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Studies on the Adaptation of Yeast to Copper

IX. Copper-Combining Capacity of Resistant Cells

By

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When a strain of *Saccharomyces ellipsoideus* is cultured in a medium enriched with $CuSO_4$, the population becomes very tolerant to copper (1, 2). Copper may directly inhibit many enzymes and may also interfere with a wide range of vital activities by changing internal physical and chemical conditions of the cell. Hence, studies on the resistance mechanism of the copper resistant variant should begin by determining, whether the copper permeability of the resistant cell is low enough, as Pulst (3) claimed for *Penicillium*, or whether the cell has an ability to produce a sufficient quantity of substance(s) which can easily combine with copper to detoxicate it.

The present paper reports the results of determining copper binding capacities for the normal and the resistant cells, with the conclusion that the detoxication mechanism plays a role in the copper resistance.

Material and method

The yeast strain used was the same as in the previous works (1, 2). The culture medium was the modified Henneberg solution* to which 1/3 volume of malt extract (about 8 Bé) was added. The copper medium was prepared by adding a measured quantity of sterilized solution of $CuSO_4$ to the sterilized culture medium, cooled to room temperature. The incubation temperature was 30°C.

To determine the copper content of the cells, the latter were harvested and washed three times with distilled water by centrifuging at 3,000 rotations per minute. With a view to dissolve the precipitate, which slightly formed during the culture and might contaminate the harvested cells, 1/20 volume of 20% HCl was added to the broth at 50°C before the first centrifugation. The washed cells

* KH_2PO_4 5g., $MgSO_4 \cdot 7H_2O$ 2g., peptone (Funai) 5g., cane sugar 100g., distilled water 1,000 ml.

were dried at 85°C to the constant weight, and ashed in a porcelain crucible.

The ash was dissolved in 2 ml. of 8N HNO_3, and the excess acid was then evaporated on water bath. The copper from the cells, now being $Cu(NO_3)_2$, was determined by the iodometric method, using KI and 0.01N $Na_2S_2O_3$(4).

This method gives the sum of copper, iron and other oxidizing substances contained in the cell ash. But as is shown below, the values obtained with this method became remarkably higher when cells were cultured or suspended in copper-containing media. If it is assumed in this case that the increase in other oxidizing substances in the ash of the cells is insignificant compared with the supposed great increase in copper, then the increase in the analytical valne may roughly represent the increase in copper content of the cells. The aim of the present work is to catch a general trend.

For convenience, the titration values are expressed in the following in mg. of copper per g. dry weight of cells, as if the liberation of iodine was due solely to copper. The value will tentatively be called the "copper content".

Results

1. COPPER CONTENT OF CELLS. The "copper content" of the cells was 0.347 for the normal cells, and from 6.7 to 6.9 for the copper-trained cells cultured in the medium, which was enriched with 1 mM/1. of $CuSO_4$ at the start of culture (cf. table). Hence the copper-cells contained more copper than the normal cells by about 6.5 mg. per g. dry weight.

In the above mentioned copper culture, cells proliferated about 100 times in dry weight. Assuming that the inoculant cells had contained the same amount of copper per dry weight as the analysed cells, the amount of copper removed by the growing cells from the culture broth can be calculated. By subtracting this value from the amount of copper originally added to the medium, the concentration of copper in the medium at the time of harvest of cells is estimated. This value shows the copper concentration of the external medium when the analysed cells had been in equilibrium with it. For example, the above mentioned copper-cells had been holding 6.7–6.9 mg. "copper" per g. dry weight in equilibrium with 0.51 mM copper in the culture broth (cf. table).

In order to see how the "copper content" of the resistant cells rises when the external concentration of copper is increased, cells of 72-hour-old 1 mM copper culture were harvested, washed twice with a nutrient medium unenriched with copper, and resuspended in a half volume of fresh 1 mM copper medium. Cells were analysed after 24 hours of incubation, during which the dry weight of cells increased only about 20 per cent. The "copper content" was 8.36, the equilibrium concentration of copper in the medium being 0.89 mM.

