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Copper Permeability of Yeast Cells in Relation to Copper Resistance

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By culturing sensitive strains of *Saccharomyces cerevisiae* in copper-containing media, resistant substrains are obtained which differ from their parents in a single genetic factor (13). Hence it may be expected for the first place that the copper resistance does not involve diverse metabolic systems. As copper may give damage to many important components of protoplasm if it enters into the cell, a simple resistance mechanism would be to prevent its penetration. It has been found that some resistant strains produce much hydrogen sulfide using sulfate in the medium as the source (6). This may also be a simple resistance mechanism. But as hydrogen sulfide producing activity is low until late exponential growth phase (7), resistance mechanism in earlier growth phase remains to be studied.

In order to estimate copper permeability, copper content of cells was measured after treating them with solutions of copper salt. The results, however, showed that the copper content of resistant cells was not necessarily lower than that of sensitive cells. And it was confirmed that the hydrogen sulfide producing activity was so low in a very early growth phase that the measured copper content of resistant cells was not contributed by copper accumulation as sulfide and that the resistance mechanism was ascribable neither to low copper permeability nor to sulfide formation. These experiments are reported in this paper.

Materials and Methods

Strains used were a copper sensitive diploid, *c*, and a resistant diploid, *c*-43, obtained by culturing *c* in a copper-containing medium. Their genotypes were r/r and R/r, respectively (13).

The standard medium contained peptone 3.5 g, yeast extract (powder) 1.0 g, KH_2PO_4 2.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, and sucrose 40.0 g per liter of deionized water. The carbon deficient medium called SN(C^-) contained in 1 liter $(NH_4)_2SO_4$ 2.0 g, KH_2PO_4 3.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, $CaCl_2 \cdot 2H_2O$ 0.25 g, vitamins mixture (see

SENO (13)) 10 ml, and 0.25 ml each of mineral solutions A* and B**. When a carbon source was supplemented to this medium, it was indicated in parentheses. For solid media agar was added to 1.5 per cent. For preparing a copper-containing medium, a suitable volume of sterilized 100 mM $CuSO_4$ solution was added to sterilized nutrient medium after cooling to room temperature. Inoculations were invariably made to media previously brought to the incubation temperature for the experiment.

Cells for experimental use were harvested from 36-hour culture in the standard liquid medium which formed a layer of ca. 8 mm depth in a ROUX bottle and was shaken moderately at 30°. At the harvest of this culture, the number of cells forming clusters was only about 15 per cent of the population. For obtaining carbon-starved cells, cells were suspended in SN (C^-) liquid medium to a density of ca. 1.2×10^8 cells per ml and shaken for 6 hours at 30°.

Viable count of culture was assayed by plating cells on the standard agar medium, after they were rinsed three times with cold (ca. 6°) deionized water, or, after copper treatment, twice with a cold copper-combining agent (Table 2) and then once with cold deionized water.

To determine copper content of cells, cells were rinsed as just mentioned, dried at 98° to constant weight, ashed by wet combustion, and submitted to the assay by carbamate method (12), Shimazu's photoelectric spectrophotometer being used for colorimetry.

Hydrogen sulfide produced by cells was determined according to NAIKI(6), namely, air was bubbled through cell suspension to make the evolving hydrogen sulfide gas absorbed by zinc acetate solution, and concentrated hydrochloric acid was added to the cell suspension at the end of incubation to make remaining sulfide turn to gas and trapped by zinc. Zinc sulfide was changed to methylene blue, which was measured spectrophotometrically.

Results

The primary object of this study is to examine experimentally if there is any difference in copper permeability between sensitive and resistant cells. Some precausions are needed when copper permeability is estimated from copper content of cells which have been treated with copper-containing medium.

When a sensitive strain is inoculated in the standard medium to which copper is added to give a concentration about 0.1 mM, outset of growth is observed after a lag period of about 45 minutes, just as when copper is not added. Since, however, the growth is inhibited soon by copper, it is considered that copper has been entering the cells to reach an intracellular concentration sufficient to inhibit cell metabolism before growth proceeds appreciably. Hence

* 0.1% $FeCl_3\cdot6H_2O$ solution.

