Although ergothioneine is widely distributed in the erythrocytes of mammals, studies in this Laboratory have indicated that it is not synthesized by the several animal species which have so far been examined (1, 2). The ergothioneine of animal tissues (3) provides a dietary source of the compound for carnivorous species. A dietary source of greater significance has become apparent from the demonstration of its occurrence in a cereal grain (4). In this paper we report the results of further studies in a search for the origin of ergothioneine. We have found that ergothioneine is a common constituent of many microbial cells and that it is synthesized by several fungi but not by bacteria.

**EXPERIMENTAL**

Ergothioneine Synthesis by Aspergillus niger—A preliminary experiment indicated that ergothioneine was present in hot water extracts of A. niger mycelium, as determined colorimetrically after chromatographic analysis on alumina (3). Proof for the identity of the compound and for its biosynthesis was obtained by growing the fungus in a chemically defined medium containing S35-sulfate.

A culture of A. niger NRRL strain 3 was grown from a spore inoculum in 150 ml. of CZapek solution (5), consisting of sucrose and inorganic salts, to which had been added 0.1 mc. of carrier-free S35-sulfate. After 11 days the mold was removed, dried, and extracted with hot water. The extract was chromatographed on alumina (3), and alternate 1 ml. fractions of the column effluent were evaporated to dryness in steel planchets and examined for radioactivity with a mica window counter. The remaining fractions were analyzed for ergothioneine by means of the color reaction with diazotized sulfanilic acid (6). The results of the determinations of radioactivity are shown in Fig. 1. The intensely radioactive peak between the effluent of 12 and 24 ml. contained no ergothioneine. (The substance responsible for this radioactivity was identified as the sulfate ester of choline (7) by the production of radioactive sulfuric acid

* This work was aided by grants from Swift and Company and the National Science Foundation.
on acid hydrolysis, the ability to replace choline during the growth of a cholineless *Neurospora* mutant, and inseparability of the radioactivity from added carrier choline sulfate. Ergothioneine was detected colorimetrically in the second, smaller peak. These fractions were combined and chromatographed on alumina. The results (Fig. 2) revealed excellent correspondence between the radioactivity and ergothioneine determinations.

The radioactive fractions from the diazo-positive area of the column effluent were combined. This material, showing a radioactivity of 2600 c.p.m., was dissolved in water and treated with a slight excess of bromine water for 30 minutes. Barium chloride was then added, and the precipitated barium sulfate was collected on a filter paper disk and examined for radioactivity. A value of 2300 c.p.m. was obtained. The ready formation of inorganic sulfate on treatment with bromine is a characteristic reaction of ergothioneine (8).

**Ergothioneine Synthesis by Neurospora crassa**—A cholineless mutant of *N. crassa* (ATCC 9277) was grown for 12 days in the Czapek solution (5), to which had been added 1 γ of choline and 0.005 γ of biotin per ml., plus 0.1 mc. of carrier-free S\(^{35}\)-sulfate. After chromatography of a hot

![Figure 1. Distribution of radioactivity in alumina chromatogram of aqueous extract of *A. niger* cells grown in the presence of S\(^{35}\)-sulfate. Positive diazo tests for ergothioneine were given by fractions from the small radioactive peak between the effluent of 24 and 32 ml.](http://www.jbc.org/)

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*Fig. 1. Distribution of radioactivity in alumina chromatogram of aqueous extract of *A. niger* cells grown in the presence of S\(^{35}\)-sulfate. Positive diazo tests for ergothioneine were given by fractions from the small radioactive peak between the effluent of 24 and 32 ml.*
water extract of the mycelium, the radioactive ergothioneine-containing fractions of the effluent liquid were combined and diluted with 50 mg. of non-isotopic ergothioneine. Crystallization from aqueous ethanol yielded ergothioneine with a radioactivity of 1000 c.p.m. Repeated crystallizations of this material failed to remove the radioactivity. Treatment of an aliquot with bromine water, followed by benzidine in acetone (9), gave a benzidine sulfate precipitate which contained 95 per cent of the radioactivity.