The main purpose of this study is to see if there is any difference in the copper binding capacity between the resistant and the sensitive cells. Hence,

Table. Amount of oxidizing substances in the ash of the sensitive and the resistant cells, expressed as mg. of copper per g. dry cells. Cells were cultured or suspended in the various media for the periods indicated. Estimates of the copper concentration in the media at the harvest of cells are given. The cases where significant growth had occurred are italicised. The values calculated on assumptions are bracketed.

Strain	Medium	Incubation period (hour)	Living Cells		Killed Cells	
			" Cu " in cells (mg./g.)	Final Cu in medium (mM/l.)	" Cu " in cells (mg./g.)	Final Cu in medium (mM/l.)
Sensitive	Nutrient	48	*0.347*	—	—	—
	1mM Cu in nutrient	6	(*1.1*)	—	(1.55)	—
		24	—	—	1.92	0.87
	1mM Cu in water	6	—	—	2.99	0.73
		24	—	—	3.83	0.69
	0.2mM Cu in nutrient	72	*1.40*	*0.15*	—	—
Resistant	1mM Cu in nutrient	72	*6.7-6.9*	*0.51*	—	—
		24	8.36	0.89	7.5	ca. 0.9
	0.2mM Cu in ntrient	72	*2.07*	*0.06*	—	—

it is desirable to compare the two kinds of cells under the same conditions. This, however, is not possible with living cells :—Owing to the low permeability of cell boundary to copper, many hours' contact of cells with the environmental copper seems to be needed for the copper-binding of cells to be equilibrated with the environment. However, the sensitive population usually becomes copper resistant in a number of hours when the medium contains sufficient nutrient, and many cells are injured by copper when the medium is poor.

Therefore, the comparison of the copper binding capacities of the normal and of the resistant cells was made in two ways: 1) with freshly killed cells, and 2) by culturing cells in a copper concentration which was too low to train them to be copper resistant.

For the first method, the parent strain was inoculated in five flasks containing 100 ml. each of the normal nutrient solution. After 48 hours of incubation they were heated for 10 minutes on water bath at 80°C. And the killed cells of all five containers were collected together. After being washed twice with the normal nutrient solution, the cells were resuspended in 500 ml. of the nutrient solution to which $CuSO_4$ was added to give the concentration of 1mM.

The suspension was kept for 24 hours at 30°C. In a similar way, the copper resistant strain was cultured in the nutrient solution containing 1mM $CuSO_4$. Cells were harvested after 72 hours of incubation, as the growth seemed to be a little slower. They were washed and were heat-killed in the nutrient solution not supplemented with copper. The killed cells were suspended in 1 mM copper-nutrient, and kept for 24 hours.

The "copper content" of cells was 1.92 for the parent strain and 7.5 for the copper resistant strain. The copper concentration in the medium was not much lowered even in the case of resistant cells, because more than 6 mg. "copper" was carried in by 1g. dry weight of the cells. So the final copper concentrations in the media are roughly the same in the two cases (cf. table). Hence it is inferable that the resistant cells can combine more copper than the sensitive cells, at least after they were heat-killed.

The table may suggest that the "copper content" of the living resistant cells is higher than that of the heat-killed ones. This may be accounted for, partly by heat denaturation of cellular substances, and partly by the fact, found by an unpublished experiment, that some copper binding substances get away from the resistant cells with relative ease when cells are injured.

For the second method, the nutrient broth supplemented with 0.2mM copper was inoculated with the sensitive strain. When the cultured cells at the stationary phase of growth were streaked on the nutrient agar slant containing 1 mM copper, colonies did not grow rapidly and confluently, but grew only scatteringly after one day's delay, just as the original sensitive strain does. Hence, the training by this concentration of copper, if at all, is much less effective than that by 1mM copper.

Two flasks, each containing 100ml. of 0.2mM Cu-nutrient broth, were inoculated respectively with the resistant strain harvested from 1ml. of 1mM Cu-broth, and with the sensitive strain harvested from the same volume of the normal broth. The "copper content" of cells after full growth were 1.40 for the sensitive cells, and 2.07 for the resistant cells. Since the final copper concentration of the medium was very low in the latter case (cf. table), it is undoubted that the copper binding power of the resistant cells is higher than the sensitive cells.

