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# Adaptation of Yeast to Cadmium
## I. An Introductory Approach to the Resistance Mechanism

By

**Hakobu NAKAMURA[1] and Joji ASHIDA**

Botanical Institute, College of Science, University of Kyoto

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When yeast is cultured with the medium which contains a certain concentration of cadmium a strain resistant to cadmium is obtained. The present paper deals with experiments which may contribute to disclosing the process of the adaptation and the resistance mechanism acquired. Comparisons are made with the case of copper resistance of yeast, since there are a bulk of informations on the latter.

### Methods and Materials

*Strain* *Saccharomyces ellipsoideus* strain B (Kyoto) was used as the parent strain, to be abbreviated as *Par.* The cadium resistant substrain, which will be designated as *R*, was obtained by successive subcultures in the PGY liquid medium containing 0.4 mM cadmium chloride. When *R* was subcultured in the cadmium-free medium the culture obtained was designated as $R_0$.

Cells to be used in the experiments were harvested at 48 hr of the preceding culture, washed three times with M/15 $KH_2PO_4$, and suspended in it so as to make the cell concentration the same as before the harvest. This suspension was stored at 5°C until uses.

*Culture* The broth medium (PGY), the synthetic medium (SG), and the medium (D) which is used in starvation experiments are shown in Table 1. For solid cultures 1.5% of agar was added. For cadmium-containing media, 100 mM cadmium chloride solution and nutrient solution were sterilized separately and mixed together after cooling to room temperature, or at 40° to 45°C in the case of agar medium.

The aerobic cultures reported in this paper were standing cultures with liquid media 1 cm deep. For anaerobic culture the atmosphere was replaced by nitrogen gas. Incubation temperature was 30°C throughout.

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1) Present address: Department of Biology, Faculty of Science, University of Konan, Kobe, Japan.

Table 1. Compositions of nutrient media (g/*l*).

| | PGY | SG | | D |
|---|---|---|---|---|
| $KH_2PO_4$ | 3 | 3 | $KH_2PO_4$ | 9.1 |
| $MgSO_4 \cdot 7H_2O$ | 1 | 1 | $K_2SO_4$ | 1 a) |
| $(NH_4)_2SO_4$ | 1 | 2 | $MgCl_2$ | 0.81 |
| $CaCl_2$ | 0.25 | 0.25 | $NH_4H_2PO_4$ | 4 b) |
| Cane sugar | 50 | 50 | Cane sugar | 50 c) |
| Na-glutamate | 1 | 2 | | |
| Peptone | 2 | − | | |
| Yeast extract | 2 | − | | |
| Micro-elements | − | + | | |
| Vitamins | − | + | | |

Micro-elements (μg/*l*): B 10, as acid, and Mn 10, Zn 70, Cu 10, Mo 10, and Fe 50, as chlorides.

Vitamins (μg/*l*): Thiamine 200, pyridoxine 200, nicotinic acid 200, pantothenic acid 200, biotin 2 and inositol 10,000.

a), b), c): To be omitted for the deficiencies in sulfur, nitrogen, and the energy source, respectively.

*Viability determination* a) *Pour plate.* Cells were mixed in molten nutrient agar media at 40° to 45°C. Ten ml aliquot was poured in each Petri dish, 9 cm in diameter. Colonies were counted after 48 hr of incubation. In cadmium-containing media the colony count continued to increase after this period. However, for the purpose of determining relative resistance it was found convenient to count colonies at this definite period. The viable count in control plate was about 400 per dish in every experiment.

b) *Gradient plate.* Gradient plate was prepared in a Petri dish, 12 cm in diameter, using 40 ml each of the normal and the cadmium-containing PGY agar media, the latter making the upper wedge. Two hours after the preparation of the gradient plate, 20 ml of molten cadmium-free agar medium in which cells were suspended was overlaid on the surface of the plate. This way of inoculation made the distribution of cells more uniform than to smear with a glass rod. After incubating for 48 hr visible colonies were counted for each of eleven 1 cm-wide zones to represent the change of colony count along the gradient of cadmium concentration. The bow-shaped areas at the two extremities were not counted. The colony count in each zone was expressed in percentage of that in the first zone where the cadmium concentration was the lowest.

*Respiration and fermentation measurements* Gas exchanges were measured by usual Warburg respirometer. Harvested cells were washed three times with M/15 $KH_2PO_4$ solution, centrifuged, and placed on filter paper to remove excess of water for fresh weight determination. Cells were suspended in deionized water, 1 g per 30 ml.