*Isolation of Crystalline Ergothioneine from N. crassa*—15 liters of medium (10) in a 5 gallon carboy were sterilized and inoculated with spores of wild type *N. crassa* (ATCC 10336). The culture was aerated for 11 days; then the mycelium was harvested, washed with water, and dried. It weighed 82 gm. and contained 44 mg. of ergothioneine, as determined by chromatographic analysis. This was extracted three times with 2 liter portions of boiling water. The combined extracts were concentrated to 500 ml., adjusted to pH 2 with sulfuric acid, and treated with cuprous oxide to precipitate the ergothioneine (11). The precipitate was decomposed with H$_2$S, sulfate was removed from the solution with barium hydroxide, and the filtrate was evaporated to dryness. The residue (about 1 gm.) was mixed with 20 ml. of cold water; an insoluble fraction was discarded.
The water-soluble material (about 0.5 gm.) was chromatographed on 20 gm. of alumina with 75 per cent ethanol as the solvent (3). The effluent fractions which contained ergothioneine were combined and evaporated to dryness. The residue weighed 161 mg. and contained 27 mg. of ergothioneine. Crystallization of this material from 1 ml. of water yielded 100 mg. of needles which did not give a diazo test. The solid material from the mother liquors, which contained all the ergothioneine, was crystallized twice from aqueous ethanol to yield 25.9 mg. of needles which were pure ergothioneine, as judged by the quantitative diazo test and the ultraviolet absorption spectrum (2).

**Ergothioneine in Micrococcus pyogenes var. aureus**—In view of the finding of ergothioneine synthesis by fungi, it was of interest to investigate bacteria. A culture of *M. pyogenes* prepared in nutrient broth (peptone 0.5, yeast extract 0.3, beef extract 0.15, and glucose 0.1 per cent) was analyzed chromatographically and found to contain 54 mg. of ergothioneine per 100 gm. of dried cells. However, when this organism was cultured in nutrient broth in the presence of 0.1 mc. of $^{38}$-sulfate, the ergothioneine which was obtained contained no radioactivity. The *M. pyogenes* was then grown in Gladstone's chemically defined medium, modified by the addition of biotin (12) and Tween 80. No ergothioneine could be detected in hot water extracts of the cells.

It appeared likely that the ergothioneine present in the cells grown in nutrient broth was derived from some component of the medium. The suspected materials were tested by growing *M. pyogenes* in the chemically defined medium to which had been added the substance under test, in the concentration used in the nutrient broth. When beef extract, peptone, and yeast extract were tested individually in this manner, ergothioneine appeared in the bacterial cells in all three cultures, at levels of 23, 8, and 4 mg. per 100 gm. of dried cells, respectively. That the cells of *M. pyogenes* were in fact capable of assimilating ergothioneine from the medium was confirmed by growing the bacterium in the chemically defined medium in the presence of ergothioneine. The addition of 1 mg. of ergothioneine to 900 ml. of medium resulted in the uptake of 300 $\gamma$ of ergothioneine into the cells during a 23 hour growth period.

**Survey of Microorganisms for Ergothioneine**—Several fungi and bacteria were examined for their ability to synthesize ergothioneine. In general, this was done by chromatographic analysis of cell extracts from cultures grown in synthetic media. Most of the organisms were purchased from the American Type Culture Collection. Strains of *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, and *Streptococcus salivarius* were obtained from Dr. James M. Neill, and *Streptococcus pyogenes* from Dr. Ralph Tompsett, of Cornell Medical College. *A. niger* and *Aspergillus carbonarius* were pro-
vided by C. W. Hesseltine of the Northern Utilization Research Branch, United States Department of Agriculture.

Bacteria were grown without agitation in 900 ml. of media in Erlenmeyer or Florence flasks, and cultures prepared in defined media were inoculated with organisms which had been grown for at least one passage in the defined media. The temperature was usually 37° and the cultures were allowed to grow until 150 mg. or more (dry weight) of cells had been formed. Cells were collected by centrifugation, suspended in 2 ml. of water, autoclaved if pathogenic, and dried at 80°. Fungi were grown at 25° in Roux bottles in 150 ml. of media, for periods up to 4 weeks. The mycelial mats were separated, washed with water, and dried at 80°.

The dried organisms were extracted for 15 minutes each with three 40 ml. portions of boiling water. The aqueous extracts were filtered if necessary, and then passed through a column of 10 gm. of Amberlite IRA-410 resin in the acetate form. The effluent was evaporated to dryness in vacuo. An aliquot of this residue not exceeding 200 mg. was chromatographed on 20 gm. of alumina with 75 per cent ethanol containing 1 per cent formic acid as the solvent (3). 5 ml. effluent fractions were collected and analyzed for ergothioneine by means of the diazo color reaction (6). With a large majority of the organisms, little difficulty was experienced in obtaining fractions free from substances which interfered with the characteristic magenta color given by pure ergothioneine. These organisms are listed in Tables I and II; a few which gave results of doubtful value, owing to incomplete separation of interfering substances, have been omitted.

