ADAPTATION OF YEAST TO COPPER

XI. CONDITIONS FOR THE ACTION OF A SPECIFIC RIBONUCLEATE IN INCREASING THE COPPER RESISTANCE OF YEAST

by

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INTRODUCTION

When a strain of *Saccharomyces ellipsoideus* was plated in the medium called MH (Henneberg's medium supplemented with malt extract) to which $CuSO_4$ was added to give concentration of 1 mM/l, about 70% of the viable cells grew to form visible colonies. When, however, cells which had grown on the MH medium containing 1 mM/l $CuSO_4$ were plated again on copper-containing media, the survival ratio was 100% even in the medium containing 2 mM/l $CuSO_4$.

A copper-resistant substrain was obtained by inoculating the normal strain on MH agar slant containing 1 mM/l $CuSO_4$, followed by successive subculturing on the same medium. It was designated as R_{1b}, because of the copper concentration to which it has been acclimatized and the brown colour of its colonies. If cells of R_{1b} are transferred to copper free medium, the resulting colonies are white in colour. But the cells exhibit in the plating test almost the same copper resistance as those of R_{1b}. The substrain obtained by subculturing R_{1b} once on, or in, the copper-free medium is designated as $R_{1b(0)}$.

MINAGAWA *et al.*[1] found that, when the same numbers of cells of the parent strain and of R_{1b} were mixed in $M/15$ KH_2PO_4 and incubated for a while, 100% of the cells proved to be viable, instead of giving the average of the survival ratios of the two substrains, in the plating test using 1 mM/l $CuSO_4$-MH. This result led them to suspect that some matter diffusing out of R_{1b} cells elevated the viability of the parent strain in the copper medium.

In order to know the nature of the matter several experiments were made on the extract from copper-resistant cells. As R_{1b} accumulated much copper in the cells, $R_{1b(0)}$ was used as the source of cell extract in order to avoid any effect, at all, of the accumulated copper. The copper concentration contained in the extract from $R_{1b(0)}$ was found to be too low to give any training effect to the cells of the parent strain.

After a series of experiments it was found that the sodium ribonucleate, which had been isolated from $R_{1b(0)}$ after CLARKE AND SCHRYVER[2] and then deproteinized using chloroform-amylalcohol[3], had the effect of increasing the copper resistance of the parent strain, whereas neither the corresponding fraction, nor the crude extract, from the parent strain cells had any such effect. The activity of the ribonucleic acid (RNA) fraction from

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$R_{1b(0)}$ was destroyed if the fraction had been digested by ribonuclease before acting upon the parent strain cells.

The authors[4] further demonstrated that the crude extract of $R_{1b(0)}$ lost its activity completely by the action of ribonuclease, just as in the above mentioned case of the purified RNA, while the crude extract, as well as the RNA from the parent strain, underwent through the action of the enzyme no significant change in the effect upon cells. They also confirmed that the RNA from $R_{1b(0)}$ did not lose its particular activity even after being heated twice at 100 °C for 30 minutes.

The authors[5] determined also that the cell extracts from the copper-trained substrains, as well as their parent strains, of seven other yeast species did not increase the copper resistance of the wild type cells of *S. ellipsoideus*. Hence, so far as tests have been carried out, the only source of the active principle was the copper-trained substrain which had been derived from the strain to be treated by the extract.

Facts are being accumulated that desoxypentose nucleic acids (DNA) transform bacterial properties, as reviewed by AUSTRIAN[6] and HOTCHKISS[7]. But few cases have been ascribed to the action of pentose nucleic acids. The factor which is responsible for the sensitization of penicillin resistant bacteria through the mixed culture with sensitive bacteria was ascribed to the RNA by GEORGE *et al.*[8] AKIBA *et al.*[9], however, in a similar experiment in which a penicillin-resistant strain of *Staphylococcus aureus* was sensitized by culturing together with the sensitive strain of *Diplococcus pneumoniae*, asserted that the substance participating in this phenomena was DNA, and not RNA, of *D. pneumoniae*. ODA[10] reported that the adaptive enzyme formation of a strain of *Pseudomonas aeruginosa* was markedly accelerated, in the presence of glucose, by the RNA of the strain itself, but not by that of another strain. In this case, the strain-specific RNA has the same effect whether it is obtained from the adapted cells or unadapted ones. Hence there are no cases so far reported that are similar to the author's finding. However, owing to repeated experiments, it is certain that the RNA from the copper-resistant substrain of yeast causes an increase in copper resistance of its parent strain.

It has often been inferred that RNA might participate in protein synthesis. Yet it is hardly known how it functions. The study of the action of RNA in modifying the nature of cells might serve as an approach to the physiological function of RNA. From this point of view, the author is studying both the RNA, and the crude extract, of the copper-resistant substrain. The present paper reports the following two series of experiments:

1. Necessary, as well as inhibitory conditions of cell treatment by which RNA made cells more resistant to copper were determined. This might be a first step to understanding how the RNA functions in the cell.

2. In the experiments so far mentioned, the rise in copper resistance was determined by counting visible colonies, using the conventional pour plate method. It can not be decided by this method whether the treated cells are directly made resistant by the RNA or whether they are altered so that they might become resistant through cell divisions in the presence of copper. Hence we determined the copper inhibition of fermentation to test if cells treated by the extract of the resistant substrain had become more resistant to copper before cell division had occurred. As this proved to be the case, some conditions of the cell treatment were studied by the manometric method.