For $O_2$ uptake 0.3 ml of the cell suspension, 0.5 ml of 4/15 M $KH_2PO_4$ and 1.1 ml of deionized water were pipetted into the manometer flask with air in the gas

phase. A side arm contained 0.1 ml of a glucose solution, the other 0.1 ml of a cadmium chloride solution, and the center well 0.2 ml of 20% KOH. Evolution of $CO_2$ was measured after flushing with nitrogen gas, 0.2 ml of cell suspension being used in this case.

*Analyses* For dry weight determination, an aliquot of harvested cells was washed three times with deionized water and dried at 100°C to constant weight. Cells were oxidized with $H_2SO_4$ and $H_2O_2$. Total nitrogen was determined with the micro-Kjeldahl method modified by Y. YAGI (1, 2), as the following: Nitrogen compound was oxidized down to $(NH_4)_2SO_4$. After cooling excess of sulfuric acid was neutralized with NaOH and sodium borate. Ammonium was converted to nitrogen gas by NaOBr. Excess of NaOBr was converted to NaBr by addition of KI. And $I_2$ was titrated with sodium thiosulfate employing starch solution as the end point indicator. Trouble of distillation is saved in this method. An accuracy over 98% is reported expectable in the range 5–50$\mu$g nitrogen per test aliquot.

The cadmium content was determined by the dithizone method of CHURCH (3).

## Results

1. *Viability in cadmium medium.*

Growth inhibition by cadmium differs according to the composition of basal medium. Using PGY medium the viability-cadmium concentration relation of *Par* was determined by counting colonies at 48 hr of incubation of pour plates. As shown in Fig. 1, 50% viability was at about 0.4 mM cadmium. Hence this concentration was used as a standard in later experiments.

Most of the inoculated cells were not dead even in the 0.8 mM cadmium plate in which no colonies were visible under a magnifying glass at 48 hr. A number of colonies appeared gradually after a long delay. This situation differs from the case with copper. When copper concentration is higher than the half suppression value, most of the microcolonies which are not visible after 3 days do not grow later.

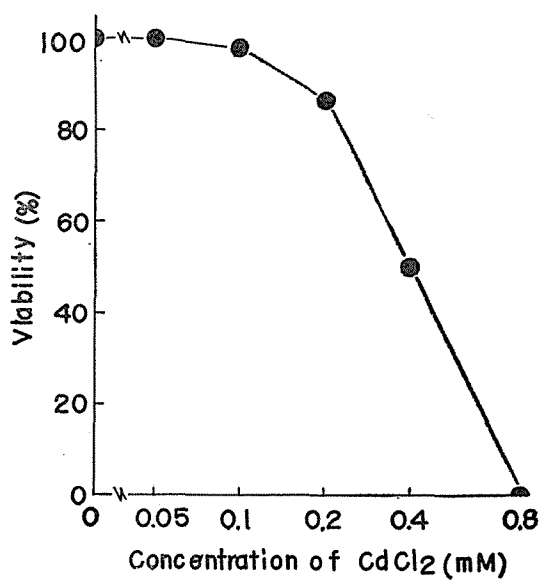


Fig. 1. Viability of the parent strain in the PGY medium containing $CdCl_2$.

Viability of *R*, which had

been serially subcultured in the 0.4 mM cadmium medium, was 100% at 0.4 mM and was nearly so even at 0.8 mM. When *R* was successively subcultured in the cadmium free medium, the resistance to cadmium began to decline after about five passages and went down to the same level as *Par* after about 17 passages. The cadmium resistance is lost more rapidly than the copper resistance.

2. *Growth in the cadmium medium.*

Growth processes of *Par* and *R* in absence and presence of cadmium are shown in Fig. 2. When *Par* is inoculated in a copper medium, the growth, which begins after a long lag, goes on at almost the same rate as a well-adapted resistant strain. In the case of cadmium, however, the inclination of curve B is smaller than that of curve C. This fact will be discussed at the end of the next section.

Anaerobic growth was compared with aerobic growth both in absence and in presence of cadmium, by measuring the yield at 40 hr of incubation (Table 2).