Although the copper-containing culture medium was never warmed above 30°C before the harvesting procedure, some portion of added copper may be bound by components of the medium with respective strengths. So it is interesting to see how much more copper the cells can take in from the plain aqueous solution of $CuSO_4$ than from the standard copper nutrient broth. The normal cells were killed, as described above, and were left suspended in a 1mM $CuSO_4$ solution for 24 hours at 30°C. The "copper content" of the cells was 3.83, or about 1.9 higher than when suspended in 1mM Cu-nutrient broth. Hence it is obvious that the nutrient medium retains copper with some force. When

the dead cells suspended in the plain copper solution were analysed after 6 hours of incubation, the value was 2.99. Hence the copper binding power was not satisfied in 6 hours at 30°C even for the killed cells.

When the sensitive cells, gathered from 500ml. of the normal culture medium, were resuspended in 250 ml. of 1mM Cu-nutrient broth, only a very slight growth was observed in the first 6 hours, followed by a gradual death of cells. The "copper content" of the cells determined at 1.5 and 6 hours of incubation were 0.52 and 1.23, respectively. According to the method of staining with Fink's methylene blue, 2.3 and 29.7 per cent, respectively, of the sampled cells were dead.

Now, if it is assumed that the "copper content" of the dead cells in this case is the same as the heat-killed cells, and further that the ratio of the "copper contents" of the killed cells at 6 and 24 hours in the copper-nutrient broth, is the same as the corresponding ratio in the plain copper-solution as for the present case, the "copper content" of the dead cells among the living cells in the 1mM Cu-broth at 6 hours of incubation is estimated to be 1.55.* The value for the living cells at the same moment is calculated to be 1.1**. Provided that the assumptions be true, the amount of copper bound by the living cells in 6 hours is less than that by the dead cells. This, however, by no means implies a difference in the binding capacity. A difference in permeability is suspected to be more probable as the cause of the difference.

2. Change of copper content during growth. One hundred ml. of the 1mM Cu-nutrient medium was inoculated with 0.3ml. of a 48-hour-old culture of the parent strain. The primary growth, which is sometimes called the residual growth, stopped within 6 hours after inoculation. Following a period of decrease in number of living cells, an active growth, so to speak the secondary growth, ensued. The progress of the secondary growth, as determined by the dry weight of cells per unit volume of culture, is represented by the curve A in Fig. 1. This curve is inferred, from various grounds, to be connected to the inoculation size as shown by the broken lines. More accurately, however, the horizonal part may be a little higher than indicated, due to the slight primary growth at the beginning of the culture.

The "copper content" of cells was not measured until 66 hours of incubation, when the population size had grown large enough to permit the analysis by the present method. The "copper content" determined is represented by curve B in Fig. 1. The value at 6 hours of incubation, calculated in the preceding section, is shown by B′.

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* Cu in dead cells at 6 hours period: $(1.92-0.35)\times\frac{2.99-0.35}{3.83-0.35}+0.35=1.55$