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MATERIAL AND METHODS

Strain. The strain of *S. ellipsoideus* used in this experiment was the same as in the previous papers[1,4,5]. It was kept on malt extract agar at low temperature. The copper resistant substrain, R_{1b}, was obtained and kept by serial passages on the MH agar medium supplemented with 1 mM/l $CuSO_4$. When R_{1b} was passed through MH not supplemented with copper, it was denoted as $R_{1b(0)}$. The parent strain to be treated by cell extract or RNA was prepared by inoculating the stock culture to liquid or solid MH medium and incubating for 2 days at 30° C.

Culture medium. Components of MH medium are as follows: sucrose 100 g, peptone 5 g, KH_2PO_4 5 g, $MgSO_4 \cdot 7H_2O$ 2 g, distilled water 1 l, malt extract (8 Bé) 360 ml. The precipitation formed through autoclaving at 20 lb. was filtered. For solid medium, 1.5% of agar was added.

Preparation of cell extract. Cells of the parent strain and R_{1b} were incubated respectively in 1.5 l of normal MH liquid medium contained in 2 l Erlenmeyer flasks for 5 days. After harvesting and washing with distilled water, cells were dried at room temperature under reduced pressure. Of each dry powdered yeast, 200 mg was suspended in 10 ml of distilled water, heated on a boiling water bath for 30 minutes, cooled, and the supernatant was separated by centrifugation. The extracts from the parent strain and $R_{1b(0)}$ will be denoted as E_P and E_R, respectively, and the corresponding ribonucleic acids, as RNA_P and RNA_R, respectively. E_R contained a small and equal amount of copper as E_P, although the extract from R_{1b}, which had been growing in the copper medium, contained more.

Treatment of the parent strain with extract. Washed cells of the parent strain were suspended in distilled water to give a titer of about $5 \cdot 10^8$ cells/ml.

For the aerobic treatment, aliquots of the cell suspension were mixed respectively with equal volumes of extracts and incubated at 30° C. The period of treatment was 90 minutes, unless otherwise specified. This period of treatment was chosen according to the result of the following experiment.

RNA was extracted as its sodium salt from $R_{1b(0)}$ after CLARKE AND SCHRYVER[2] and purified by chloroform-amylalcohol[3]. Parent strain cells were suspended in the RNA solution in M/15 phosphate buffer (pH 5) and incubated at 30° C. Aliquots were removed at 30 minute intervals and plated by the method described below. As is shown in Fig. 1, the effect of the RNA can be fully exhibited within from 60 to 90 minutes. The concentration of RNA_R in the treating medium was chosen to be a little over 100 μg/ml, because this concentration was sufficient for the maximal effect. Below 100 μg/ml, the more concentrated the RNA_R, the higher the survival ratio of treated cells. RNA_P, on the other hand, did not influence the survival ratio even up to 2000 μg/ml.

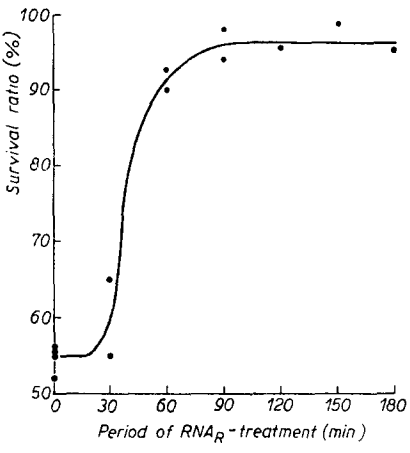

Fig. 1. Relation between the period of contact of the parent strain to a solution of 1 mg/ml RNAR and its survival in the MH-medium containing 1.2 mM/l $CuSO_4$.

For the anaerobic treatment, a Warburg vessel, which contained 0.5 ml of cell suspension in the main compartment and 0.5 ml of treating extract in a side arm, was thoroughly flushed with N_2 gas. After equilibrium was reached at 30° C, the extract and the cell suspension were mixed together. As shown in Fig. 7 C and D, the CO_2 evolution was small during the period of treatment, presumably because reserve substrates for fermentation, if they had remained, were consumed during the flushing procedure.

Method of plating. After the treatment, cell suspensions were diluted 400 times with distilled water. Then 0.05 ml of this diluted suspension was dropped into 100 ml of melted MH agar medium at 45° C. After mixing well, with care to avoid foaming, an aliquot of 20 ml was poured into a Petri dish, as a control plate. Then an aliquot of 50 ml was dispensed in a sterilized Erlenmeyer flask which contained a measured volume of M/10 $CuSO_4$ to give a final concentration of 1.2 or 1.3 mM/l. Two 20 ml aliquots of this mixture were poured into plates. Then, another control plate was made. The Petri dishes used were 9 cm in diameter. Plates were read after 2 or 3 days of incubation at 30° C. The *survival ratio* was given by the ratio of the means of duplicate plates, copper medium: control.

Measurement of respiration and fermentation. Using the usual Warburg apparatus, measurements were made by direct method at 30° C. The treated cell suspension was diluted 5 times with distilled water. Except where otherwise specified, the composition of the reaction mixture was 1 ml of cell suspension plus 1 ml of M/7.5 phosphate buffer (pH 5.0) which contained 10% glucose. A side arm of the vessel contained 0.14 ml of 5.5 mM/l $CuSO_4$ solution, and this was run into the main compartment after the equilibration, the resulting concentration of $CuSO_4$ being 0.36 mM/l. The center

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well contained 0.3 ml of 20% KOH solution for estimating O_2 uptake. Flask constants were from 1.6 to 1.8. Anaerobic measurements were carried out in N_2 or H_2 gas.

Ribonuclease (RNase). This enzyme, prepared from pancreas[11], was kindly supplied by Dr. F. EGAMI, Nagoya University.