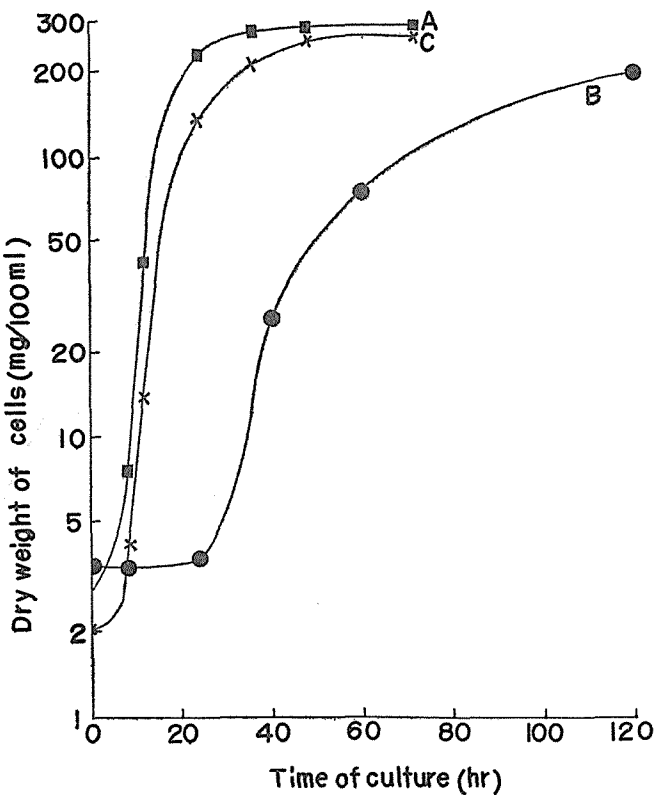


Fig. 2. Growth of the parent and the resistant strains. A: Parent strain in the normal medium; B: parent strain in the medium containing 0.4 mM $CdCl_2$; C: resistant strain in the medium containing 0.4 mM $CdCl_2$.

Table 2. Effect of 0.4 mM $CdCl_2$ and anaerobiosis on the growth of *Par* and *R* in 40 hr. Dry weight (mg) of cells per 100 ml culture. Inoculum size: *Par* 2.3 mg, *R* 3.8 mg.

| | *Par* | | *R* | |
|---|---|---|---|---|
| | No Cd | With Cd | No Cd | With Cd |
| Aerobic | 365 | 39.5 | 362 | 338 |
| Anaerobic | 120 | 2.9 | 88.9 | 41.3 |

The aerobic cultures of *Par* and *R* without cadmium had reached the stationary phase. The yields of these cultures were higher than in Fig. 2, possibly because the liquid medium was thinner, namely 0.7 cm deep, in the present case.

The anaerobic cultures and the cultures containing cadmium had not reached the stationary phase at the time of the harvest. Hence the values of these cultures reflect the relative growth rates under those conditions. Even the growth of *R* is inhibited by cadmium under anaerobiosis. The yield of *P* in the presence of cadmium depends largely on the emergence of resistant cells in the culture, while the emergence may depend largely on the multiplication of *Par* type cells from which resistant cells originate. The result in Table 2 shows that resistant cells had probably not yet emerged under anaerobiosis.

3. *Changes in cadmium resistance during training culture.*

When *Par* is inoculated in the cadmium medium the growth proceeds as represented by curve B, Fig. 2. And the population eventually obtained is such as to be capable of growing without a long lag when transferred to fresh cadmium medium. Hence the cadmium resistance must be changing during the training culture. This was observed by using gradient plates.

*Par* was inoculated in 0.4 mM cadmium medium which contained 0.1% of Tween 80 to prevent the formation of clusters of cells. Samples were withdrawn at proper intervals and mixed with the non-cadmium agar medium. This was layered on the gradient plate of which the upper wedge contained 2 mM of cadmium chloride and the lower wedge no cadmium. Colonies visible at 48 hr of incubation were counted for every 1 cm zone of the plate. The distribution of colonies along the gradient may represent to some extent the distribution of cadmium resistance in the population.

A representative set of results is shown in Fig. 3. The half inhibition of the inoculant population (denoted as 0 hr) looks to situate in a zone of cadmium concentration higher than to be expected from Fig. 1. This may be, at least partly, because cells in the overlay agar are affected by cadmium which comes diffusing from below.

Cells seem to be gradually weakened in the first 24 hr of incubation. Resistant cells are found in the 48 hr sample, to increase conspicuously thereafter (*cf.* 74 hr

