MEMOIRS OF THE COLLEGE OF SCIENCE, UNIVERSITY OF KYOTO, SERIES B,
Vol. XXIV, No. 3 (Biology), Article 5, 1957

Studies on the Adaptation of Yeast to Copper

XVI. Effect of Copper on Amino Acid Pool*

By

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(Received Oct. 9, 1957)

It was reported earlier (1) that the amino acid pool of yeast was disturbed when the growth was inhibited by copper, but not when the growth was not inhibited. The copper inhibition was paralleled by a decrease in serine and glycine and an increase in peptide-like substances. It is feasible to assume that the amino acid metabolism is disturbed by copper. The experiments reported here were designed to see the interference of copper with the amino acid metabolism.

Methods

The strain used was the same as in the previous reports (1, 2, 5). The stock was kept on the MH agar or the malt extract agar, as before. In the present case the synthetic medium as represented in Table 1 was used for the experimental cultures,

Table 1. Composition of the standard synthetic medium.

Cane sugar	50 *g*	Vitamins B_1, B_2 & B_6	each 200 μg
$(NH_4)_2SO_4$	6	Ca-Pantothenate	200
KH_2PO_4	3	Nicotinamide	200
$MgSO_4 \cdot 7H_2O$	1	p-Aminobenzoic acid	200
$CaCl_2$	0.3	Biotine	2
KI	0.0001	Inositol	10000

Trace elements: B, Mn & Cu 0.01 ppm; Fe 0.05 ppm, Zn 0.07 ppm.
Distilled water up to 1000 *ml*; pH: 5.2 with NaOH.

to make ammonium the sole nitrogen source. Cells to be used in the experiments were precultured for 48 hours on the agar slant containing the same synthetic medium. For the copper medium, copper sulfate was added to the medium to make

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

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the copper concentration 0.15 *mM*. The survival ratio of the parent strain was even lower with this copper medium than with the MH medium containing 1 *mM* of copper, since copper inhibition is generally stronger in simple media than in rich media. The incubation temperature was 30°C.

Harvested cells were washed five times with *M*/15 KH_2PO_4 solution and extracted with water over boiling water bath for 20 minutes, as reported elsewhere (1).

Amino acids were separated by paper chromatography, the first solvent being phenol containing 15% of water and the second, water-saturated buthanol containing 1/5 volume of glacial acetic. Procedures were as described in the previous report (1) except that ninhydrine acetone solution was 0.15% in the present case.

Cells not stained by TOWNSEND & LINDEGREN's methylene blue (8) were counted as living, using haematimeter.

Results

1. Amino acid pool of cells in the copper medium.

When one volume of the parent strain culture was inoculated in 10 volumes of nutrient medium containing 0.15 *mM* copper sulfate and incubated at 30°C, living cells first increased and then decreased, as shown in Fig. 1. The cell concentration remained at a low level untill about 50 hours of culture, then to increase rapidly

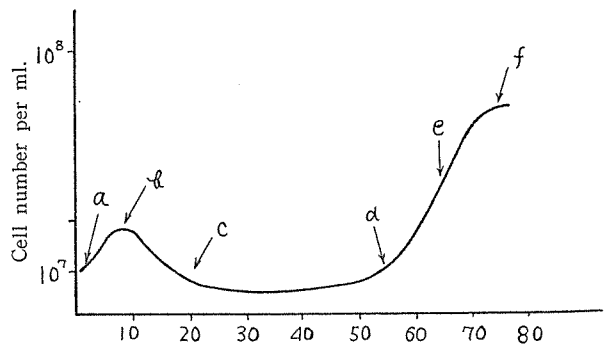
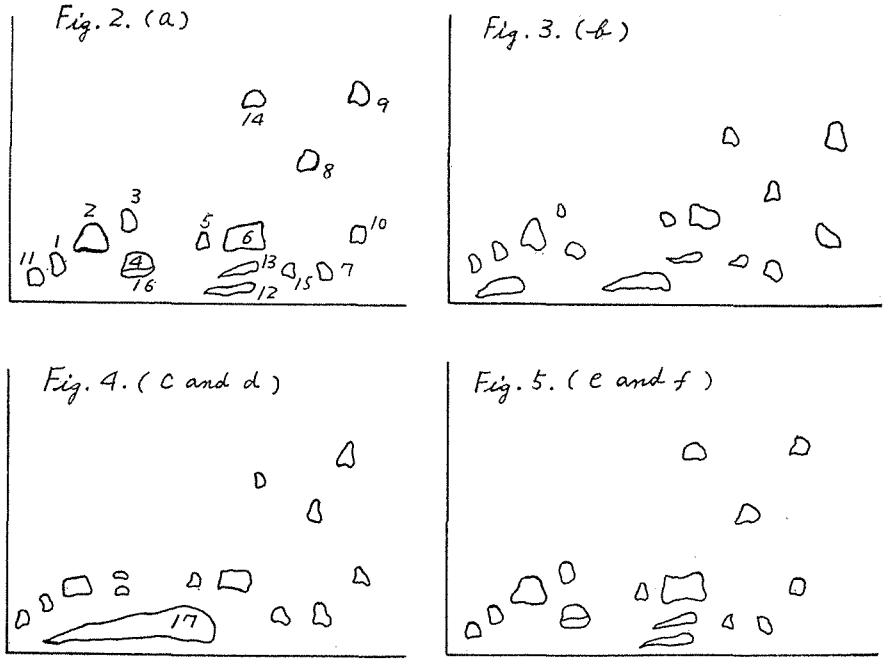


