MEMOIRS OF THE COLLEGE OF SCIENCE, UNIVERSITY OF KYOTO, SERIES B,
Vol. XXIV, No. 3 (Biology), Article 6, 1957

Studies on the Adaptation of Yeast to Copper

XVII. Copper-Binding Nitrogenous Substances of the Copper-Resistant Substrain*

By

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(Received Oct. 9, 1957)

It was reported by the author and others (1) that copper was accumulated by cells of the stable copper-resistant substrain, R_{1b}, which was established by training *Saccharomyces ellipsoideus* in a medium containing 1 *mM* $CuSO_4$. And an assumption was proposed that the resistant substrain can combine copper in the cell and detoxify it.

It is desirable to identify the substances which serve as the detoxifier. At first proteinous matter was suspected. And the present paper reports the nature connected with copper of the fractions extractable and not extractable by dilute alkali from the copper-grown cells.

Material and Method

The copper resistant substrain, R_{1b}, of *S. ellipsoideus* used was the same as in the previous paper (1). R_{1b} was cultured at 30°C in the liquid MH** medium containing 1 *mM* of $CuSO_4$. To prepare the cell material for extractions and analyses, the culture about 48 hours of age, i.e. at the stationary phase of growth, was harvested, washed and dried to constant weight at 85°C.

Nitrogen was determined by micro-Kjeldahl method, and copper by carbamate method (2), Shimadzu's spectro-photometer being used for the colorimetry.

Results

Copper-cultured cells were washed three times by distilled water. The suspension was divided into two lots. Cells in one of them were analyzed for the copper

* Supported by the Grant in Aid for Scientific Research of Education Ministry.

** KH_2PO_4 5*g*, $MgSO_4 \cdot 7H_2O$ 2*g*, peptone 5*g*, cane sugar 100*g*, malt extract (Bé. 8) 360*ml* and distilled water 1000 *ml*.

content at once, and those in the other were analyzed after overnight stay in a refrigerator. The copper content was 8.41 and 8.06 *mg/g* dry cells, respectively. Hence the copper leached out from the cells during a night was only 4% of the copper content.

In order to compare the extractions on alkaline and acidic sides, 48 hours old cells were harvested, washed, and divided into three aliquots. One was used to measure the dry weight. Another was suspended in 0.1 *N* solution of NaOH, and the thrid in 0.1 *N* acetic acid. The suspensions were shaken for 15 minutes, and then centrifuged to renew the media. Each series of extraction was repeated 6 times in this way, suspensions being shaken for 30 minutes in the last two times. Then, the cells which had been treated by alkali were extracted with the acid solution repeatedly, and the acid-treated cells by the alkali solution. The 12 extracts of each series were analyzed for nitrogen and copper. The amounts of nitrogen and of copper extracted each time was summed up successively. And the extraction curves as represented in Fig. 1 were obtaind.

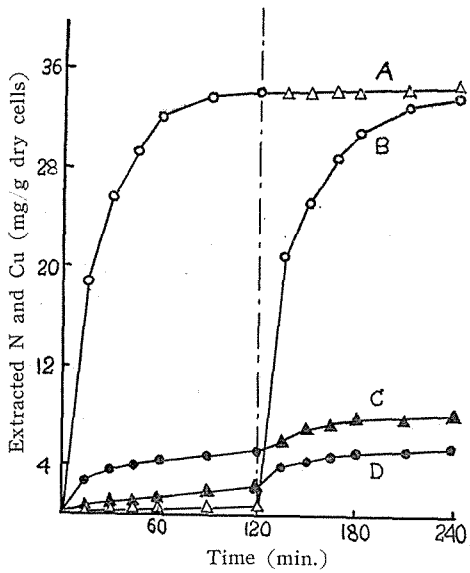

Fig. 1. Nitrogen and copper extracted from resistant cells by 0.1 *N* NaOH and by 0.1 *N* acetic acid.
○: Nitrogen extracted by 0.1 *N* NaOH; ●: copper extracted by 0.1 *N* NaOH; △: nitrogen extracted by 0.1 *N* acetic acid; ▲: copper extracted by 0.1 *N* acetic acid.

About 55% of the nitrogenous matter of cells was extracted by the repeated alkali treatments, and no more by the following acid treatment (curve A). Even when the order of the treatments were reversed, the nitrogenous matter was extracted chiefly by the alkali treatment (curve B).