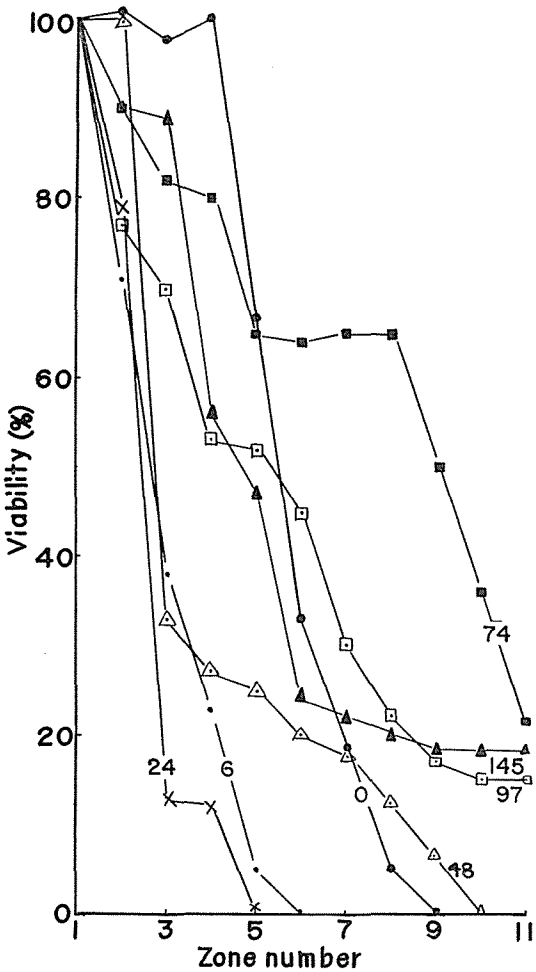


Fig. 3. Change during incubation in the distribution of cellular resistance in the culture. Parent cells inoculated at 0 hr in 0.4 mM $CdCl_2$ PGY medium. The time (hr) of sampling indicated on each curve.

curve). Later, however, cells seem to be somewhat weakened.

By referring to Fig. 3 the form of curve B, Fig. 2, may be explained as follows: The logarithmic growth of resistant cells began after 24 hr, then the apparent growth rate waned gradually as cells became less and less vigorous.

4. *Effect of cadmium on respiration and anaerobic fermentation of* Par.

Culture of *Par* in the normal PGY medium was harvested at 24 hr of age, namely toward the end of the logarithmic phase. And effects of 0.4 mM cadmium on the gas exchanges were observed.

As shown in Fig. 4, inhibition was relatively stronger in respiration than in fermentation. It is noteworthy that the fermentation of *Par* cells is not severely inhibited even in 1.2 mM of cadmium, at least in 60 min. The gas exchanges of $R_0$ are hardly inhibited by 1.2 mM of cadmium.

5. *Starvation and cadmium injury.*

It was reported that yeast cells were more sensitive to copper when they were in shortage of sulfur rather than in shortage of energy source (4). In order to see such relations in the case of cadmium, cells of *Par* and $R_0$ were washed and suspended ($10^6$ cells/ml) in nitrogen-, sulfur-, and carbon-deficient D media (*cf.* Table 1). After incubation for 20 hr the starved cells were suspended in the agar media of the respective deficiencies and layered on the gradient plates (2 mM Cd/no Cd) of the same deficiencies. After incubation for 6 hr, the plates were covered by 1/15 M $KH_2PO_4$ solution and shaked, the