Fig. 1. Growth process in the copper medium.

owing to the growth of copper resistant cells. Such a two-step type of growth is not uncommon when inoculated sensitive microbes are not injured rapidly by the toxic agent contained in the medium (2, 8).

Figs. 2, 3, 4 & 5 represent the paper chromatograms of amino acids extracted from the cells harvested at the respective growth phases as indicated on the curve in Fig. 1. Compared with the chromatogram at phase a (Fig. 2), the spots corresponding to serine and glycine became smaller in phase b (Fig. 3), and still smaller



Figs. 2, 3, 4 & 5. Amino acid paper chromatograms of extract of cells harvested at the growth phases, a–f in Fig. 1, indicated in brackets. The first (horizontal) run by phenol, the second (vertical) by buthanol. 1. Aspartic acid, 2. glutamic acid, 3. serine, 4. glycine, 5. threonine, 6. alanine, 7. methionine sulfoxide, 8. leucine & isoleucine, 9. norleucine and/or phenylalanine, 10. proline, 11. cysteic acid, 12. arginine, 13. glutamine, 14. tyrosine, 15. histidine, 16. asparagine, 17. peptide-like substances.

in phases c and d (Fig. 4). The spot which was presumed to represent peptide-like substances in the previous report became apparent in phase b, and more striking in phases c and d. Then, in phases e and f where the culture was composed of resistant cells (2), the spots of glycine and serine were recovered and the peptide-like substances almost disappeared. Thus the inhibition of growth by copper is accompanied by the disturbance in the amino acid pool. The pattern of disturbance is the same as that observed when the copper resistant strain was inhibited by a very high concentration of copper (1).

2. Effect of copper on nitrogen starvation and recovery.

When the parent strain was inoculated in the synthetic medium lacking in ammonium sulfate, the cell number increased about 4 times as many, accompanied by a marked decrease in the cell size. When cells were kept nitrogen-starved in this way for 24 hours, spots of amino acids disappeared, except those of aspartic acid,

glutamic acid and alanine, which also became small (Fig. 6 B). On the other hand, when 0.15 *mM* of copper sulfate was added to the nitrogen deficient medium, the inoculated cells did not grow. In this case, the spots did not disappear as in the foregoing case, although some spots, including those of serine and glycine, became smaller. (Fig. 6 C). Utilization of amino acids in the pool seemed to be inhibited by copper. The decrease in glycine and serine was observed even under the supply of ammonium. But the peptide-like substances were not formed in the present case. Hence, the formation of peptide-like substances in the presence of copper seems to be conditioned by the supply of nitrogenous matter, including ammonium.