DISCUSSION

The foregoing demonstration of the synthesis of ergothioneine by common fungi is gratifying in that it reveals for the first time a primary source which makes more comprehensible the extensive distribution of ergothioneine in animal species. That ergothioneine has heretofore escaped detection in these fungi can probably be ascribed to the analytical difficulties associated with the detection of small amounts of ergothioneine in many natural materials. In this connection, it is of interest that a bromine-oxidizable sulfur fraction was observed by Plumlee and Pollard (13) in Penicillium chrysogenum broths; it seems likely that ergothioneine could account for this.

The values for ergothioneine given in Table I must be considered as minimal figures, inasmuch as the completeness with which ergothioneine is extracted from the organisms has not been precisely established. Other data which we have obtained with isotope dilution techniques suggest that the over-all recovery of free ergothioneine present in N. crassa is 80 to 85 per cent. It is obvious that the ergothioneine concentrations are appre-
ERGOTHIONEINE IN MICROORGANISMS

ciably less than those found in ergot (Claviceps purpurea). Hunter et al. (14) have given values in the range of 200 to 500 mg. of ergothioneine per 100 gm. for various samples of ergot sclerotia.

The lower limit of sensitivity of the analytical method is such that ergothioneine values below 2 to 6 mg. per 100 gm. would not be readily detected. The sensitivity for any one organism depends on the amount of organism used and the degree of separation of interfering substances by the chromatographic procedure. The results obtained with Alternaria tenuis are worthy of note in this respect. First analyses of cultures of this organism gave no indication of the presence of ergothioneine. To increase the sen-

Table I

Ergothioneine Levels in Fungi Grown in Chemically Defined Media

Ergothioneine values are given as mg. per 100 gm. of dried cells; growth period in days. The numbers given in parentheses are bibliographic references.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Medium</th>
<th>Growth period</th>
<th>Ergothioneine</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. tenuis ATCC 11612</td>
<td>(10)</td>
<td>8*</td>
<td>4</td>
</tr>
<tr>
<td>Alternaria zinniae ATCC 11786</td>
<td>(5)†</td>
<td>67</td>
<td>2</td>
</tr>
<tr>
<td>A. carbonarius NRRL 369</td>
<td>(5)</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>&quot; niger NRRL 3</td>
<td>(5)</td>
<td>28</td>
<td>12</td>
</tr>
<tr>
<td>&quot; ATCC 1027</td>
<td>(5)†</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>Mucor mucedo ATCC 9836</td>
<td>(5)†</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>N. crassa ATCC 10337</td>
<td>(5)†</td>
<td>11</td>
<td>85</td>
</tr>
<tr>
<td>Neurospora tetrasperma ATCC 9457</td>
<td>(5)</td>
<td>11</td>
<td>27</td>
</tr>
<tr>
<td>Penicillium notatum ATCC 8537</td>
<td>(5)‡</td>
<td>28</td>
<td>13</td>
</tr>
<tr>
<td>Pullularia pullulans ATCC 9348</td>
<td>(5)‡</td>
<td>14</td>
<td>34</td>
</tr>
</tbody>
</table>

* Rotary shaker.
† Czapek's solution.
‡ Czapek's agar.

sitivity of the analytical method, radioactive sulfur was used. A. tenuis was grown in the chemically defined medium in the presence of approximately 2 μc. of S35-sulfate. The dried cells were extracted with water containing 1 mg. of carrier ergothioneine. The effluent from the chromatographic column showed radioactivity in the ergothioneine area. These fractions were chromatographed twice, first with 75 per cent ethanol and then with 80 per cent ethanol. The final fractions contained a radioactive peak which coincided exactly with the ergothioneine peak. The original ergothioneine content of 4 mg. per 100 gm. (Table I) was calculated from the specific radioactivity.

In sharp contrast to the fungi, none of the bacteria which were examined showed any evidence of ergothioneine synthesis (Table II). Two of these bacteria were studied with radioactive sulfur. M. pyogenes var. aureus
was grown in the modified Gladstone medium to which had been added L-cystine-S\(^{38}\), L-methionine-S\(^{35}\), and S\(^{35}\)-sulfate. *E. coli* was grown in a medium (15) in which the sole sulfur source was S\(^{35}\)-sulfate. In neither case was radioactive ergothioneine found in aqueous extracts of the cells. The level of radioactivity used with the *E. coli* was such that the synthesis of 30 \(\gamma\) of ergothioneine per 100 gm. of cells would have been detected.

