THE INCORPORATION OF RADIOACTIVE SULFUR INTO THIAMINE BY SACCHAROMYCES CEREVISIAE

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The biosynthesis of thiamine has been believed to be accomplished with the initial formation of the pyrimidine and thiazole moieties by independent routes, followed by a final step in which pyrimidine and thiazole are jointed together to form thiamine. Recently, Nose (1-3), Brown (4-7), and Leder (8, 9) separated the enzyme system from cell-free extracts of bakers' yeast which is capable of synthesizing thiamine from pyrimidine and thiazole. The following sequence of reactions on the biosynthesis of thiamine and thiamine pyrophosphate was established by them.

(a) Pyrimidine + ATP → pyrimidine pyrophosphate
(b) Thiazole + ATP → thiazole monophosphate
(c) Pyrimidine pyrophosphate + thiazole monophosphate → thiamine monophosphate
(d) Thiamine monophosphate → thiamine
(e) Thiamine + ATP → thiamine pyrophosphate

However, there is little information available concerning the biosynthetic pathways of pyrimidine and thiazole moieties themselves. On the other hand, nutritional experiments on thiamine-requiring Neurospora mutants by Harris (10) suggest that there are two alternative pathways of thiamine biosynthesis, but that the predominant pathway involves the condensation of pyrimidine with a precursor of thiazole to form a thiamine-like intermediate which is secondarily changed to thiamine. Nothing has been reported, however, on the nature of the thiamine-like intermediate. Several hypotheses on the biosynthetic route of thiazole have been presented, but the biosynthetic pathway of thiazole has not been established yet.

The detailed study was undertaken to obtain the information on the precursor of thiazole. The present paper deals with the incorporation of radioactive sulfur into thiamine by Saccharomyces cerevisiae.

EXPERIMENTAL

1. Organism

Saccharomyces cerevisiae AUH 3114 was used in this work. This strain was kindly supplied by Dr. T. Sasaki (Department of Agriculture, Hokkaido University).
Stock cultures were maintained on agar slants and were subcultured bimonthly (48 hours at 30°C).

2. Media

The medium employed in these experiments was a synthetic medium similar to that of Olsen and Johnson (11). It had the following composition: glucose, 20.0 g; (NH₄)₂HPO₄, 3.5 g; KH₂PO₄, 0.2 g; MgSO₄·7H₂O, 0.5 g; sodium citrate trihydrate, 1.0 g; L-asparagine monohydrate, 2.5 g; biotin, 20 µg; calcium pantothenate, 0.5 mg; inositol, 10 mg; Zn²⁺ (as sulfate), 400 µg; Fe²⁺ (as ferrous ammonium sulfate), 150 µg; Cu²⁺ (as sulfate), 25 µg; H₂O, 1,000 ml; pH 5.0. Radioactive sulfur (5 mc/1,000 ml) as carrier-free sulfate was added. Glucose, phosphates, and other salts were separately autoclaved at 120°C for 15 minutes. Medium of stock culture was composed of dried yeast extract 1%, peptone 0.5%, glucose 1%, and agar 2%.

3. Condition of Culture

The inoculum was the cells derived from a 48-hour culture on the agar slant. The inoculated medium was incubated for 48 hours at 30°C on a shaker. The resulting cells were centrifuged at 6,000 rpm and were washed twice with water.

4. Determination of Cell Weight

To determine the dry weight of the cells, an aliquot of the cell suspension was centrifuged and cell pellet was washed twice with water. The supernatant solution was discarded and the cells were suspended in a small amount of water and transferred quantitatively to a previously weighed bottle. The cells were dried at 110°C until constant weight had been attained and then weighed.

5. Extraction of Thiamine and Its Phosphates

The weighed cells were extracted with 50 ml of 2 N perchloric acid by vigorously shaking for 30 minutes at room temperature and the cell debris was then centrifuged and extracted with alcohol. The clear supernatant fluid was adjusted to pH 4.5 with conc. NaOH. Two g of fuller's earth was added and the mixture was shaken vigorously for 10 minutes. After centrifugation, the supernatant was discarded and the fuller's earth was washed twice with water. Thiamine and its phosphates were then eluted from the fuller's earth by shaking with 5 ml of a mixture of pyridine : water : acetic acid (4.0 : 1.0 : 0.1 in volume) for 10 minutes. After centrifugation, the eluate was concentrated below 40°C in vacuo to approximately 0.3 ml and was then separated from the resulting solid by centrifugation. The concentrated supernatant was subjected to paper chromatography.