Comparisons of numerical values obtained in different series of experiments should be avoided, for the survival ratio and the inhibition in fermentation in the presence of a certain concentration of copper are not always strictly constant. Comparisons were made between the copper tests and the controls made simultaneously.

RESULT

1. *Effect of conditions of cell treatment as tested by the plating method.*

By supplementing the cell extracts with sugar and peptone, it was tested how sugar and peptone modify the effect of RNA_R in increasing copper resistance. Table I shows typical results of an experiment in which a culture of the parent strain was treated by six kinds of solutions before plating. When 10% sucrose was present, the increase in surival ratio by E_R did not appear, its value remaining at the same level as in the case of treatment by E_P. On the other hand, the values of cells treated by E_P did not differ, irrespective of the supplementation with sucrose. But the inhibition of the E_R action by sugar was relieved by the coexistence of 0.5% peptone. Sugar and peptone in the concentrations used above had no effect upon cells.

TABLE I

Survival ratios in 1.2 mM/l $CuSO_4$-MH medium, of the parent strain treated by E_P and E_R which were and were not supplemented with sucrose and peptone. S: 10% sucrose; N: 0.5% peptone.

Treating medium	*Survival ratio (%)*
E_P	77.5
E_P + S	79.8
E_R	103
E_R + S	77.9
E_R + S + N	101
S + N	77.0

TABLE II

Survival ratios, in 1.3 mM/l $CuSO_4$-MH, of the parent strain treated by E_R supplemented with 10% sucrose and various concentrations of $(NH_4)_2SO_4$.

Concentration of $(NH_4)_2SO_4$ (%)	0	1/16	1/8	1/4
Survival ratio	49.2	69.0	98.0	97.6

Peptone, which antagonized the sugar inhibition, could be replaced by $(NH_4)_2SO_4$. Table II shows that the antagonizing action of $(NH_4)_2SO_4$ increased as the concentration was raised up to 0.125%, above which no further increase was observed. The total nitrogen content of the treating extract was 45 μg/ml, while nitrogen in 0.125% $(NH_4)_2SO_4$ solution was 265 μg/ml. The number of cells viable in the copper-free medium did not differ whether the treating extracts contained sugar or not. Moreover, cells treated by E_P with and without added sugar did not differ from each as to their viability in the copper medium. Added sucrose, therefore, did not decrease the copper resistance of treated cells, but seems to have interfered with the development of the effect of RNA_R.

The fact that sugar renders the action of RNA_R ineffectual, and that nitrogen compounds prevent it may suggest that carbohydrate and nitrogen metabolisms are involved in the alteration of the cell nature by RNA_R. Hence in order to reduce a major way of sugar break-down, fermentation inhibitors, NaF and monoiodoacetic acid (IAA), were added to E_R which contained 10% sucrose. Effects of azide and 2,4-dinitrophenol (DNP) were also examined.

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Table III A represents the survival ratios of the parent strain treated by E_R which were supplemented with 10% sucrose and the inhibitors. Each inhibitor was used in the highest concentration that did not affect the survival ratio of the parent strain. The Table indicates that the interfering action of sugar was blocked by the presence of either of the fermentation inhibitors, while the other two enzyme inhibitors inhibited the increase in survival ratio by E_R.

TABLE III

Influence of enzyme inhibitors upon the effect, exerted on the parent strain, of E_R containing 10% sucrose (S) and 0.5% peptone (N). Cells were treated for 90 minutes at 30° C and plated to read the survival ratio in 1.2 mM/l $CuSO_4$-MH medium.

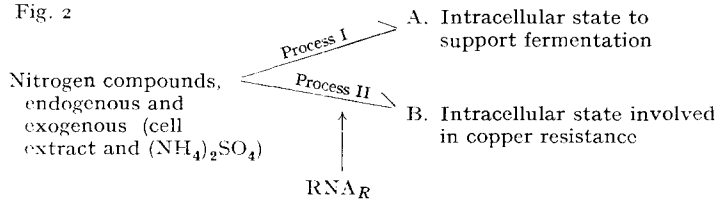
	Treating medium	*Enzyme inhibitor added to treating medium (mM/l)*		*Survival ratio (%)*
A	E_RS	—	—	75.0
		NaF	10.0	95.5
		IAA	0.1	94.4
		NaN_3	0.01	74.6
		DNP	0.1	73.8
B	E_RS	—	—	71.0
	E_RSN	—	—	97.6
		NaN_3	0.01	70.4
		DNP	0.1	84.4

The latter two enzyme inhibitors interfered with the action of E_R even in the presence of peptone, as shown in Table III B. This fact can be accounted for by a direct or indirect inhibition of nitrogen metabolism, or as will be discussed later, by an inhibition of the energy supply to the metabolism which leads to an increase in copper resistance. These inhibitors are known to inhibit phosphorylation in low concentrations where they do not depress rates of respiration and fermentation. Their inhibiting effect on adaptive enzyme formation has been explained in this connection[12].

Increasing evidence is favourable to the hypothesis that RNA is involved in protein synthesis or enzyme formation in the cell. Supposition might be allowed, therefore, that some qualitative and/or quantitative change in the plasmatic state of the treated cell is controlled by RNA_R and results in an increase in copper resistance.

A hypothetical scheme might be presented to account for the above-mentioned effects of sugar and nitrogen source (Fig. 2). Let state A represent an intracellular state

Fig. 2



which supports fermentation, and state B another one which, when present in the cell, makes it more resistant to copper. Suppose state A develops when fermentation occurs,

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and state B is established by an action, not of RNA_P, but of RNA_R. If nitrogen is needed for states A and B to develop (or to maintain themselves), the processes I and II, which lead to A and B, respectively, may compete with each other.