** KI 20 mg, H_3BO_3 100 mg, $MnCl_2\cdot4H_2O$ 60 mg, $CuCl_2\cdot2H_2O$ 40 mg, and $ZnCl_2$ 250 mg were contained per 100 ml of deionized water.

even if the cells inoculated in a copper medium are harvested during the lag phase, their copper content may reflect the copper accumulation by cells on the verge of injury rather than that by healthy cells. If some cells are killed, they will probably make the average copper content of cells higher. A resistant strain, on the other hand, grows so rapidly in the copper-containing medium that the average copper content of cells does not increase remarkably until cells produce hydrogen sulfide in a late log phase (9). Hence, under the growing condition, the resistant cells will show a lower copper content per unit weight of cells than the sensitive ones, even if copper permeability is the same for both kinds of cells or is higher in the former than in the latter. Thus, for the present purpose, it was desirable to use experimental conditions under which both strains would neither grow nor be much injured in the presence of copper. Preliminary experiments were conducted in order to find such conditions.

1. *Effect of carbon source on copper-sensitivity of cells.*

Cells of the sensitive strain harvested from a 36-hour culture in the standard medium were suspended in the non-copper SN(C^-) liquid medium to a density of ca. 1.2×10^7 cells per ml. The culture medium was 30 mm deep in a Roux bottle and shaken moderately at 30°. During 12 hours of incubation, the number of budding cells did not increase appreciably, and total and viable cell number did not increase or decrease. The same was true when the medium was supplemented with up to 0.01 per cent of sucrose, and most cells began to bud in 6 hours when sucrose concentration of the medium was 0.05 per cent or higher. An increase in budding cells was observed after 12 hours when SN(C^-) was supplemented with 0.5 per cent of sodium acetate, but not when the concentration of acetate was 0.05 per cent.

A series of copper concentrations was prepared using SN(C^-) liquid medium supplemented and unsupplemented with 0.01 per cent sucrose or 0.05 per cent sodium acetate. Cells carbon-starved in the way as described above were suspended in these media. Cells not subjected to the starvation pretreatment were also suspended in the carbon-deficient medium. The cell density was ca. 1.2×10^7 cells per ml in all cases. After 6 hours of incubation at 30°, the cells were washed twice with cold M/15 KH_2PO_4 solution by centrifugation, and aliquots were diluted and plated on the standard agar medium. The viable counts thus obtained are represented in Table 1.

In the absence of available carbon source, cells were not killed appreciably even in the presence of 1.2 mM of copper. When cells were not carbon-starved beforehand, copper injury was significant even when the medium contained no carbon source. And the injurious effect of copper was more pronounced when the medium contained a small amount of sucrose or acetate. That cells are less susceptible to copper in carbon-deficient condition was observed also by ASHIDA and NAKAMURA(1).

Table 1. Toxic effect of copper in relation to carbon source in medium. Viable count in percentage of that of each cell suspension before treatment.

Copper concn. (mM)	Carbon source			
	None (Cells prestarved)	None (Cells not prestarved)	0.01% Sucrose	0.05% Na-acetate
0.0	104	92	98	85
0.4	106	85	53	43
0.8	104	61	41	29
1.2	98	51	25	24

2. *Viability estimate as affected by method of cell washing.*

If the washing of copper-treated cells does not remove copper well enough from the cells, even those which are viable at the time of plating would be injured on the test plate by the copper they carry with them. This possibility can not be disregarded because cells become more susceptible to copper when they enter into growing condition, as pointed out in the preceding section. Accordingly, it was tested whether the viable count could be raised by washing copper-treated cells with some copper-binding agents prior to plating.

Cells of the sensitive strain precultured in the standard medium were

Table 2. Recovery of viable count by removing copper from copper-treated sensitive cells. Viable count in percentage of that of cells incubated 3 minutes in the non-copper medium.

Washing agent	Cu concn. (mM) in the medium	Incubation period in the medium	
		3 min.	6 hours
SN(C⁻)	0.0	100	105
	0.8	100	73
M/15 KH_2PO_4	0.0	100	100
	0.8	100	72
1 mM EDTA*	0.0	100	99
	0.8	100	68
1 mM DDC**	0.0	100	102
	0.8	100	90
1 mM KCN	0.0	100	109
	0.8	100	92
1 mM Salicyl-aldoxime	0.0	100	99
	0.8	83	66
1 mM Thiourea	0.0	100	105
	0.8	100	61