Copper was also extracted more easily by the alkali treatment than by the acid treatment. When the cells were treated first by alkali, about 60% of the copper contained in them was extracted, the remainder to be totally extracted by the following acid treatment (curve C). On the other hand, when the cells were treated first by acid and then by alkali (curve D), the total amount of the extracted copper hardly exceeded the amount in the first half of the curve C, namely, that extracted by alkali only.

It might be suspected that insoluble hydroxide of copper was formed in the cell when copper could not be dissolved out by alkali. So the alkali solution in the above-mentioned experiment was replaced by 0.1 *N* NH_4OH. But the process and

yield of copper extraction was practically the same as before. When ammonium was used, the determination of extracted nitrogenous matter was impossible.

How much of the nitrogen and copper of cells was extracted by alkali is shown in Table 1. Copper was extracted relatively more than nitrogen, the ratio of copper to nitrogen being 0.126 (*mg/mg*) in the original cell and 0.144 in the extract.

Table 1. Nitrogen and copper contained in, and extractable by alkali from cells of R_{1b} and of the parent strain, *mg* per *g* dry weight of original cells.

	Nitrogen			Copper		
	Cell	Extracted	Extraction ratio (%)	Cell	Extracted	Extraction ratio (%)
Resistant strain	64.5	35.5	55.0	8.14	5.1	62.5
Parent strain	86.6	32.7	37.7	—	—	—

The nitrogen content of the cell and of the alkali extract was determined also for the parent strain cultured in the normal medium. The lower nitrogen content of the copper-cultured R_{1b} cells may partly be due to their smaller size compared with those cultured without copper, the cell wall forming a higher percentage of dry weight in the former case. It is to be noted that the absolute amount of nitrogen extracted from unit dry weight of R_{1b} cells was not less than that from the parent strain cells.

R_{1b} cultured in the copper medium was colored brown. The brown-colored matter moved entirely into the alkali extract. And it was precipitated when acetic acid was added to the extract to make pH 4.0–4.2, the supernatant being light bluish. Thus the three fractions were examined separately, namely, 1) the brown-colored precipitation, 2) the blue colored supernatant, and 3) the fraction remaining in the cell not extracted by alkali.

1) The precipitate was dialyzed against running water for 24 hours. Little copper seemed to be washed out. Then the precipitate was dissolved by adding dilute NaOH, and pH was adjusted to 7.0 by acetic acid. This solution was colored deep brown. For comparison, cells of the parent strain cultured without copper were similarly extracted, and the extract was treated just in the same way. This solution was also colored brown, though very lightly.

The solution prepared from the copper-cultured cells had the maximum absorption between 360 and 400 mμ, varying according to samples. Copper-sulfhydryl linkage of albumin is reported to have an absorption at 375 mμ, which disappears when mercuric chloride is added (3). But the absorption of the present preparation was not much influenced by the mercuric ion. On the other hand, the brown precipitate of CuS formed by bubbling H_2S gas through a dilute solution of $CuSO_4$ had an absorption something between 360 and 400 mμ. Hence the nature of the brown colored fraction from the copper-cultured cell could not be guessed by the absorption only. The light brown fraction from the normal cells had an absorption at about 400 mμ. But it contained no detectable copper.

In oder to see the copper-binding capacity of the proteinous matter contained in the alkali extract, saturated ammonium sulfate was added at pH 7.2. The proteinous precipitate obtained was dialyzed against running water overnight. Then the precipitate was dissolved by adding dilute NaOH, and aliquots were dispended in cellophane bags. They were soaked overnight in the NaCl–HCl buffer solutions at pH 6.9 which contained a series of copper concentrations. The bags were then dialyzed against running water for 24 hours. The content of each bag was precipitated by 80% acetone. And copper was determined referred to the dry weight of the precipitate.

The results, as represented in Fig. 2, show that the copper content of the proteinous fraction from the copper-cultured cells is always higher than that from the normal cell by a constant difference about 9 *mg/g*, namely, the amount of copper originally contained in the former fraction. Hence proteinous fraction from the copper cell has a capacity to combine copper in addition to the amount already bound, and this capacity is roughly equal to that of the corresponding fraction from the normal cell. The fraction is of protein nature. But the matter which had orginally combined copper in the cell either may be proteinous or may be some other substances that comes along with protein through the procedure mentioned above. The preparation from the normal cells was bluish green after the dialysis against copper, but the color change was not observable with that from the resistant cells because of the brown color.