When no sugar is present in the treating E_R solution, process I is weak, and II can proceed at the expense of nitrogen compounds in the cell and in the extract. But if much sugar is present in the treating medium, process I goes so vigorously as to put process II on short nitrogen allowance, and thus the effect of RNA_R fails to be realised. Process II can proceed in the presence of much sugar, however, either when process I is inhibited by NaF or IAA, or when enough nitrogen is supplied exogenously to relieve the competition. This idea is supported by the following experiment.

Table IV depicts typical results of a set of experiments performed simultaneously. In the Table, *c* and *d* represent successive treatments by two kinds of solution, namely E_R supplemented with 10% sugar and E_R supplemented with 10% sugar and 0.125% $(NH_4)_2SO_4$. In the case of *c*, cells in which state A had been attained by the first treatment, were introduced to the treating medium which can make state B develop. In the case of *d*, on the contrary, cells in state B were subjected to a condition which accelerated process I. The survival ratio in *d* was higher than in *c*, both being intermediate between those of single treatments, *a* and *b*. The results may indicate that, when state A has once been developed by the first treatment, the formation of state B is weak even in the presence of ammonium salt, and that state B once established is not easily destroyed, at least within 90 minutes, even if fermentation may occur significantly.

TABLE IV

Effect of successive treatments by different media. S: 10% sucrose, N: 0.125% $(NH_4)_2SO_4$. Numbers in parentheses indicate the duration in minutes of each treatment.

Treatment	*Survival ratio (%)*
a. E_RS (90)	70.6
b. E_RSN (60)	97.8
c. E_RS (90) —— E_RSN (60)	81.1
d. E_RSN (60) —— E_RS (90)	88.0
e. E_R (120) —— E_P (120)	102
f. E_RSN (60) —— E_PSN (270)	91.8

Experiment *e* in Table IV shows that the survival ratio of cells in state B does not lower even if they are suspended in E_P for 120 minutes. This may indicate that E_P has no power to revert the established state B to the sensitive state, and that state B can persist unless some process which requires a nitrogen source proceeds vigorously, as in *d*.

It was ascertained that cells hardly proliferated during treatments in the above experiments. Experiment *f* in Table IV was carried out in order to see how the established state B is impaired if the cells treated with E_R proliferate in the absence of RNA_R. Cells treated with E_R were transferred to E_P which was supplemented with sugar and $(NH_4)_2SO_4$. The number of cells increased 4 times in 4.5 hours. But the survival ratio did not decrease to the level of cells treated with E_P. Hence it can be stated that E_P does not decrease the survival ratio of cells in B and that a few generations of cell proliferation in E_P do not result in a remarkable decrease of survival ratio. However, when cells were left to proliferate until they became 30 times increased, in an experi-

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ment similar to *f* in Table IV, the survival ratio was lowered to the level of control cells, while cells having proliferated to the same extent in the presence of E_R, supplemented with sugar and $(NH_4)_2SO_4$, survived 100%.

On the other hand, treated cells proliferate very much when they form visible colonies in copper-containing plates. And cells composing those colonies are copper resistant. Hence the copper resistance is not lost by multiplication in the presence of copper. So it may be concluded that the cell-proliferation without either E_R or copper "dilutes" state *B*, though it is uncertain whether *B* is destroyed or not.

2. *Effect of E_R as tested prior to cell proliferation.*

Of the parent strain treated by E_P and E_R, respiration as well as aerobic and anaerobic fermentation in the presence of 5% glucose were measured. But no difference was found between the effects of the two kinds of extract.

When, however, the copper inhibition of aerobic fermentation was measured, a significant difference was noticed between the treatments by E_P and E_R. As shown in Fig. 3, fermentation of cells which has been treated by E_P ceased almost completely in 40 minutes following the introduction of $CuSO_4$ solution into the cell suspension, while with cells treated by E_R, fermentation was not completely inhibited. In many cases, a difference in copper inhibition of fermentation between the two treatments could be noticed even in 10 minutes after the addition of copper. As to copper inhibition of O_2 uptake, cells treated by E_R suffered copper inhibition to a lesser extent than those treated by E_P in every experiment, but the difference was small as seen in Fig. 3.

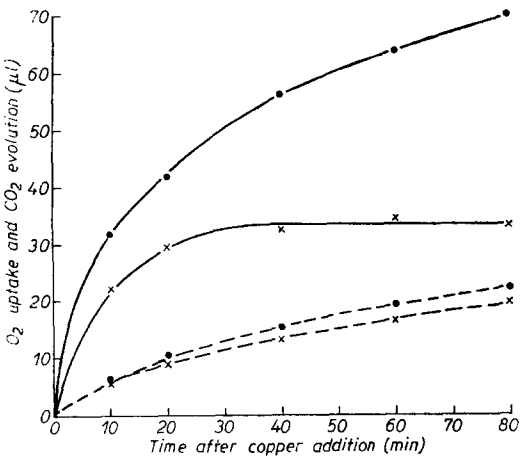

Fig. 3. Effect of copper on respiration (– – –) and aerobic fermentation (——) of the parent strain which has been treated with E_R(●) and with E_P(×). $CuSO_4$ solution was added at zero time to give concentration of 0.34 m*M*/l.

Results analogous to the case of aerobic fermentation were obtained also with anaerobic fermentation (in N_2 or H_2 gas), as illustrated in Fig. 4.

Since no difference in the rate of fermentation in the absence of copper was caused by the treatments with E_P and E_R, the alteration caused in cells by E_R-treatment must be such as to let fermentation proceed in spite of the presence of copper.

