out such a possibility. The results obtained with C¹⁴-glycine and C¹⁴-formaldehyde, as well as with methyl-labeled methionine, suggest that single carbon compounds are not involved. In the experiment with S³⁵-cystine in the human, the negative results can be regarded as only suggestive of the non-synthesis of ergothioneine, because of the short term nature of the experiment and the high dilution factors which are involved.

It would seem reasonable to conclude that ergothioneine is not synthe-
sized in the tissues of the rat, chicken, or guinea pig. The possibility that
the compound might arise from synthesis by microorganisms of the intes-
tinal tract seems doubtful, since we have found that blood ergothioneine
levels in the rat are not significantly altered by the feeding of sulfasuxidine
and have shown, by studies on germ-free chickens, that the intestinal flora
do not contribute significant amounts of ergothioneine to the blood in this
species (7).

It appears most likely, therefore, that ergothioneine is of dietary origin
in these animals. There is evidence in the literature to indicate that
cereal grains are effective to varying degrees as dietary sources of ergothio-
neine (8); the recent work of Baldridge and Lewis (15) and Baldridge (16)
implicates oats as being a good source. The failure so far to identify ergo-
thioneine as a constituent of plant materials may be due to low concentra-
tions of the substance or possibly to its occurrence in a bound form.

The work presented by Heath and coworkers (9) to show that S\textsuperscript{35}-me-
thionine gives rise to radioactive ergothioneine in the pig is unexpected
in view of our present results and indicates the need for further studies of
species differences in the ability to synthesize ergothioneine.

The authors express their appreciation to Dr. Aaron Bendich and Dr.
Jacques R. Fresco for supplying the blood from glycine-treated animals,
to Dr. Bertram A. Lowy for blood from formaldehyde-treated animals,
and to Dr. Laurance Goodwin for blood from the cystine-treated human
subject.

SUMMARY

By the administration of isotope-labeled compounds considered to be
likely precursors, studies on the possible synthesis of ergothionine in ani-
mals have been carried out. No evidence for the formation of isotope-
labeled ergothioneine has been obtained after the administration of C\textsuperscript{14}-
labeled histidine to the rat, methyl-labeled C\textsuperscript{14}-methionine to the rat and
the chicken, S\textsuperscript{35}-labeled methionine to the rat and the guinea pig, C\textsuperscript{14}-
labeled glycine or C\textsuperscript{14}-labeled formaldehyde to the rat, and S\textsuperscript{35}-labeled
cystine to the human.

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