6. Paper Chromatography and Detection of Thiamine

Paper chromatograms (Toyo filter paper No. 51 A) were developed by the ascending technique. The solvents used will be described in results. The zones of thiamine and its phosphates were located by bioautography, radioautography, and detection of thiochrome fluorescence. For bioautography, the developed chromatograms were placed with the surface of a solid medium (the medium of Davis and Mingioli (12) with 2% agar, in which a thiazole-less mutant of Escherichia coli (designed as 26–43) kindly supplied by Dr. B. D. Davis had been inoculated), 5 mm thick, in a sterile 30 × 20 cm dish. After about 5 minutes, the chromatograms were removed and the plate was covered and incubated for 18 hours at 37°C. Radioautograms were prepared by contact with Fuji X-ray film for 3 days. For detection of thiochrome
fluorescence, chromatograms were sprayed with a mixture of 55% ethanol, 10% NaOH, and 2.5% K$_2$Fe(CN)$_6$ (5.0:5.0:0.1 in volume).

7. Assay of Thiamine

An aliquot of the eluate of the thiamine zone on the chromatogram was used to determine the thiamine content by microbiological assay with *Lactobacillus fermenti* according to the directions described by Sarett and Cheldelin (13). Another aliquot of the eluate was used to estimate the radioactivity of thiamine.

8. Estimation of Radioactivity

The radioactive sulfur in organic compounds was degraded by oxidation with concentrated HNO$_3$ and 30% H$_2$O$_2$ to inorganic sulfate and separated as benzidine sulfate. The radioactivity was estimated with the flow counter, CE-14 low-background beta counter (Tracer Lab. Inc., U.S.A.).

**RESULTS**

To obtain radioactive thiamine, and other sulfur-containing compounds such as cystine and methionine with high specific activity, the growth response of *Saccharomyces cerevisiae* to the concentration of sulfate was investigated. The results were shown in Fig. 1. At concentrations slightly in excess of 0.5 g of sulfur as magnesium sulfate per 1,000 ml medium, no further increase of yeast cells occurred. At lower concentrations, the cells depleted the sulfur of the medium and the final cellular yield was dependent on the total sulfur available. These data showed that the sulfur content of the medium employed in these experiments was adequate.

In Table I was shown the incorporation of radioactive sulfate sulfur into yeast cells. The distribution of radioactive sulfur among the fractions was shown in Table II. Forty-three % of radioactive sulfate sulfur in the medium was incorporated into the yeast cells. Basic sulfur-containing compounds adsorbed on fuller's earth contributed

\[
R_f
\]

![Detection of thiamine, thiamine phosphates and thiazole by bioautography](image1)

![Detection of $S^{35}$ spots by radioautography](image2)

![Detection of thiamine zones by thiochrome fluorescence](image3)

**Fig. 2** Paper Chromatograms Developed with Solvent A of Yeast Extracts

Similar results were obtained with paper chromatograms developed with solvent B.
a small amount of the perchloric acid-soluble fraction. Thiamine contributed only 0.026% of the total radioactivity, approximately $6.4 \times 10^4$ cpm/μmole.

**Table I**

*Radioactive Sulfate-Sulfur Uptake by Saccharomyces cerevisiae*

<table>
<thead>
<tr>
<th>Component</th>
<th>Radioactivity per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original medium</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant fluid</td>
<td>51</td>
</tr>
<tr>
<td>Cells after washing</td>
<td>43</td>
</tr>
</tbody>
</table>

**Table II**

*Distribution of Radioactive Sulfur Among the Fractions Extracted from the Cells of Saccharomyces cerevisiae*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Weight</th>
<th>Radioactivity per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original washed cells</td>
<td>5.330</td>
<td>100.0</td>
</tr>
<tr>
<td>Perchloric acid-soluble</td>
<td></td>
<td>28.1</td>
</tr>
<tr>
<td>Supernatant fluid after adsorption</td>
<td>24.8</td>
<td></td>
</tr>
<tr>
<td>with fuller’s earth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total thiamine</td>
<td>$163.2 \times 10^{-4}$</td>
<td>0.026 (3.1 $\times 10^4$ cpm)</td>
</tr>
<tr>
<td>Alcohol-soluble</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>Residue protein</td>
<td>62.7</td>
<td></td>
</tr>
<tr>
<td>Percentage accounted for</td>
<td>102.1</td>
<td></td>
</tr>
</tbody>
</table>

$R_F$ values of sulfur-containing compounds present in yeast cells was shown in Table III. In Fig. 2 were shown the results of the bioautogram with *Escherichia coli* 26–43, radioautogram, and the detection of thiochrome fluorescence. By these means, the zones of thiamine and its phosphates were located. However, the presence of thiazole, thiazole monophosphate, and sulfur-containing compound considered to be its precursor was not observed.