In an attempt to find out if the above mentioned effect of E_R is attributable to RNA_R, cell extracts were digested by RNase. Each of E_P and E_R was mixed with two aliquots of phosphate buffer at pH 7.2, the one containing the enzyme and the other not. The four kinds of mixtures were kept at 60° C for 120 minutes, and then heated at 100° C for 30 minutes with the object of inactivating the enzyme, which was contained in two of them. After cooling, the pH was regulated to the original value of 4.8. Fig. 5 represents the copper inhibition of glucose fermentation under anaerobic conditions of the parent strain, which has been treated by those solutions. Cells treated with E_R (A), not affected by RNase, suffered less copper inhibition than those treated with E_R(B), digested by the

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enzyme. And the copper inhibition of the latter was of the same degree as those treated with E_P either with (D) or without (C) RNase digestion.

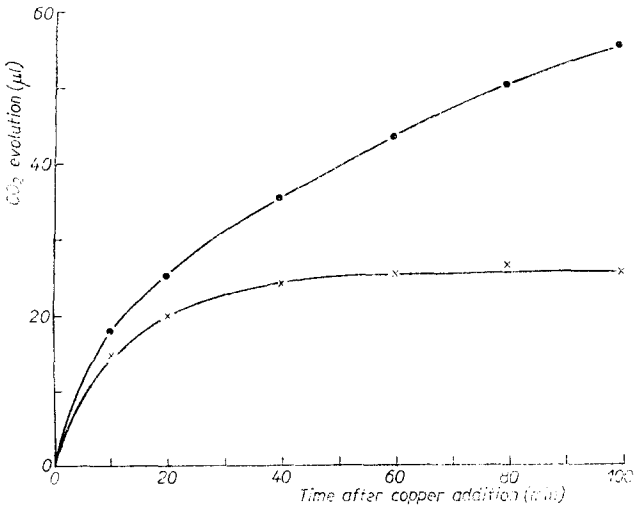

Fig. 4. Effect of copper on CO_2 evolution in N_2 gas of cells treated with E_R(●) and with E_P(×). $CuSO_4$ solution was added at zero time to give 0.34 mM/l.

Thus it was proved that E_R alters the parent strain and makes it less susceptible to copper inhibition by the specific RNA contained in it, just as was found with the plating method. It is to be noted that the use of the manometric method allows copper sensitivity of cells to be detected before they multiply when brought in contact with copper, while, with the plating method, copper resistance is estimated by cell proliferation in the copper containing medium.

The cell treatments were carried out under aerobic conditions in the above experiments. When, however, cells were treated under the anaerobic condition, E_R had no beneficial effect compared to E_P, as illustrated in Fig. 6. By aerobic treatment made simultaneously, the effect of E_R was evident. In the three cases, anaerobic treatment with E_R, and aerobic as well as anaerobic treaments with E_P, there was no significant difference in the effect on cells of changing the copper sensitivity of fermentation.

It was determined with the plating method that E_R failed to be effective when cells fermented vigorously during the treatment, provided that there was no supply of nitrogen compound. Hence in order to ascertain if the ineffectiveness of the anaerobic treatment is due to nitrogen deficiency, $(NH_4)_2SO_4$ was supplemented to E_R. But E_R was still ineffective.

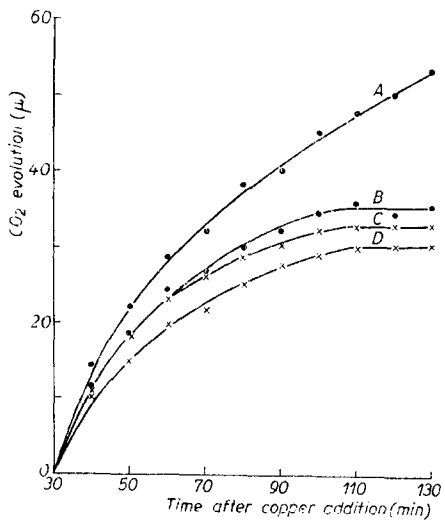

Fig. 5. Effect of RNase digestion on the activity of cell extracts. Fermentation in N_2 gas, in the presence of copper, of the parent strain which has been treated by E_R not digested (A) and digested (B) by RNase, and of that treated by E_P not digested (C) and digested (D) by RNase.

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Aerobiosis seems to be needed for the cell alteration which is induced by RNA_R and by which the parent strain becomes less susceptible to copper. During treatments with extracts, fermentation under the anaerobic condition was very low compared with respiration, as shown in Fig. 7. (In this measurement, the density of the cell suspension was 10 times as high as in the other experiments.) A possibility is suggested that sufficient energy necessary for the realisation of the effect of RNA_R can not be furnished under the anaerobic condition, while it can be in the aerobic treatment. If this is the case, the cellular alteration would be induced by RNA_R even under the anaerobic condition, if an available energy source is supplied.

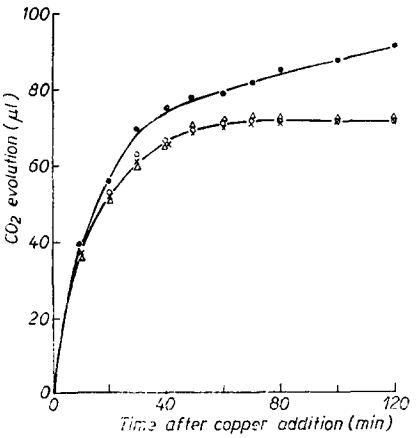

Fig. 6. Significance of aerobiosis during the cell treatment. Fermentation in N_2, in the presence of copper, of the parent strain which has been treated with E_R in air (●) and in N_2 (○), and with E_P in air (×) and in N_2 (△).