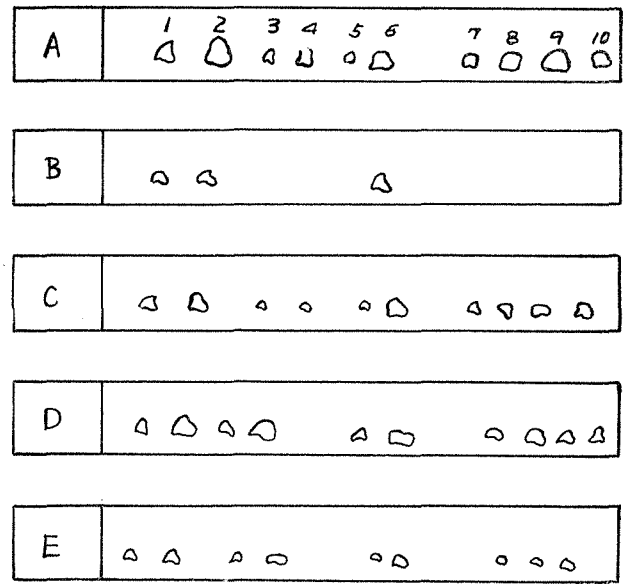


Fig. 6. One-dimensional paper chromotograms of cell extracts rum by phenol-water. A: Normal; B: Nitrogen starved for 24 hours; C: Nitrogen starved in the presence of 0.15 *mM* $CuSO_4$ for 24 hours; D: Nitrogen starved cells (as in B) treated with the synthetic medium for 30 minutes; E: Nitrogen starved cells treated for 30 minutes with the synthetic medium containing 0.15 *mM* $CuSO_4$. 1. Aspartic acid, 2. glutamic acid, 3. serine, 4. glycine, 5. threonine, 6. alanine, 7. valine, or methionine sulfoxide, 8. leucine and/or isoleucine, 9. norleucine and/or phenylalanine, 10. proline.

In order to see the effect of copper on the assimilation of ammonium, cells were nitrogen-starved for 24 hours. They were, then, suspended in the complete synthetic medium both with and without addition of copper sulfate. When the medium was not poisoned, the pool components were recoverd almost completely within 30 minutes (Fig. 6 D). But when 0.15 *mM* copper sulfate was contained the recovery of amino acid pool was incomplete even after 60 minutes or more (Fig. 6 E). Hence, copper possibly inhibits the formation of amino acids from ammonium.

3. Effect of amino acid supply.

The amino acid pool was poor when the growth was inhibited by copper (Fig. 3 & 4). If the growth inhibition in the copper medium is due to the inhibition by copper of the synthesis of a certain amino acid from ammonium, the growth would recur when that amino acid is supplied.

The parent strain was inoculated in the synthetic medium containing 0.15 *mM*

copper, and harvested after 8 hours. The cells were in the phase as represented by b in Fig. 1. It is supposed that the cells were penetrated by an amount of copper sufficient to stop growth. They were washed and divided into several aliquots. Each aliquot was introduced in a 0.15 *mM* copper-containing synthetic medium in which ammonium sulfate was replaced by a kind of amino acid for the same amount of nitrogen.

Since the activity of the cupric ion may be differently affected by different amino acids, the effect of amino acids on the growth was observed by returning the treated cells into the original copper medium, of which the nitrogen source was ammonium.

Cells were harvested after 30 minutes of the amino acid supply, washed three times with *M*/15 KH_2PO_4, and returned to the original medium. The cell multiplication ceased within 8 hours after the returning to the original medium. The effect of the preceding amino acid treatment on the multiplication in the ammonium

Table 2. Effect of amino acid supply on the following cell multiplication in the copper-containing standard medium.

Amino acid (conc. *g/l*)		Multiplication factor	Amino acid (conc. *g/l*)		Multiplication factor
dl-Phenylalanine	(1.25)	2.0	*dl*-Isoleucine	(1.0)	1.1
dl-Methionine	(1.0)	1.6	*l*-Glycine	(0.57)	1.1
dl-Serine	(0.8)	1.5	*dl*-Proline	(0.87)	1.0
dl-Lecucine	(1.0)	1.4	*l*-Asparagine	(0.5)	1.0
dl-Threonine	(0.91)	1.3	*l*-Lysine	(0.55)	1.0
dl-Aspartic a.	(1.0)	1.2	*dl*-Valine	(0.88)	1.0
dl-Norleucine	(1.0)	1.2	$(NH_4)_2SO_4$	(0.5)	1.0

medium is represented in Table 2. The most effective was phenylalanine, and next to it, methionine, serine and leucine. Glycine was not as effective as serine, even though it decreased as the latter at phase b.

To test the replenishment of amino acid pool in the presence of copper, nitrogen-starved cells were suspended in copper media containing each amino acid. After 30 minutes, the composition of amino acid pool was examined. Cells treated with phenylalanine, methionine and serine possessed the complete amino acid pocl, so far as the chromatographic spots concerned. By the treatment with leucine, many spots were recovered except those of serine and glycine. On the other hand, treatments with other amino acids could hardly improve the pool, only the spots of the exogenously supplied amino acids growing large. These amino acids did not seem to serve as the source of many components of the amino acid pool in the presence of copper. The experiment again showed the parallelism between the temporary cell multiplication and an integrity of the amino acid pool.