** Cu in living cells at 6 hours period: $\frac{(1.23-0.35)-0.297\times(1.55-0.35)}{0.703}+0.35=1.1$

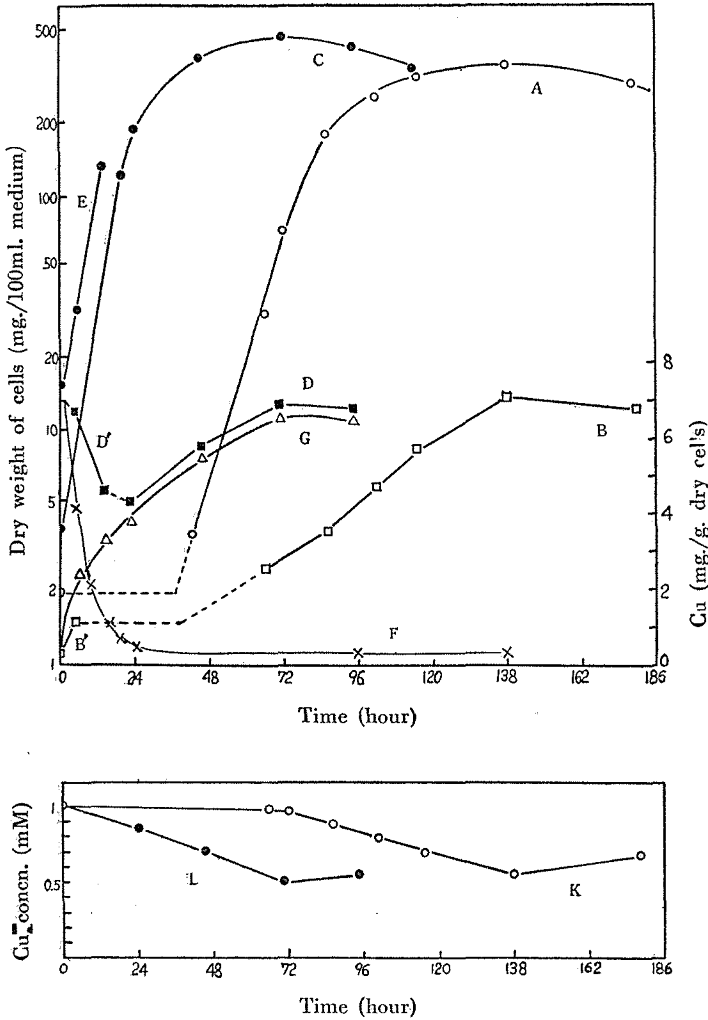

Fig. 1. Growth and copper content of cells when the sensitive and the resistant strains were inoculated in the medium containing 1 mM $CuSO_4$.

A, C, E: Growth in dry weight of cells per 100ml. of culture. A, sensitive strain inoculated; C, resistant strain; E, resistant strain more heavily inoculated.

B, B′, D, D′: Changes in copper content per unit dry weight of cells. B′–B, for culture represented by A; D′–D, for cultures represented by C and E.

F: Dilution of intracellular copper by cell growth, no absorption of copper being assumed.

G: Amount of copper having entered the cells during the growth represented by C.

Fig. 2. Concentration of copper in the medium left unabsorbed by the cells.

K: in the case of A and B, Fig. 1; L: in the case of C and D, Fig. 1.

It may be said that the extrapolation of B intersects with the level line of B′ roughly at the time of beginning of the secondary growth (cf. curve A). It can be inferred with high probability, that the copper binding power of cells begins to increase when they commence to grow invested with the copper resistance. The steady increase in "copper content" is probably not due to the accumulation of dead cells and their fragments, because the copper fixation by killed cells was inferred in the preceding section not to be higher than the living cells.

If the strain kept culturing in 1mM Cu-medium is inoculated with a fresh medium of the same composition, growth proceeds in about the same way as when the parent strain is inoculated in the normal medium. Curve C represents the growth in dry weight, when 0.9ml. of 48-hour-old copper-culture of the resistant strain was inoculated in 100ml. of the fresh 1mM Cu-medium. The "copper content" of cells changed with time as shown in D. And it is to be noted that the relation of D to C well corresponds to that of B to A.

Now, it is interesting to note that the first measured point of curve D is lower than the maximum level. The inoculated cells must have contained nearly the maximum amount of copper, because they had been in 1mM Cu-medium. In order to see the change in "copper content" in the early phase of growth, the quantity of medium and the inoculation size were made large enough to make the determination possible.

In this case, the 72-hour-old culture was used as the inoculant, since the "copper content" had been found to reach the maximum within this time. Into 480ml. of 1mM Cu-medium, 20ml. of 72-hour-old copper-culture was inoculated. The dry weight of cells increased as represented by E in Fig. 1. The growth rate was only a little lower than in the case of smaller inoculation size. The "copper content" of cells decreased with time, as shown by curve D′.