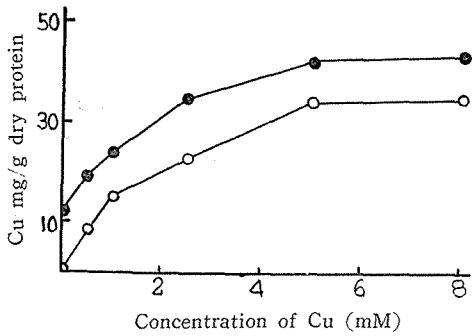

Fig. 2. Amount of copper bound by the proteinous matter of the alkali extract of cells, through the dialysis against variou concentrations of $CuSO_4$ solution buffered at pH 6.9 by NaCl–HCl, *mg* Cu per *g* dry proteinous matter.
●: Protein from the resistant strain;
○: protein from the parent strain.

The brown coloration of the alkali extract from the copper-grown cell was bleached easily when a small quantity of hydrogen peroxide or potassium cyanide or concentrated ferric chloride was added. Hence the presence of copper sulfide was suspected. Experiments on this point will be reported elsewhere.

2) Brown coloration of the alkali extract became weaker and weaker as the extraction was repeated. The sixth extracts was colored light brown with dark violet tint. Brown matter was precipitated by making the solution pH 4.0–4.2 with acetic acid. By adding 4 volumes of acetone to the supernatant, proteinous precipitate colored light blue was obtained. It was dissolved again in a weakly alkaline solution.

When pH of this solution was adjusted to 8.6, 6.2 and 4.5 by acetic acid, it became colored violet, green and light blue, respectively. The spectral absorptions

were as shown in Fig. 3. They correspond to the absorptions obtained by KLOZT *et al.* (4) for bovin albumin, β-lactoglobulin, bovin serum-γ-globulin and β-casein to which the cupric ion was added. They think that copper binds with protein in the amine type at pH 8.6 and in the carboxyl type at pH 4.5 while the tetracoördination linkage composed of two carboxyl residues and two amino residues is formed at pH 6.2.

3) Cells were colored pale violet after the repeated extraction. Suspension of these cells was divided into equal aliquots, centrifuged and resuspended in a series of buffer solutions from pH 2 to 9. After one hour, cells were washed with distilled water, and the copper and the nitrogen remaining in the cells were determined. The results are shown in Fig. 4.

The loss of nitrogen was little even in the acidic reaction. Copper, on the other hand, was lost conspicuously in the acidic side, to remain little in the cell at pH 2. This corresponds to the experiment represented in Fig. 1. The kind of buffer solution may have some effect on the copper extraction, in addition to the pH effect (5).

The color of cells was pale violet, green and light blue, respectively, when the treating solution was alkaline, neutral and acidic. Cells became colorless at pH 2.

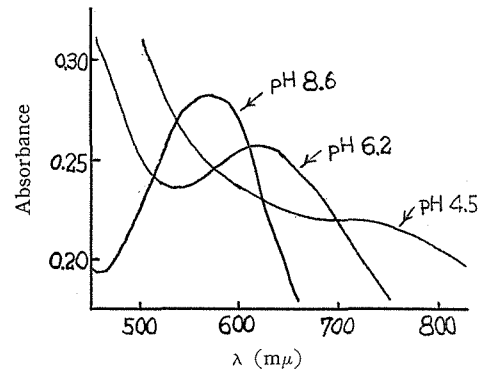

Fig. 3. Absorption spectra, at pH 8.6, 6.2 and 4.5, of the copper-containing proteinous fraction obtained by 80% acetone from the alkali extract of resistant cells after rejecting the brown fraction. The solution used for the measurements contained 4.9 *mg/ml* of nitrogen and 0.4 6*mg/ml* of copper.

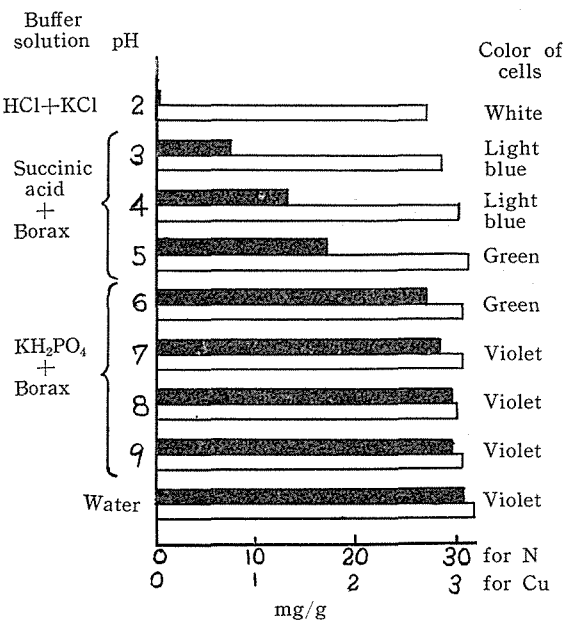

Fig. 4. Nitrogen and copper remaining in the cells suspended in a series of buffer solutions for 1 hour after repeated alkali extraction, *mg* per original weight of cells. □ : Nitrogen; ■ : copper.