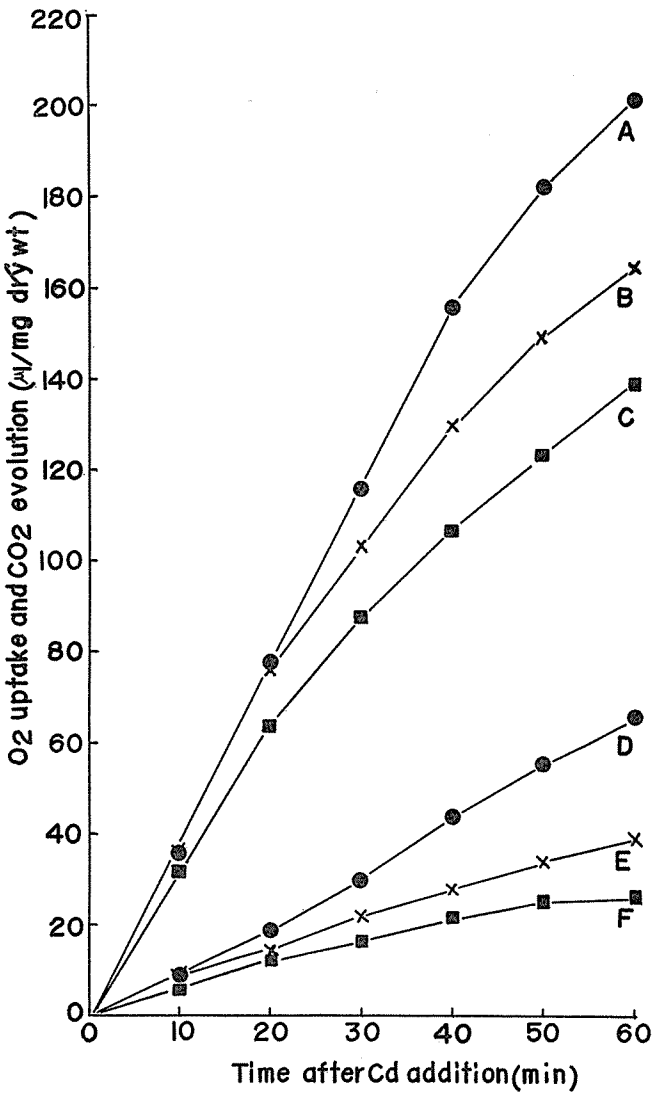


Fig. 4. Effect of cadmium on respiration and anaerobic fermentation of the parent strain.

Keys to curves:

| | No Cd | 0.4 mM Cd | 1.2 mM Cd |
|---|---|---|---|
| Anaerobic $CO_2$ output | A | B | C |
| $O_2$ uptake | D | E | F |

washing solution being renewed frequently. Diffusible cadmium in the plates thus being washed out, PGY liquid medium was flooded on the plates and discarded after 6 hr. The colonies which grew visible in 48 hr of incubation were counted.

It may be expected that the more badly the cell is injured, the more difficult the growth of it to reach the visible size in the definite duration. Hence the distribution of visible colonies as shown in Fig. 5 may indicate that, under the experimental conditions, cells both of *Par* and $R_0$ were the most sensitive to cadmium when they were nitrogen starved. $R_0$ is far more resistant than *Par* in any of the deficiencies tested.

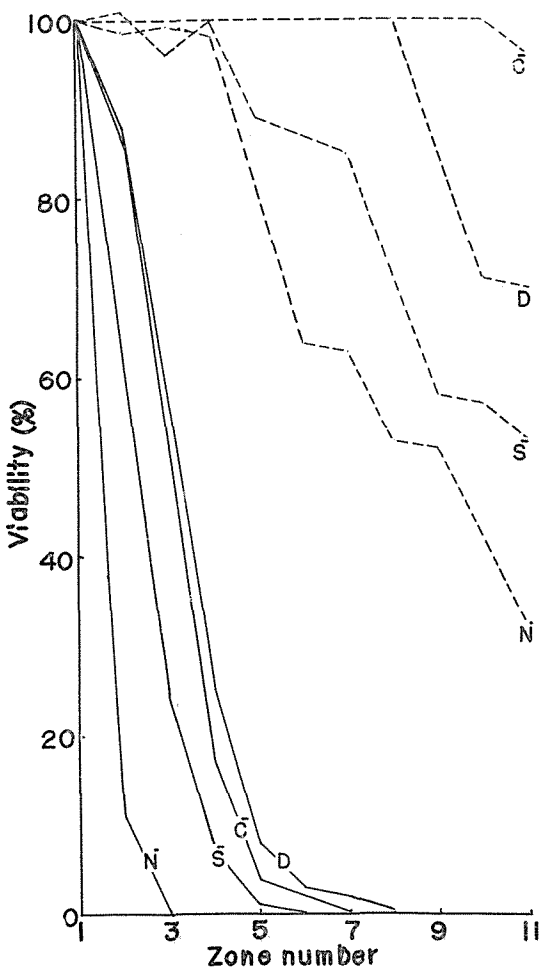


Fig. 5. Viability on the Cd-gradient plate of the parent strain and $R_0$ previously made deficient in the various elements. $N^-$ : Nitrogen starved; $S^-$ : sulfur starved; $C^-$ : carbon starved; and D: control. ——: Parent strain; – – –: $R_0$.

The following experiment was undertaken in order to see how the nitrogen starved cells restore the normal cadmium tolerance by the supply of nitrogen source. Nitrogen starved cells were suspended in the molten nitrogen-deficient agar medium at 42°C, and aliquots were poured in Petri dishes. The agar layer thus formed in each dish was 1.2 mm thick, namely thin enough for the rapid penetration of cadmium and ammonium to be supplied from solutions poured on it. The dishes were divided into six groups for the treatments with (a) the complete D medium for scoring viable cells, (b) the nitrogen-deficient D medium containing 0.5 mM $CdCl_2$, (c) the complete D medium containing 0.5 mM $CdCl_2$, and (d, e, f) the normal D medium for 1, 2, and 4 hr, respectively, followed by the treatment with the complete medium containing 0.5 mM $CdCl_2$. These solutions were discarded after 3 hr. The plates were washed with 1/15 M $KH_2PO_4$ and fed with PGY. Colonies were counted after 48 hr.