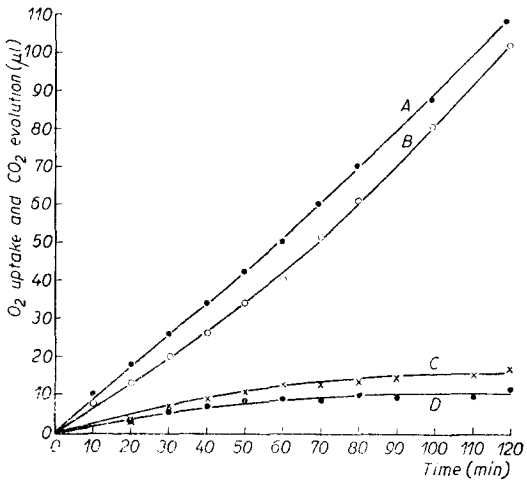

Fig. 7. Respiration and anaerobic fermentation in the treating extracts. CO_2 evolved (A) and O_2 taken up (B) in air by cells being suspended in E_R; and CO_2 evolved in N_2 by cells being suspended in E_P (C) and E_R (D). Density of cells was ten times as high as other experiments reported in the present paper.

Anaerobic treatments were carried out with and without the addition of 0.05 ml of 0.06 *M*/l glucose solution to 0.95 ml of cell suspensions in E_P and E_R. Among the four kinds of treatment, the one by E_R supplemented with the small amount of glucose was effective, as shown in Fig. 8.

Here the question arises whether a small amount of sugar, necessary for the anaerobic treatment, is required for providing some metabolic product or energy. SPIEGELMAN *et al.*[13] using yeast cells proved that esterification of inorganic phosphate was inhibited by a low concentration of NaN_3, without fermentation being inhibited. LOOMIS *et al.*[14] reported that a low concentration of DNP, which did not inhibit oxidation, interfered with phosphorylation. Hence these agents were used to inhibit phosphorylation occurring during treatments with the extracts.

Anaerobic fermentation of cells of the parent strain was inhibited very slightly by 32 m*M*/l NaN_3, and not at all by 16 m*M*/l. Hence, when the latter concentration of the inhibitor was added to cell extracts together with a small amount of glucose,

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phosphorylation may be inhibited without fermentation being interfered with during the anaerobic treatment.

The parent strain cells were anaerobically treated by E_R and E_P, supplemented with 3 μM of glucose, with and without addition of 16 mM/l NaN_3. The anaerobic fermentation of treated cells was measured in the presence of copper. In contrast to the cells treated by E_R without NaN_3 (Fig. 9 A), those treated by E_R in the presence of NaN_3(B) remained copper sensitive, just as those treated by E_P (C). Hence it seems most reasonable to suppose that sugar is needed for the anaerobic treatment as the energy source to form high energy phosphate bond which is to be needed for the process II in Fig. 2.

In the aerobic treatment, on the other hand, sugar, the supplementary energy source, is not necessary. Since the cell extracts, as used for treatments, contained no

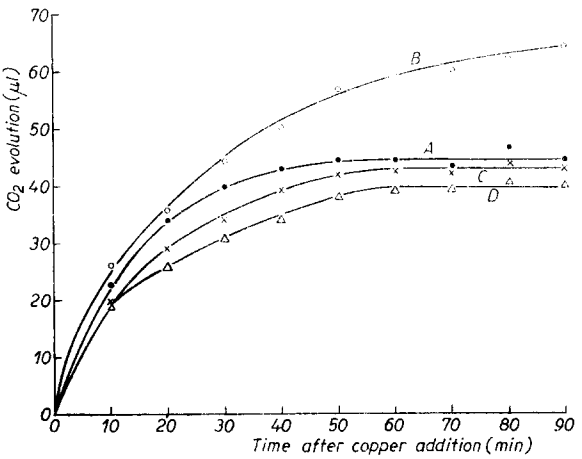

Fig. 8. Significance of a trace of sugar in the anaerobic treatment of cells. Fermentation in N_2, in the presence of copper, of the parent strain which has been anaerobically treated by: A: E_R; B: E_R + 3 μM of glucose; C: E_P; D: E_P + 3 μM of glucose.

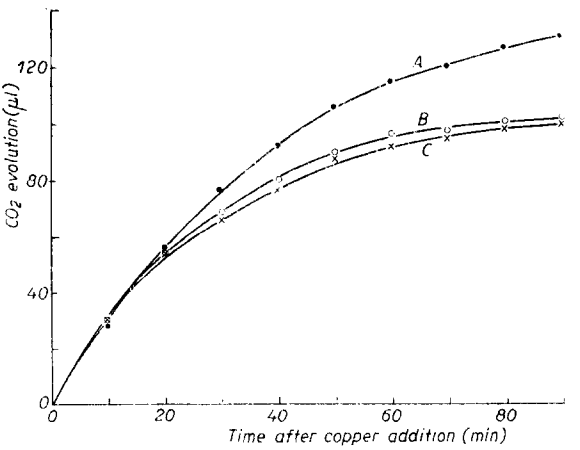

Fig. 9. Azide inhibition of the action of E_R. Fermentation in N_2, in the presence of copper, of the parent strain which has been anaerobically treated by: A: E_R; B: E_R + $1.6 \cdot 10^{-2}$ M/l NaN_3; and C: E_P. Each extract contained 3 μM of glucose.