These colors of cells exactly corresponded to the dependence on pH of the color of the fraction described in the foregoing section. The fact that cells lost more copper as pH was lowered may be explained by the tetracoördinate complex described above being less stable as pH decreases. The extinction of coloration at pH 2 corresponds to the loss of copper.

Thus it is presumed that, in the copper cultured cell, an amount of copper is bound by some constituents of protoplasm which is not dissolved out by 0.1 *N* NaOH. And there is an indication of a coördination complex of copper formed.

Discussion

Some enzymes, such as those having the thiole group as their integral part, are known to be more sensitive to copper than some others. However, copper may be considered to render general injury to the cell, by forming complexes with most of important cell constituents and also by modifying the colloidal state of the protoplasm. Hence the resistant cell may perform its whole metabolism by enzyme systems relatively insensitive to copper, or may have some mechanism to keep low the activity of the copper ion inside it. In the latter case, the cell has to keep out copper, or has to precipitate the copper that enters it. R_{1b} which is colored brown when cultured with certain copper media contains much copper bound by some matter (1).

For the copper-binding substance, which perhaps can be produced more by the resistant cell than by the normal cell, nitrogenous matter was suspected in the first place, and experiments were made to examine that.

When the copper-cultured cell was extracted by 0.1 *N* NaOH, some copper of the cell comes out, the ratio of copper to nitrogen being larger in this extract than in the cell. The copper in the extract was contained in most part in its brown-colored fraction which was precipitated on the acidic side. Much copper was contained in this proteinous fraction. But there were some indications that the brown color is attributable to copper sulfide. Research on the relation of copper to sulfur-containing substances in the copper-cultured cell are in progress. The proteinous matter which was not preoccupied by copper showed the same copper-binding capacity as the corresponding fraction obtained from the normal cell.

The alkali extract, after the rejection of the brown fraction, showed spectral absorptions which suggested that copper was bound by amino and carboxyl radicals of proteins. A considerable amount of copper remains in the cell even after repeated extraction by 0.1 *N* NaOH. This copper dissolves out by acid treatment. The color changes of the cell depending on pH suggest again that copper is combined in a tetracoördinate type by proteinous matter. It was not determined whether this type of coördination had been present as such in the living cell or was formed as an artifact through the procedure of preparation.

Thus the cell grown in the copper medium accumulates copper largely in three types of compound, roughly divided: 1) As the brown-colored compound which pre-

cipitates from the alkali extract when the pH is made 4.0–4.2 by acetic acid, 2) as the proteinous tetracoördinate compound which precipitates from the alkali extract by 80% acetone, and 3) as the proteinous complex which is not dissoloved out of cells by 0.1 *N* NaOH. The latter two are colored green in the neutral medium.

When the parent strain is cultured with media containing copper in concentrations which permit its growth, the culture becomes colored light brown. Hence the parent strain is not lacking in the ability of producing the brown colored matter. However, this ability is very much pronounced in R_{1b}. On the other hand, the parent cells suspended in copper solutions become colored green at neutral reaction.

The brown-colored substance, therefore, is a product by cell metabolism, and its direct bearing on copper detoxification may be suspected. Hence the relation of sulfur to copper was chosen as the next step of approach to the mechanism of copper resistance.

Summary

1. Copper resistant cells cultured in a copper medium lost only 4% of copper contained in them, when they were kept overnight in distilled water.

2. By 0.1 *N* NaOH, much copper was extracted from the cell together with nitrogenous substances. Most of the extracted copper was contained in the brown-colored fraction.

3. The brown substance in the fraction is suspected to be sulfide, on which a report is to follow.

4. The proteinous fraction of the alkali extract of the copper-cultured cells and the corresponding fraction of the normal cells were dialyzed against a concentration series of $CuSO_4$ solution. The amounts of copper bound during the dialysis were roughly the same for both fractions.

5. The tetracoördinate type of copper complex was suggested for the non-brown copper-containing fractions according to their spectral absorption.

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