The rapid cell growth, in comparison with the rate of penetration of copper into the cell, may account for the decrease in the "copper content". If the cells grew without absorbing copper from the environment, the "copper content" would have decreased in an inverse proportion to the dry weight of the cells. Curve F represents the case, the final level being the basic value of the normal cells. Subtracting F from D′ and D, curve G is given, which represents the course of entrance of copper into the cells.

The amount of "copper" remaining in the culture medium was calculated, as before, along the curves B and G. Curves K and L, in Fig. 2, illustrate the results. The absorption and fixation of copper by cells, as represented by the curves B and G in Fig. 1, proceeded when the copper content of the medium was decreasing as those curves show.

Discussion

Iodometry gives the sum of copper, iron and other oxidizing substances

present in the ash of cells. However, this method was used in this preliminary study for determining how much more copper the yeast cells combined when they were kept in copper-containing media, since the values found were considerably higher than the cells cultured in the normal medium. The disclosed enormous capacity of the copper-resistant cells to combine with copper suggests strongly that they can produce a considerable amount of substance(s) which can easily fix copper and protect important enzymes and other cell components from being poisoned by copper.

It was also found that the cell permeability to copper was so low that the copper content of cells significantly decreased even in the copper medium, when the growth proceeded rapidly (cf. D′ in Fig. 1). Hence it is inferred that the resistant cells grow fast since they can detoxicate the copper which enters into them. And, that they can grow fast is, in turn, effective in reducing the intracellular concentration of copper which enters them slowly.

The resistant cells accumulate very much copper when they are in contact with media supplemented with copper. But the copper content of them is considered by Minagawa *et al.* (5) to be at the same level as the sensitive cells when they have been cultured in the normal medium without added copper.

In case the sensitive cells are inoculated to 1 mM Cu-nutrient broth, the over-all growth stops before the intracellular "copper" amounts to about 1mg. per g. dry cells, while the resistant cells can grow even when they contain far more copper. In a period of 1 to 2 days, after which the secondary growth occurs, the number of living cells slowly decreases as a result of the death of cells insufficiently compensated by slower budding. Detailed descriptions of this phase will be published elsewhere.

The lines connecting B′ to B in Fig. 1 are not well substantiated. It is true, however, that the first measured point of B at 66 hours is lower than what would be expected if copper had been entering into cells during this period with a rate similar to that observed for the resistant cells. This may be explained in two ways: 1) The permeability of the sensitive cells to copper is lower than the resistant cells; and 2) a thermodynamic equilibrium had been reached with respect to the diffusible form of copper between the medium and the cell inside, which had a much lower copper-binding power than the resistant cells. According to the rapid intake of copper by sensitive cells at the earliest period, as represented by B′ in Fig. 1, the first alternative seems to be improbable.

After the cells had begun to grow rapidly, invested with the resistance mechanism, the copper content of cells increased in spite of the high growth rate (B in Fig. 1). This differs from the case when copper-trained cells were inoculated (D′ in Fig. 1), where the copper content decreased at the early phase of growth possibly because the cells had contained very much copper. In the present case, on the other hand, the copper content of cells was low when the rapid growth began. The rate of increase in copper content of cells is a little

lower in the logarithmic growth phase than in the phase of negative growth acceleration. It is likely that the rate of copper accumulation of cells changes in the course of growth conditioned by an increase in the intracellular copper, changes in the rate of production of copper binding substances, a decrease in copper in the medium, and a decrease in the growth rate.

Although the analytical method used was primitive, it can be concluded from the results so far obtained that the copper resistant cells combine far more copper than the sensitive cells, and that this is an important mechanism which makes cells viable in the copper medium. The present study, however, by no means excludes other possibilities of mechanism of copper resistance.

Summary

1. The copper resistant cells accumulated far more copper than the sensitive cells, living or dead, when they were cultured or suspended in copper-containing media.

2. When the sensitive strain was inoculated in the copper medium, the copper content of cells markedly increased side by side with the growth of adapted cells.

3. The detoxication of copper by substances adaptively produced in the cell is inferred to be a mechanism of copper resistance.

4. The cell permeability to copper is so low that the copper content of cells decreases even in the copper medium when the growth is rapid.

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