Table 3. Effect of nitrogen replenishment on the recovery of cadmium tolerance of nitrogen starved cells.

| | *Par* | $R_0$ |
|---|---|---|
| | % | % |
| a) Control (No Cd treatment) | 100 | 100 |
| Cd treatment: | | |
| b) Without nitrogen supply | 1.4 | 81 |
| c) Without previous, but with concomitant nitrogen supply | 86 | 97 |
| d) After 1 hr of nitrogen supply | 81 | 100 |
| e) After 2 hr of nitrogen supply | 86 | 98 |
| f) After 3 hr of nitrogen supply | 87 | 100 |

Results as shown in Table 3 revealed that the nitrogen-starved cells can exhibit almost the complete cadmium tolerance even when nitrogen was supplied at the same time with the exposure to cadmium.

When *Par* is inoculated on a gradient plate of copper, colonies composed of resistant cells grow colored brown in a certain zone, owing to the accumulation of copper sulfide. When, however, the gradient plate of cadmium was inoculated by *Par*, the resulting colonies did not show the coloration to suspect cadmium sulfide at any part on the gradient plate. Hence the cadmium resistant strain does not seem to produce significant amount of hydrogen sulfide as the copper resistant strain of brown type does.

6. *Sulfur and nitrogen contents of cadmium resistant cells.*

It is suggested above that the nitrogen metabolism has more important relation to cadmium tolerance than the sulfur metabolism has, contrary to the case of copper. The copper resistant strain cultured in the copper medium contains more sulfur and less nitrogen than the parent strain (5). Hence the sulfur content and the nitrogen content of cadmium-cultured cells were determined.

For sulfur, cells were melted with metallic sodium, subjected to nitroprussid reaction, and the color densities were compared. The *R* cells harvested from the cadmium culture did not differ in the color reaction from the *Par* cells harvested from the normal culture, while the reaction was very strong with the copper resistant cells harvested from a copper culture.

The total nitrogen was determined with the cells harvested from aerobic and anaerobic cultures with and without addition of cadmium. The results are presented in Table 4.

When the inoculant was *Par*, the nitrogen content of cells was lower with the cadmium culture than the normal one, either aerobic or anaerobic. It is guessed from Table 2 that, at 40 hr of the cadmium culture inoculated by *Par*, the growth of resistant cells had proceeded only a little under the aerobic conditions and no significant growth thereof had occurred under the anaerobic conditions.

Table 4. Total nitrogen content of cells cultured for 40 hr under the various conditions, mg/100 mg dry cells.

| | *Par* inoculated | | *R* inoculated | |
|---|---|---|---|---|
| | No Cd | 0.4 mM Cd | No Cd | 0.4 mM Cd |
| Aerobic | 8.95 | 8.30 | 9.25 | 9.60 |
| Anaerobic | 9.70 | 8.17 | 10.4 | 10.2 |

The nitrogen content of the resistant strain is not affected by the presence of cadmium. The nitrogen content of *Par* was lower than that of the resistant strain even in the cadmium-free medium. In each column of the table, the nitrogen content was lower under aerobiosis than under anaerobiosis, except the second column where only little growth had occurred.

As preliminaries of approaching the nitrogen metabolism of the resistant strain, the recovery from the nitrogen starved state was studied. *Par* cells and $R_0$ cells harvested from the stationary phase of the cultures were washed with deionized water, suspended in the nitrogen deficient medium, and incubated for 20 hr. The starved cells were washed, suspended in the complete D medium, incubated under aerobic and anaerobic conditions, and the changes in the dry weight and in the nitrogen content were followed.

It is seen in Table 5 that $R_0$ retains more nitrogen than *Par* under the nitrogen starvation, and that the rate of recovery of the nitrogen content is higher with *Par* than with $R_0$.

The nitrogen content on the dry weight basis reflects the proportion of the nitrogenous matters to the non-nitrogenous matters of cells. The nitrogen content may be low in such cases as when much glycogen or lipid is accumulated, when the relative amount of wall substance is high, and so forth. Hence the results reported above serve only for introducing further studies on the nitrogen metabolism in relation to the cadmium resistance.