**Table III**

*RF Values of Thiamine, Its Related Compounds, and Sulfur-Containing Compounds Present in Yeast Cells*

Solvents used were the following (ratios are given in terms of volumes); Solvent A, *n*-propanol, iso-amyl alcohol, H$_2$O, *iso*-butyric acid, NH$_4$OH (28%) (7.2 : 2.5 : 7.5 : 12.0 : 0.2); Solvent B, *iso*-butyric acid, 1 N NH$_4$OH, 0.1 M EDTA (25.0 : 15.0 : 0.4).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent A</th>
<th>Solvent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine</td>
<td>0.70</td>
<td>0.87</td>
</tr>
<tr>
<td>Thiamine monophosphate</td>
<td>0.45</td>
<td>0.77</td>
</tr>
<tr>
<td>Thiamine diphosphate</td>
<td>0.19</td>
<td>0.68</td>
</tr>
<tr>
<td>Thiamine triphosphate</td>
<td>0.09</td>
<td>0.55</td>
</tr>
<tr>
<td>Thiazole</td>
<td>0.78</td>
<td>0.86</td>
</tr>
<tr>
<td>Thiazole monophosphate</td>
<td>0.53</td>
<td>0.78</td>
</tr>
<tr>
<td>Thiochrome</td>
<td>0.56</td>
<td>0.82</td>
</tr>
<tr>
<td>Thiamine disulfide</td>
<td>0.74</td>
<td>0.92</td>
</tr>
<tr>
<td>5’-Deoxy-5’-methylthioadenosine</td>
<td>0.23</td>
<td>0.62</td>
</tr>
<tr>
<td>Adenosylmethionine</td>
<td>0.08</td>
<td>0.25</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.17</td>
<td>0.48</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.51</td>
<td>0.75</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.08</td>
<td>0.40</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.65</td>
<td>0.78</td>
</tr>
<tr>
<td>Coenzyme A</td>
<td>0.38</td>
<td>0.03</td>
</tr>
</tbody>
</table>
To find a synthetic medium that would support yeast growth equivalent to that obtained on a natural medium, Olsen and Johnson (11) had investigated in detail the effects of glucose, asparagine, vitamins (thiamine, pyridoxine, inositol, biotin and calcium pantothenate) and cation concentrations on the cellular yield of a yeast. However, the effect of the concentration of sulfate on the cellular yield was not investigated. The experiments were undertaken to decide the adequate concentration. The results in Fig. 1 showed that the magnesium sulfate concentration of the medium described above was adequate. Radioactive thiamine with very high specific activity was obtained in small amounts by extraction of the resulting yeast cells, followed by hydrolysis with Takadiastase and separation. Radioactive L-cystine and L-methionine with high specific activity were also obtained by hydrolysis of the residual cellular protein from which thiamine was extracted. Perchloric acid-soluble fraction contained 28% of the total radioactivity. It was considered that this fraction might contain water-soluble compounds such as glutathione, adenosylmethionine, vitamins, and other sulfur-containing compounds. It was considered that the alcohol-soluble fraction was composed of alcohol-soluble proteins and lipids.

It was reported by Nakayama (14) that thiazole was found in the culture medium of *Neurospora crassa* 18558 A grown in a medium supplemented with cystine or thiazolidine-4-carboxylic acid. The presence of thiazole in the extracts of the
mycelium of *Aspergillus oryzae* was reported by Kamihara and Ikeda (15). In the authors’ experiments, thiazole, thiazole monophosphate, and the compound recognized as its precursor were not found by bioautography with thiazole-requiring mutant of *Escherichia coli*. Except thiamine and its phosphates, no radioactive sulfur spots corresponding to thiazole and thiazole monophosphate were found by radioautography. It was considered therefore that such intermediates of thiamine biosynthesis failed to accumulate in detectable amounts in the yeast cells.

**SUMMARY**

1. *Saccharomyces cerevisiae* growth in a medium containing radioactive sulfate produced radioactive sulfur-containing thiamine of high specific activity in small amounts.

2. Radioactive sulfur-containing thiamine, its phosphates, and its precursor were investigated on paper chromatograms. However, no intermediate of thiazole biosynthesis accumulated in detectable amounts in yeast cells.

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**REFERENCES**