The marked ability of *M. pyogenes* to incorporate ergothioneine from the growth medium is worthy of comment. This concentration effect has been useful in certain instances for the detection or semiquantitative determination of ergothioneine in materials which gave unsatisfactory separation of ergothioneine by the usual chromatographic procedure. Similar assimilatory powers are possessed by some other bacteria. For example, high concentrations of ergothioneine were found in *Mycobacterium tuberculosis* H37-RV which had been grown in the Dubos medium containing bovine albumin (Armour). When *M. pyogenes* was grown in a defined medium to which the albumin had been added, ergothioneine was found in the *M. pyogenes* cells in similar concentrations. It is evident that the bovine albumin preparation contained ergothioneine.

### Table II

**Bacterial Cultures with No Evidence of Ergothioneine Synthesis**

The numbers given in parentheses are bibliographic references.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Medium</th>
<th>Growth period</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td>(16)*</td>
<td>19</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em> ATCC 10543</td>
<td>(17)</td>
<td>22</td>
</tr>
<tr>
<td><em>Corynebacterium xerose</em> ATCC 9016</td>
<td>(16)*</td>
<td>22</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>(15)†</td>
<td>48†</td>
</tr>
<tr>
<td><em>L. casei</em> ATCC 7469</td>
<td>(16)*</td>
<td>32</td>
</tr>
<tr>
<td><em>M. pyogenes</em> var. <em>aureus</em> H.</td>
<td>(12)</td>
<td>23</td>
</tr>
<tr>
<td><em>Mycobacterium</em> spp. ATCC 607</td>
<td>(16)*</td>
<td>500</td>
</tr>
<tr>
<td><em>P. vulgaris</em> OX19</td>
<td></td>
<td>26</td>
</tr>
<tr>
<td><em>P. fluorescens</em> ATCC 11251</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td><em>S. pyogenes</em> C203MV</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td><em>S. salivarius</em></td>
<td></td>
<td>19</td>
</tr>
<tr>
<td><em>Vibrio metchnikovii</em> ATCC 7708</td>
<td>(16)*</td>
<td>21</td>
</tr>
</tbody>
</table>

* Modified by the omission of norleucine and norvaline, replacement of 200 mg. of aspartic acid by asparagine, and addition of 500 \(\gamma\) of pyridoxamine, 500 \(\gamma\) of pyridoxal, 0.8 \(\gamma\) of vitamin B\(_{12}\), 10 \(\gamma\) of nicotinamide, and 0.5 ml. of Tween 80 per liter.

† Modified by the omission of norleucine and norvaline; used double strength.

‡ Rotary shaker.

§ Difco brain-heart infusion broth.

|| Nutrient broth (see the text).
Not all bacteria possess the power to incorporate large amounts of ergothioneine from their media. For example, no ergothioneine was detected in cells of *Pseudomonas fluorescens* which had been grown in nutrient broth, as compared to the 54 mg. per 100 gm. value found with *M. pyogenes* grown under similar conditions. Weak assimilatory activity was shown by *Lactobacillus casei* and *Streptococcus faecalis* ATCC 8043, which gave ergothioneine values of 1 and 5 mg. per 100 gm., respectively, after growth in nutrient broth.

The synthesis of ergothioneine by fungi has an important bearing on the fact that ergothioneine is present in oats and probably in other cereal grains (4). It has not yet been determined whether higher plants possess the ability to synthesize ergothioneine. On the other hand, it is known that fungi are commonly present in grains, and it seems likely that part of the ergothioneine has its origin in these fungi. We have isolated species of *Alternaria* and *Aspergillus* from oat grains and found that they synthesize ergothioneine. Furthermore, these and other genera of fungi are commonly found in soil in many parts of the world. It is entirely possible, therefore, that the ergothioneine of oats and other cereal grains is derived from fungi, either directly or by assimilation from the soil.

Appreciation is expressed to Dr. Ralph Tompsett for preparing cultures of *M. tuberculosis* H37-RV, and to Miss Joyce Shaver and Miss Mary Exner for aid in some phases of this work.

**SUMMARY**

With the aid of radioactive sulfate, it has been shown that ergothioneine is synthesized by fungi. Crystalline ergothioneine has been isolated from *Neurospora crassa*. Of nine species of fungi examined, all produced ergothioneine during growth in defined media. In contrast, no ergothioneine synthesis could be detected in the twelve species of bacteria which were studied. Several of these bacteria, however, possess the ability to incorporate ergothioneine from the media in which they grow. This effect, in the case of *Micrococcus pyogenes*, has been used to advantage in the detection of ergothioneine in natural materials.

The demonstration of ergothioneine synthesis by common fungi discloses a probable primigenial source for the ergothioneine present in plants and animals.

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