Table 5. Recovery in dry weight and in nitrogen content of nitrogen starved cells after the supply of $(NH_4)_2SO_4$ under aerobic and anaerobic conditions.

| | Time after $NH_4^+$ supply hr | Parent strain | | | Resistant strain | | |
|---|---|---|---|---|---|---|---|
| | | Dry wt. per culture (relative) | Cell. nitrogen per culture (relative) | Nitrogen content mg/100 mg dry cells | Dry wt. per culture (relative) | Cell. nitrogen per culture (relative) | Nitrogen content mg/100 mg dry cells |
| Aerobic | 0 | 100 | 100 | 4.63 | 100 | 100 | 5.60 |
| | 2 | 91 | 131 | 6.72 | 100 | 122 | 6.82 |
| | 6 | 160 | 304 | 8.78 | 172 | 253 | 8.20 |
| Anaerobic | 0 | 100 | 100 | 4.63 | 100 | 100 | 5.60 |
| | 2 | 92 | 134 | 6.57 | 101 | 118 | 6.60 |
| | 6 | 126 | 246 | 9.08 | 135 | 198 | 8.25 |

7. *Changes in cadmium content and nitrogen content of cells during culture.*

NAIKI *et al.* (6) found that, when the parent strain was incubated in a copper medium, the copper content of cells did not rise considerably in the lag period, but did so as resistant cells grew. For comparison the change in cadmium content of cells was followed after the inoculation of the parent strain in 0.4 mM cadmium PGY medium. Content in nitrogen, which is more important than sulfur, was also determined.

A representative set of results is illustrated in Fig. 6. The cadmium content attained its maximum at 24 hr of incubation, when the rapid growth had not yet occurred. It declined as the growth proceeded, contrary to the case of copper. The nitrogen content was low just before and during the rapid growth, and rised up as the growth rate and the cadmium content of cells fell.

Similar determinations were made by inoculating 0.4 mM cadmium PGY medium with *R* cells harvested from 48 hr preculture in the presence of cadmium. As

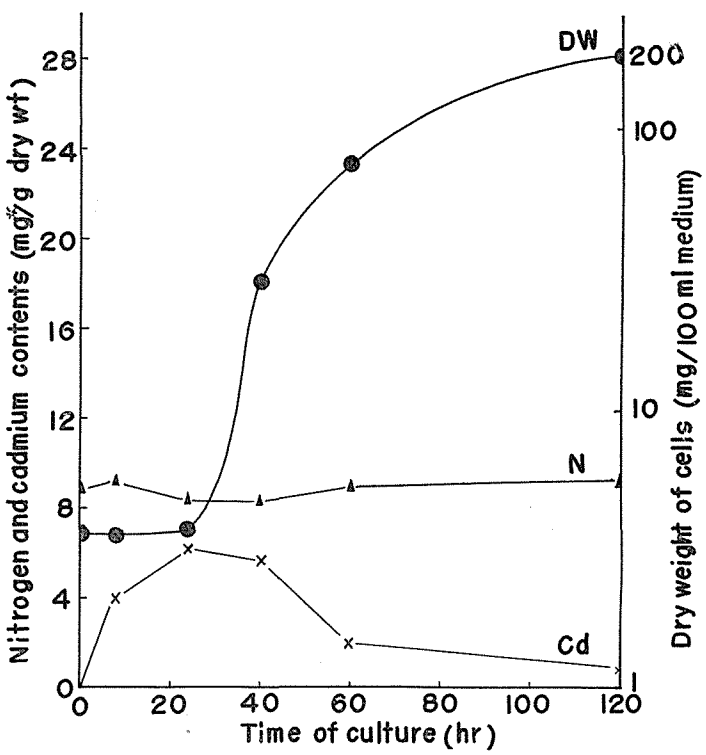


Fig. 6. Changes in cadmium and nitrogen contents of cells during the culture in 0.4 mM Cd-PGY medium, parent strain being inoculated at 0 hr. DW: Dry weight of cells; Cd: cadmium content; N: nitrogen content.

* Read ×10 mg for nitrogen content.

seen in Fig. 7, the nitrogen content of cells rised at the retardation phase of growth. The cadmium content was high at the early logarithmic phase and fell thereafter. This process is inverse of the case of copper: When the copper resistant strain is transferred from a copper culture to a fresh copper medium, the copper content of cells drops with the rapid growth, then to return gradually up to the original level as the culture reaches the stationary phase (6).