* : Ethylenediaminetetraacetic acid tetrasodium salt

** Sodium diethyldithiocarbamate

suspended in SN(C^-) liquid medium with and without addition of 0.8 mM copper. After 6 hours of shaking at 30°, each suspension was divided into 7 aliquots, to make cells rinsed in SN(C^-), M/15 KH_2PO_4, and 1 mM solutions of ethylenediaminetetraacetic acid tetrasodium salt (EDTA), sodium diethyldithiocarbamate (DDC), KCN, salicylaldoxime, and thiourea. After being rinsed again by the same solutions, the cells were rinsed once with cold deionized water and, through proper dilution, plated on non-copper standard agar plates. The results presented in Table 2 showed that the viable count of copper-treated cells was improved by washing them with the solutions of DDC and KCN. No favourable effect was observed with the other solutions, though none of the solutions used did not affect the viable count of cells treated by the non-copper medium. Thus, to wash copper-treated cells with solution of DDC or KCN seemed to be useful in the later experiments in which the copper locating outside the cellular osmotic barrier should be minimized.

3. *Relationship of metabolic activity to copper-content of cells.*

In order to expose cells to copper after they have started metabolizing in the new medium, 36-hour standard liquid cultures of the sensitive strain, *c*, and the resistant strain, *c*-43, were transferred to SN (4% sucrose) to a density of ca. 1.2×10^7 cells per ml, and after 10 minutes incubation at 30° $CuSO_4$ solution was added to the medium to make a concentration of 0.8 mM. Samples were withdrawn out of the cultures every 10 minutes, cells were washed twice with deionized water by centrifugation, and copper content of cells was determined.

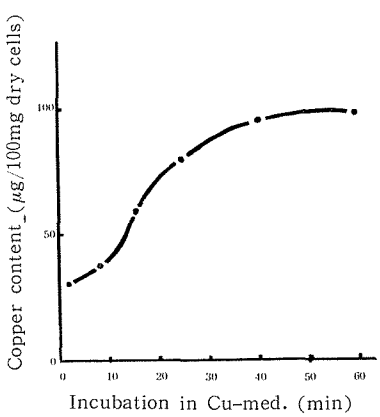

Fig 1. Increase of copper content of sensitive cells with duration of suspension in 0.8 mM copper-containing SN (4% sucrose) medium. Cells harvested from a 36-hour culture of strain *c* in the standard liquid medium were suspended to a density of 1.2×10^7 cells per ml. Sampled cells were washed twice with deionized water prior to copper assay.

Figure 1 shows the change in copper content of cells of strain *c* in the first hour of incubation in the copper medium. The result did not essentially differ for strain *c*-43. A rapid increase in copper content occurred in a minute or two followed by a slower one. Binding of copper ions by cell surface may be suggested for the first case, as by ROTHSTEIN (11).

The rate of copper accumulation became very low in 40 minutes of incubation. At 40 minutes of these cultures, viable counts of strains *c* and *c*-43 appeared

to be 85 and 93 per cent, respectively, of those at 0 minute if cells were washed by KCN solution, and 74 and 83 per cent if cells were washed only by deionized water. Cells harvested at 40 minutes were assayed for copper content after washing with deionized water and also after washing with 1 mM KCN solution from once to five times. Comparing the results shown in Fig. 2 with Fig. 1, it may be understood that nearly three fourths of the copper which was not washed out by water could be removed by three times of KCN washing. And there was no indication that the resistant cells contained less copper in the cell interior than the sensitive cells.

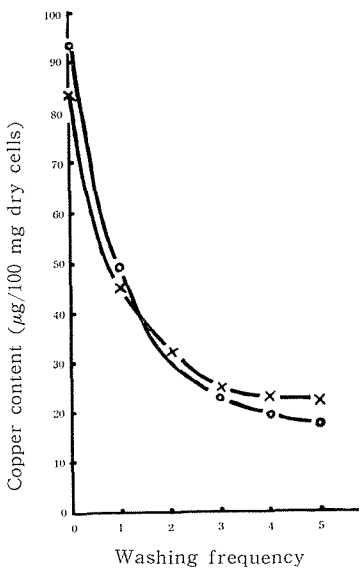

Fig. 2. Copper content of cells as affected by repeated washing using KCN solution. 36-hour cultures of *c* and *c*-43 in standard liquid medium at 30° were suspended to a density of 1.2×10^7 cells per ml in 0.8 mM copper-containing SN (4% sucrose) liquid medium and cells were harvested after 40 minutes incubation. Copper content of cells was assayed after washing them only with deionized water and after washing them with 1 mM KCN solution one or more times. ○, Strain *c*; and ×, strain *c*-43.

Table 3. Copper content of sensitive strain, *c*, and resistant strain, *c*-43, suspended in 0.8 mM copper-containing SN(C^-) medium under different conditions. Copper content expressed in μg/100mg dry cells. Cells used were from a 36-hour culture in the standard medium.