The four most effective kinds of amino acids were combined two by two for the treatment, 3 *g/l* of each being contained in the treating medium. As shown in Table 3, the combinations were not more effective, but on the contrary, less effective than each of the component amino acids.

Table 3. Effect of treatment by combination of amino acids on the cell multiplication in the copper-containing standard medium.

Combination of amino acids	Multiplication factor
dl-Phenylalanine-*dl*-Methionine	1.4
" -*dl*-Serine	1.6
" -*dl*-Leucine	1.5
dl-Methionine-*dl*-Serine	1.5
" -*dl*-Leucine	1.0
dl-Serine-*dl*-Leucine	1.5

4. Interference by an amino acid analogue.

It is shown in the preceding section that cells resumed growth in the presence of copper when the amino acid pool could be replenished by the supply of an amino acid. And in this respect phenylalanine was the most effective. The use of amino acid analogue may serve to elucidate the amino acid metabolism in the presence of copper.

p-Chlorophenylalanine (PCPA) was synthesized from p-chlorobenzaldehyde. The preparation used was not extremely pure. PCPA was added to each of the four effective amino acids at various proportions. Cells at phase b, Fig. 1, were suspended in the copper media containing these mixtures for 30 minutes, and the multiplication was observed in the ammonium medium containing copper. Factors of the cell increase determined are represented in Table 4. The presence of PCPA at 1 : 1 mol ratio annulled the effect of serine, methionine, and leucine. To antagonize phenylalanine, 5 times more of the analogue was necessary.

Table 4. Interference of p-chlorophenylalanine with the effect of amino acid on the cell multiplication in the presence of copper.

Amino acid (conc. in *mM*)	Mol ratio (amino acid : PCPA)				
	1 : 0	1 : 1	1 : 2.5	1 : 5	1 : 10
dl-Phenylalanine (10)	1.8	1.3	—	—	—
" (1)	1.3	1.3	1.3	1.0	1.0
dl-Serine (10)	1.8	1.0	—	—	—
" (1)	1.5	1.0	1.0	1.0	1.0
dl-Methionine (10)	1.5	1.0	—	—	—
dl-Leucine (10)	1.4	1.0	—	—	—

In order to see the effect of PCPA on the replenishment of the amino acid pool, cells were nitrogen starved for 48 hours in absence of copper, and then suspended in the non-toxified medium which contained 0.25 *mM* of ammonium sulfate, glutamic acid, or phenylalanine as the sole nitrogen source with and without addition of

0.25 *mM* of PCPA. The amino acid pool as detected by one-dimensional paper chromatography was replenished by each of the nitrogen source when PCPA was absent. But when PCPA was present, the paper chromatogram obtained was roughly the same as in Fig. 6 E, except that there was a large spot at the site of phenylalanine. Hence PCPA inhibits the replenishement of amino acid pool by any one of ammonium, glutamic acid and phenylalanine. It had not been expected that a low mol ratio of PCPA to phenylalanine as 1 : 1 is sufficient to inhibit the recovery of amino acid pool by phenylalanine, since five times as much PCPA was necessary to inhibit the growth to be caused by the supply of phenylalamine.

Discussion

It has been reported in the previous paper (1) that serine and glycine decreased in the extract of cells when their growth was more or less inhibited by copper, even in the copper resistant strain if the copper concentration was sufficiently high. The culture medium contained peptone and malt extract in that case. In the present investigation the untrained strain was inoculated in a synthetic medium which contained ammonium sulfate as the sole nitrogen source. A low concentration of copper was sufficient to inhibit the growth. And, just as in the foregoing case, a decrease of serine and glycine in the cell extract was observed when the growth was inhibited (Figs. 3 & 4). They recovered when the growth occurred after two days' pause (Fig. 5). On the paper chromatogram the large spot, which was reported in the preceding paper and considered to correspond to the peptide as reported by DENT (10, 11), was conspicuous also in the present case, as far as the growth was inhibited.

HALVORSON *et al.* (6) have reported that serine and glycine decrease when yeast is grown on pyruvate, lactate or glycerol as the carben source. They suspect as the cause a greater demand for porphyrin synthesis. Sucrose is used as the carbon source in the present experiment. MINAGAWA (12) has determined that, in the presence of copper, the yeast produces coproporphyrin rather than cytochromes. However, this disturbance in porphyrin metabolism is not yet studied in connexion with the elevated consumption of serine and glycine.

The decrease of serine and glycine was accompanied by the apperarance and expansion of the peptide spot, when the complete medium was used. But, when the medium was deficient in nitrogen source, serine and glycine disappeared not accompanied by a detectable appearance of the peptide spot.