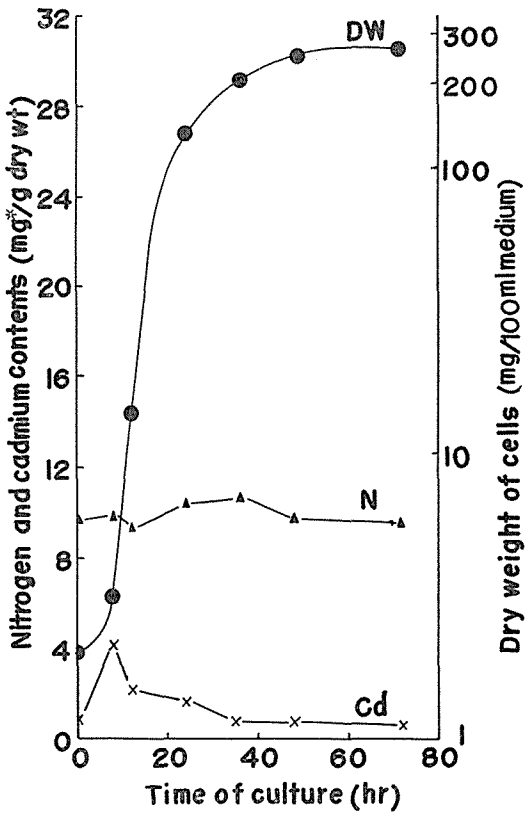


Fig. 7. Changes in cadmium and nitrogen contents of cells during the growth of resistant strain in 0.4 mM Cd-PGY medium. DW: Dry weight of cells; Cd: cadmium content; N: nitrogen content.
* Read ×10mg for nitrogen content.

## Discussion

The copper resistance of yeast has been studied to some extent by ASHIDA and his colleagues. Interest was naturally extended to the resistance to other toxic metal ions. Silver and gold, which belong to the same group of the periodic table

with copper, are not convenient for experiments in various respects. Hence cadmium was chosen for the first step, as it resembles copper in some chemical reactions.

On the basis of molar concentration the inhibiting effect of cadmium is a little stronger than copper, at least with the yeast strain used. The yeast becomes resistant to cadmium, as to copper, by a single training culture. According to NAIKI's unpublished experiment, however, the cadmium resistant substrain obtained by training is hardly more resistant to copper than its parent strain, and the copper resistant substrain which usually predominates in the copper medium is a little less resistant to cadmium than its parent strain. Hence it was expected that the cadmium resistance has features different from the copper resistance.

It was revealed, as described in section 7, that the cadmium and the sulfur contents of cadmium resistant cells cultured in the cadmium medium was considerably lower than the copper content and the sulfur content of copper resistant cells cultured in the copper medium (*cf.* 5, 6).

Yeast cells, either resistant or sensitive, are more susceptible to cadmium when they are deficient in nitrogen than when deficient in sulfur, while the case is reversed with copper. How the nitrogen metabolism makes cells tolerate cadmium remains to be studied.

Cadmium differs from copper also in the inhibition of the gas metabolism. According to Y. SHIMURA's unpublished data, respiration and fermentation of the copper resistant substrains which were obtained by training with the PGY media containing 1 mM and 3 mM of copper, are inhibited appreciably even by 0.5 mM of copper in the reaction mixture. The percentage inhibition was only a little less than with the parent strain. On the other hand, with the cadmium resistant substrain obtained by training in 0.4 mM cadmium medium, the gas exchanges were not significantly inhibited even by 1.2 mM cadmium, so far as within 60 minutes. Cadmium inhibits the respiration of the parent strain more strongly than the anaerobic fermentation, while copper inhibits the latter more strongly than the former.

When the parent strain is inoculated in the cadmium medium, the distribution of cellular resistance in the population changes with time in a way different from the case when it is inoculated in the copper medium.

Thus, physiological effects of cadmium and the resistance mechanism of the cadmium-trained substrain differ from those with respect to copper. Analogies can not generally be expected to hold between cadmium and copper. Particular approaches are to be taken for the cadmium resistance.

## Summary

1) When the sensitive strain was cultured in the medium containing 0.4 mM $CdCl_2$, the number of cadmium resistant cells became appreciable in the incubation period between 24 and 48 hr,

2) Both the parent strain and the resistant substrain were injured by cadmium more badly under nitrogen starvation than under sulfur starvation and carbon (energy) starvation. The resistant substrain tolerated much better than the other even under the starvations. Nitrogen-starved cells showed the normal tolerance soon after the supply of ammonium.

3) Cadmium inhibited respiration more strongly than anaerobic fermentation.

4) The resistant cell was higher in the nitrogen content than the parent cell, whether they may be cultured in presence of cadmium or in its absence.

5) When the parent strain was inoculated in the cadmium medium, the cadmium content of cells increased until the apparent growth owing to resistant cells occurred, to decrease thereafter.

6) Injury and resistance concerning cadmium differ in various respects from those concerning copper.

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