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significant amount of substrates, energy must be obtained chiefly by endogenous respiration. The fact that the anaerobic treatment is ineffectual when no exogenous energy source is given is understandable from this point. In order to confirm the importance of energy coupling during the aerobic treatment, DNP was added to treating extracts to which no sugar was added. Respiration of cells suspended in cell extracts was hardly inhibited by 0.11 mM/l DNP, and not at all by 0.55 mM/l. When the latter concentration of DNP was added, E_R showed no effect which distinguishes it from E_P, which is inactive if the same amount of DNP is either added or not. Fig. 10 illustrates a typical case. Sometimes the effect of E_R was not blocked so clearly by this concentration of DNP. But the copper inhibition was always weaker with cells treated by E_R without DNP than those treated in the presence of the inhibitor.

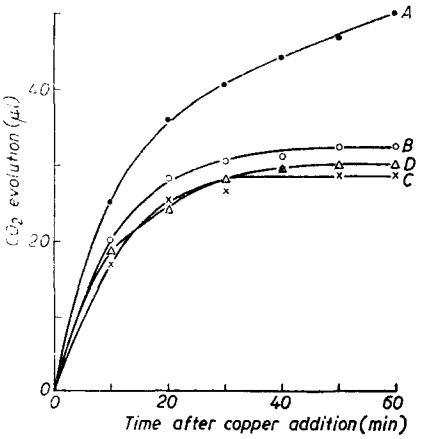

Fig. 10. Inhibition by 2,4-dinitrophenol of the action of E_R. Fermentation in N_2, in the presence of copper, of the parent strain which has been treated under aerobic condition by A: E_R; B: E_R + $5.5 \cdot 10^{-5}$ M/l DNP; C: E_P; and D: E_P + $5.5 \cdot 10^{-5}$ M/l DNP.

It may be concluded, therefore, that the effect of RNA_R can not be brought about unless sufficient phosphorylation takes place during the treatment. The alteration which is induced in the cell by RNA_R and makes the cell less sensitive to copper, most probably involves reactions which require an energy-rich phosphate bond.

DISCUSSION

According to BERNHEIMER[15], the individuality of RNA has so far been recognized only in two cases, namely the formation of streptolysin S and the inhibition of DNase. ODA[10] found that RNA was strain specific with respect to the effect on adaptive enzyme formation of *Pseudomonas aeruginosa*. He could not, however, observe differential activities between the RNA's from adapted and non-adapted cells. It is found in the present case that the RNA from the copper-trained substrain of yeast differs from that from the parent strain in the effect of decreasing the copper injury, as measured by the survival ratio and the glucose fermentation.

Since different kinds seem to be discriminated in the copper resistance, possible relations among them are represented in Fig. 11. Let B_3 designate the state involved in the copper resistance of cells which have been acclimatized in copper medium, and B_2 the state for another copper resistance with which parent strain cells are invested through the action of E_R. State B_2 can be determined by a weaker copper inhibition of fermentation measured just after the E_R-treatment, namely before the cells proliferate in the presence of copper. Fermentation even of the cells having B_3 was considerably inhibited by copper, though not so strongly as of the parent strain cells.

It is inferred from the experiments, *c* and *d* in Table IV, that a certain intracellular state related to copper resistance is already established by the E_R-treatment prior to the contact of the cells with copper. Cells must contain more nitrogen compounds at the end of treatment *c* than *d*. A high nitrogen content is favourable for E_R to be effective

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when much sugar is present, as seen in Table II. Therefore, if E_R reveals its effects only when it is coexistent with extraneous copper, the survival ratio should be higher in *c*

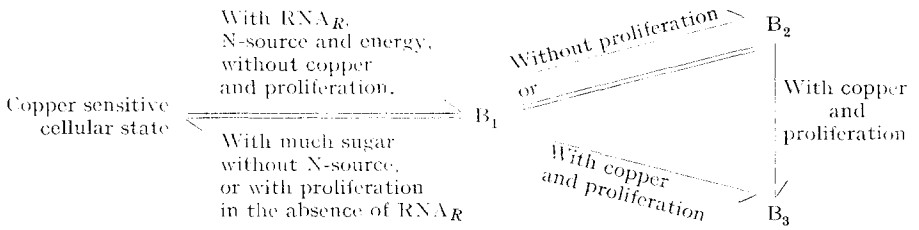

Fig. 11

than in *d*, contrary to the results obtained. Hence an intracellular state is inferable which is induced by RNA_R without any cooperation by extraneous copper. The comparison of experiment *a* with *b* in Table IV suggests the same circumstance. Hence an intracellular state, designated as B_1, must be set up by the effect of RNA_R, during the incubation period using nitrogenous matter, without cells being put in contact with copper. Copper, however, does not seem to interfere with this process, for E_R was effective even when untreated cells were plated in the copper medium to which E_R was supplemented.

For the detection of B_2, cells must be brought in contact with copper. Hence it is questionable whether B_2 is produced (1) in the presence of extraneous copper by cells in which B_1 is already formed by the action of RNA_R, or (2) solely by RNA_R without the action of extraneous copper. B_2 may be identical with B_1 in the latter case. The cell proliferation is not required in both cases, (1) and (2).

The fact that the formation of state B_1 or B_2 is interfered with by a certain amount of sugar may be explained by the spending of endogenous utilizable nitrogen compounds for the making of the fermentation system. In the anaerobic treatment, however, a small amount of sugar is indispensable for the action of RNA_R. It is proved by using uncoupling agents that energy is needed for the establishment of B_1 under the aerobic as well as anaerobic condition. The energy supplied by endogenous respiration during the aerobic treatment, and that furnished by glucose in the anaerobic treatment might be used either (1) in the uptake of RNA_R by cells, (2) in the formation of B_1 under the influence of RNA_R, or (3) in both of (1) and (2). It is surmised that high energy phosphate bond may be involved in the resistance raising effect of RNA_R.