The analysis of the substance in this spot has not yet succeeded. Hence its nature and its relation to serine and glycine are not yet known. There remains a possibility that the syntheses of serine and glycine are inhibited by copper relatively more severely than those of other amino acids. For this possibility, however, the decrease of these amino acids in the case of the broth medium should be explained properly.

When NAGAI (9) cultured yeast in media containing so much NaCl as to interfere

with growth, he found in the cell extract glutamic acid, alanine, histidine and trace of aspartic acid, all the other amino acids being extinct in the paper chromatogram. The peptide-like substance was not found in that case. Copper thus seems to cause disturbances in the amino acid metabolism different from those effected by the concentrated NaCl. ARAKATSU *et al.* (5) inoculated normal yeast on solid copper media, each containing one kind of amino acid in addition to ammonium. He determined the highest copper concentration which permitted an uninterrupted growth, not as in Fig. 1. By this method glutamic acid and aspartic acid helped the yeast growth in copper best among amino acids tested. These two amino acids were not so effective in the present experiment as phenylalanine, methionine, serine and leucine. The latter three were of the second class in the experiment of ARAKATSU, when amino acids were classified into five according to their effectiveness in antagonizing the growth inhibition by copper. Chief disagreement between the two experiments are in glutamic acid and aspartic acid on the one hand, and in phenylalanine on the other hand. No explanation seems to be available except permeability. Cells at phase b, Fig. 1, were treated by an amino acid only for 30 minutes in the present experiment. The period of the treatment was taken short in order not to let cells multiply during the treatment. So the result may differ from the case where the amino acid was supplied continuously during the culture. When there was no copper, the 30 minutes' treatment of nitrogen-starved cells by glutamic acid produced the complete content of the amino acid pool. Hence the permeability to glutamic acid should be lower in the presence of copper. Phenylalanine is supposed to be relatively permeable, since the single application of it can cause a temporary growth in copper. But perhaps phenylalanine is not one of efficient amino acids to support the continued growth as in the case of ARAKATSU *et al.*

When the cells at phase b, Fig. 1, were treated with phenylalanine, methionine or serine for 30 minutes, the amino acid pool recovered completely in the presence of copper. Leucine compared somewhat unfavourably with them in this respect, as well as in the growth effect. The former amino acids can let cells produce all the amino acids necessary and useful for growth in the presence of copper.

The equimolar PCPA could antagonize phenylalanine recovering the amino acid pool of nitrogen starved cells. However, five times more of PCPA was needed to inhibit the effect of phenylalanine making the copper-inhibited cells resume growth. The paper chromatogram of extract of the copper-inhibited cells differed from that of cells of simple nitrogen starvation in at least two points, namely, that the spot corresponding to phenylalanine was not small and that the peptide-like substance was present. Hence more experiments are needed to discuss the interference by the amino acid analogue with the recovery in growth of copper-inhibited cells in connexion with the replenishment of amino acid pool.

When the yeast growth is inhibted by copper some components of the amino acid pool become deficient, while the pool remains undisturbed in the presence of the same concentration of copper if the strain is resistant enough. The copper inhibition causes deficiency in particular amino acids, and the deficiency can be

restored in the presence of copper by each one of a few particular amino acids supplied singly. These facts may be useful for finding some metabolic steps which are relatively sensitve to copper inhibition in the parent strain.

Summary

1. The normal strain of *S. ellipsoideus* was inoculated in a synthetic medium containing 0.15 *mM* copper sulfate, and the amino acid pool of cells was examined at different growth phases. Serine and glycine decreased and peptide-like substances increased while the growth was inhibited, and these abnormal patterns disappeared when resisant cells grew preponderantly after 2 days of incubation.

2. When the copper medium was lacking in the nitrogen source, the peptide-like substances were not produced.

3. Copper inhibits the replenishment of the amino acid pool by the ammonium sulfate treatment of cells.

4. Cells whose growth was inhibited by copper in the ammonium medium were treated with each amino acid for 30 minutes, and the multiplication was observed after they were returned to the original copper medium. Phenylalanine, methionine serine and leucine were more effective than other amino acids for the multiplication. The effect of these amino acids was antagonized by coexistent p-chlorophenylalanine.

Acknowledgements

The author wishes to express his cordial thanks to Professor Joji ASHIDA for his kind guidance during the course of this research. It is also pleasure to record his thanks to the members of the research group of microbial variation for valuable advices.

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