Conditions of incubation			Washed with			
Sucrose (g/l)	Temp.	Period (min.)	Deionized water		1 mM KCN	
			c	*c*-43	*c*	*c*-43
40	30°	40	150	146	25	33
0	30°	360	37	49	10	17
0	30°	0.5	19	22	2	2
40	5°	40	36	46	4	6
40	30°	0.5	25	—	—	—

The two strains were compared for the copper content of cells after exposing them to copper under various conditions, as seen in Table 3. The cell suspensions were prepared in essentially the same way as in the preceding experiment. Copper content of cells appeared to be in parallel with the metabolic activity,* And a tendency may be observed that the amount of copper not removable by KCN washing was higher when cells underwent metabolism.

Copper content of resistant cells was not lower than that of sensitive cells in any of the cases observed. Thus, the results obtained are not favourable for the assumption that the resistant cells are less permeable to copper than the sensitive ones. If, however, the resistant cells produced more hydrogen sulfide than the sensitive ones during the experimental period, the copper content of the former would be unduely high owing to copper sulfide precipitated at the cell wall. Hence the two strains should be compared for their hydrogen sulfide production during an early short period of culture, though the results presented above is probably not misleading because the washing by KCN solution may have taken most of copper sulfide off the cell surface (5).

4. *Hydrogen sulfide production in an early phase of culture.*

NAIKI (7) measured the hydrogen sulfide producing activity of resistant cells by suspending them in a copper-free reaction mixture, and found that no measurable amount of hydrogen sulfide was produced by the cells harvested from young cultures. It was, however, not assured that these cells were not producing hydrogen sulfide while they were in the copper medium. So the hydrogen sulfide production was measured using SN(4% sucrose) liquid medium. Cells of strains *c* and *c*-43 were transferred from 36-hour non-copper cultures to SN(4% sucrose) liquid medium to make a density of ca. 1.2×10^7 cells per ml. The medium had previously been at 30°, and 10 minutes after the inoculation a solution of $CuSO_4$ was added to make a concentration of 0.4 mM**. For control cultures pure water was added in place of copper solution. Three hours after the copper (or water) addition, an equal volume of concentrated hydrochloric acid was poured into each culture. The evolving hydrogen sulfide gas was trapped, and the total hydrogen sulfide produced during the incubation was determined. The results as exemplified in Table 4 show that, during 3 hours, dry weight increased in the non-copper medium but decreased in the copper-containing medium even with the resistant cells. In either strain hydrogen sulfide production was lower in the copper medium. And it was shown that there was practically no difference in hydrogen sulfide production between the two strains whether copper was present or not.

* Some activity was suspected in the sugar-deficient medium as cells were not prestarved.

** Cells fix copper as expected from Fig. 1, but the copper concentration is not lowered appreciably.

Table 4. Hydrogen sulfide evolution by lag phase cultures of sensitive strain, *c*, and resistant strain, *c*-43, in non-copper and in 0.4 mM copper-containing media.

Strain	*c*		*c*-43		
Incubation period (hr)	3		3		0
Cu concn. (mM)	0.0	0.4	0.0	0.4	0.0
H_2S (μgmole/100mg dry cells)	0.24	0.07	0.13	0.06	0.03
Cell yield (mg dry cells/culture)	49.8	34.5	46.1	35.9	44.7

As is shown in Table 4, the amount of hydrogen sulfide produced during 3 hours incubation was 0.06-0.07 μgmole per 100 mg dry cells in either strain. According to Table 3, on the other hand, copper content of cells incubated under similar conditions for 40 minutes was 0.4-0.5 μg-atom per 100 mg dry cells even after washing by KCN solution. The total amount of copper entering into the cell seems to be much larger than to be precipitable by hydrogen sulfide produced by the cell. And, at 3 hours of incubation, resistant cells are going to grow while sensitive cells are not, in spite of that the hydrogen sulfide production and the copper content of cells do not differ significantly between the two kinds of cells. Hence it may be concluded that the higher copper resistance of *c*-43 in the early phase of culture is owing neither to low copper permeability, nor to detoxification by hydrogen sulfide production. As to the latter point, NAIKI, who found that a copper-resistant strain produced much hydrogen sulfide (6), has reported that the hydrogen sulfide producing activity of its cells becomes striking after 12 hours or so in a copper culture (7).

The respiratory deficient variant clones of *Sacch. cerevisiae* produce hydrogen sulfide only little even if they become copper resistant through training cultures (KIKUCHI, in preparation). Hence the cellular copper content without hydrogen sulfide production may be known by using these strains. Non-copper cultures of strains, *c* and *c*-43, were spread on the copper-free standard agar medium, and 3 clones each for the two strains were picked up from among colonies of spontaneous respiratory deficient variant. The clones showed the same copper resistance as their respective parent strains on copper gradient agar plates (Fig. 3). Cells of these clones were suspended in 0.8 mM copper-containing SN (4% sucrose) liquid medium as indicated in Table 3, and copper contents of cells were assayed after 40 minutes of incubation at 30°. The results gave 57 and 61 μg per 100 mg dry cells for sensitive and resistant clones, respectively, when washed with deionized water, and 11 and 24 μg per 100 mg dry cells, respectively, when washed with 1 mM KCN solution. Thus, the copper content of resistant cells was not lower than sensitive cells even though the former cells had no higher hydrogen sulfide producing activity.

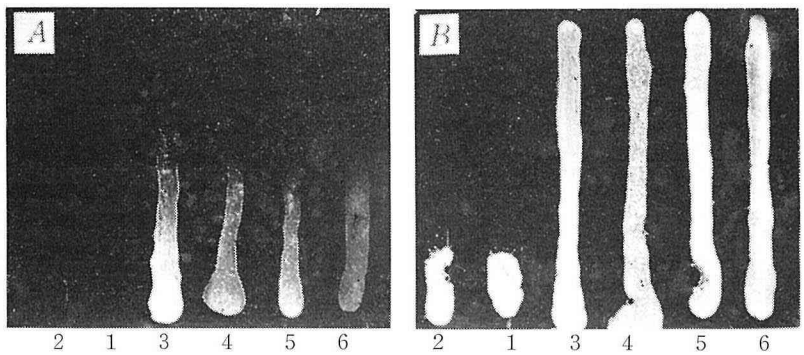

Fig. 3 Comparison of respiratory deficient clones with their parent strains for copper resistance. Respiratory deficient clones were obtained by picking up spontaneous variant colonies from copper-sensitive *c* and copper-resistant *c*-43. Cell suspensions were streaked on 6/0 (*A*) and 3/0 (*B*) LS copper-gradient plates.

1, *c*; 2, a respiratory deficient clone derived from *c*; 3, *c*-43; 4-6, three respiratory deficient clones derived from *c*-43.

Discussion

When a strain resistant to a toxic substance is derived from a sensitive strain, following changes may be suspected to occur (2): (a) The part of cell metabolism most sensitive to the toxic substance becomes less so by a change in nature of enzyme(s) or by development of alternative pathway(s); (b) the cell get, or increase, an ability to produce metabolite which detoxifies the toxic substance; and (c) the cell permeability to the toxic substance becomes lower.

For the first case, MURAYAMA (4) has obtained an indication that, in the copper resistant strain of *Sacch. ellipsoideus*, relative magnitudes of different pathways of organic acid metabolism, which underlie syntheses of amino acids, are shifted so that less copper-sensitive one is relatively more active. Since, however, the genic basis of copper resistance is rather simple in *Sacch. cerevisiae* (13), hypotheses which do not assume changes in many kinds of enzyme protein are preferable for the first step approach to the resistance mechanism.

As for the second possibility, NAIKI (6) has reported that the copper-resistant strain which usually predominate in copper-containing cultures has a high activity of producing hydrogen sulfide by reducing sulfate present in the medium. This activity, however, develops only in the late exponential growth phase (7). And in the lag phase of copper culture no difference was observed between sensitive and resistant strains in the hydrogen sulfide production (Table 4). Hence the hydrogen sulfide production is effective in copper detoxification only in a late phase of growth, if at all. Genetical analysis has shown that the activity of hydrogen sulfide production can be segregated from

copper resistance (14). Therefore, if the second category of resistance mechanism is to be considered, there should be some copper detoxifying substance(s) other than hydrogen sulfide. This must also be the case with respiratory deficient strains, which do not produce hydrogen sulfide when they have become copper resistant (KIKUCHI, in preparation).

The present study was intended to examine the third possibility. In *Escherichia coli*, streptomycin-resistant cells do not uptake the drug significantly, while sensitive cells do (15). KUSHNER (3) suggests presence of a barrier to chloramphenicol at the cell surface of chloramphenicol resistant variant of *Pseudomonas fluorescens*. When one wishes to compare copper permeability of two kinds of cells by measuring their copper content, following considerations are needed : (i) The copper which has passed the osmotic barrier should be discriminated from that bound by cell wall and protoplasmic surface, and also from extracellular copper complexes centrifuged down together with cells. (ii) Copper permeability of sensitive cells may become higher when they are injured by copper. On the other hand, (iii) a higher copper content will be recorded with cells less injured by copper, if copper uptake by cells is promoted by metabolic activity. (iv) A lower copper content per dry weight of cells may be observed for resistant cells which grow in copper-containing medium than for those which do not grow in it, even if the velocity of copper penetration into cells is the same in both types of cells. And (v) if resistant cells produce and contain in them more copper-binding substance(s) than sensitive cells, copper content of cells will reflect it rather than copper permeability. Thus, the copper permeability of resistant cells, in comparison with that of sensitive cells, may be estimated to be lower than reality in the cases (ii) and (iv), and to be higher in the cases (iii) and (v).

In the present study cells were brought in contact with copper in carbon-starved condition, in which viable count did not appreciably decrease even with sensitive cells (Table 1). The possibility (ii) thus seems to be eliminated, but there is no convincing proof that the permeability of cells was not changed at all by the effect of copper. Without carbon source, the possibility (iv) is eliminated.

The possibility (iii) is suggested by ROTHSTEIN's finding (11) that resting cells of *Sacch. cerevisiae* take in bivalent cations of the medium against concentration gradient when phosphate is added together with glucose. In the present case, too, higher copper content was recorded under the conditions promoting metabolic activity, as shown in Table 3. Accordingly, the possibility (iii) may be excluded for the values obtained with cells prestarved for carbon and copper-treated without sugar.

The copper not in the cell interior, as mentioned in (i), seems to have been almost removed by washing the cells with KCN solution (Table 3). And after the KCN washing, higher copper contents of cells were recorded for the resistant strain than for the sensitive strain, both in the presence and in the absence of sugar during the copper treatment (Table 3 and Fig. 2). Hence,

the assumption that the permeability might be lower in resistant cells than in sensitive cells was not warranted. QUAGLIOZZI and RESCIGNO (10) estimated copper permeability of yeast cells to be 7.2×10^{-6} g copper/g yeast/hr, although the conditions are not described fully. This value does not differ very much from the present results obtained with cells copper-treated in absence of sugar and washed with KCN.

If it is assumed that copper permeability does not differ between the two types of cells, the possibility (v) is tempting for the explanation of the results that the copper content after KCN washing was higher with the resistant cells than with the sensitive cells. The difference between the two kinds of cells in this case, however, was not affected much by the presence of sugar in the medium (Table 3). Since only a negligible amount of hydrogen sulfide can be produced even by the resistant cells in the absence of carbon source (8), and since the hydrogen sulfide production did not differ between the two strains under the experimental conditions (Table 4), the presumable copper-binding substance may not be hydrogen sulfide. The substance may have been present in the cells when they were suspended in the medium for copper treatment.

Cells both of the resistant and the sensitive strains were less susceptible to copper in carbon deficiency than in its presence (Table 2). It is not certain, however, whether this effect of carbon source comes from that the cell takes up more copper in the active state than in the inactive state (Table 3), as suggested by ROTHSTEIN (11), or that it is more liable to copper injury in the active state.

Cells in the lag phase were observed in the present study, because resistant cells begin to grow towards the end of this phase, while sensitive cells do not, and if growth once starts, the average copper concentration inside the cells does not increase appreciably, or it may even decrease, depending on the ratio of the rate of copper penetration to the rate of cell growth.

Summary

1) Copper permeability of cells was investigated in relation to their copper resistance, using lag phase cultures of *Saccharomyces cerevisiae*.

2) Both the copper resistant strain and its parent sensitive strain were less susceptible to copper injury in carbon deficiency than under sugar supply.

3) Viable count of copper-treated sensitive cells was recovered significantly by washing them with KCN solution prior to plating.

4) Remarkably higher copper content was recorded with both of the strains when the treating copper medium contained sugar than when it did not.

5) Cells being copper-treated and washed under various conditions, copper content of resistant cells was invariably rather higher than that of sensitive cells. The possibility that the resistant cells have lower copper permeability

than the sensitive cells was eliminated.

6) Hydrogen sulfide production by cells does not seem to contribute significantly to copper resistance during the lag phase of copper-culture.

7) Possible mechanisms of copper resistance are discussed in relation to the experimental results obtained.

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