Through the proliferation in the presence of copper, B_3 is eventually established by progeny cells of the parent strain, regardless of whether these have been treated with E_R or E_P, the difference between the two cases being only in the degree of ability of making up B_3. YANAGISHIMA *et al.*[16] reported that when the parent strain was spread on solid medium containing 1 m*M*/l $CuSO_4$, a thin white film grew at first, discontinued brown colonies then growing on it secondarily. When the parent strain treated by E_R or RNA_R is spread on the copper medium, the growth usually proceeds in the former type. Its survival ratio in copper medium is also much lower than $R_{1b(0)}$. Hence the cells having B_1 or B_2 cannot grow in the copper medium so well as $R_{1b(0)}$ does. However, it may be that the cells having B_1 or B_2 suffer copper inhibition less than E_P-treated cells, the residual growth thus being favoured. And when the residual growth in the copper

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medium is better, there is a higher possibility that B_3 is built up in individual cells, or there is a greater chance of B_3-cells being produced. Such will be proved before long. ASHIDA[17], by tracing microscopically the growth of individual clones starting from sensitive cells spread on the copper-agar medium, has shown that fast-growing cells arise eventually while cells of the sensitive type grow with much trouble.

The copper resistance induced by E_K is not lowered by a subsequent treatment by E_P (Table IV *e*). In other words, the induction is possible only in the one direction. While, however, the properties transformed by DNA are reported to be inheritable, the copper resistance induced by RNA_R is lowered by a few hours' proliferation (without copper) in the absence of E_R (Table IV *f*). And the resistance induced by E_R eventually disappears after further proliferation. Hence the intracellular state induced by RNA_R can be transmitted only partly to daughter cells.

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SUMMARY

A cell extract of a copper-resistant substrain, which has been obtained by training a parent strain of *Saccharomyces ellipsoideus* on a copper-containing medium, can alter the parent strain cells to be more viable in the copper medium. It was further found that the cells of the parent strain, treated by the extract of the resistant substrain, are less sensitive to the inhibition of fermentation by copper than those treated by the extract of the parent strain.

The factor responsible for the above mentioned alteration is the ribonucleic acid, which differs in action from that contained in the extract from the parent strain.

The parent strain cells which have been acted upon by the ribonucleic acid of resistant cells have become less sensitive to copper inhibition before any significant cell divisions occur, and probably before cells come in contact with copper.

The alteration in copper sensitivity of cells by ribonucleic acid does not occur when much sugar is added to the treating extract, if nitrogen source is insufficient. But a very small amount of sugar is necessary as energy source in case of the anaerobic treatment.

The ribonucleic acid of the resistant substrain is ineffective in the presence of NaN_3 or 2,4-dinitrophenol, which may inhibit the synthesis of energy rich phosphate bond.

RÉSUMÉ

L'extrait de cellules d'une souche résistante au cuivre obtenue en cultivant une souche mère de *Saccharomyces ellipsoideus* dans un milieu renfermant du cuivre, peut rendre les cellules de la souche mère plus aptes à vivre dans le milieu au cuivre. Nous avons trouvé, de plus, que les cellules de la souche mère traitées par l'extrait de la souche résistante sont moins sensibles à l'inhibition de la fermentation par le cuivre que celles, traitées par l'extrait de la souche mère.

L'agent de la modification ci-dessus est l'acide ribonucléique, qui diffère par son action de l'acide ribonucléique contenu dans l'extrait de la souche mère.

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Les cellules de la souche mère, traiteés par l'acide ribonucléique des cellules résistantes, sont devenues moins sensibles à l'inhibition par le cuivre avant que des divisions de cellule eussent lieu, et probablement avant que les cellules fussent en contact avec le cuivre.

Le changement de sensibilité au cuivre provoqué par l'acide ribonucléique n'a jamais lieu lorsque beaucoup de sucre est ajouté à l'extrait de traitement, à moins qu'une quantité suffisante de source d'azote ne soit ajoutée. Mais une très petite quantité de sucre est nécessaire comme source d'énergie en cas du traitement anaérobique.

L'acide ribonucléique de la souche résistante est inefficace en présence de NaN_3 ou de 2,4-dinitrophénol, qui pourrait inhiber la synthèse du composé phosphate riche d'énergie.

ZUSAMMENFASSUNG

Ein Zellextrakt eines kupferwiderstandsfähigen, durch Züchtung des Mutterstammes von *Saccharomyces ellipsoideus* in kupferhältigem Nährboden erhaltenen Stammes kann die Zellen des Mutterstammes so verändern, dass sie in einem kupferhältigen Medium lebensfähiger werden. Es wurde ferner gefunden, dass die mit dem Extrakt aus dem widerstandsfähigen Stamm behandelten Zellen des Mutterstammes gegenüber der Hemmung der Gärung durch Kupfer weniger empfindlich sind, als die mit dem Extrakt aus dem Mutterstamm behandelten Zellen.

Der für die oben erwähnte Veränderung verantwortliche Faktor ist die Ribonucleinsäure, die sich von der, im Extrakt aus dem Mutterstamm erhaltenen, in ihrer Wirkung unterscheidet.

Die Zellen des Mutterstammes, die mit der Ribonucleinsäure des widerstandsfähigen Stamms behandelt worden waren, sind gegenüber der Hemmung durch Kupfer weniger empfindlich geworden, bevor die Zellteilung stattfindet, und wahrscheinlich bevor die Zellen mit dem Kupfer in Berührung kommen.

Wenn viel Zucker zu der zu behandelnden Lösung hinzugesetzt wird, findet keine Veränderung der Empfindlichkeit gegen Kupfer durch die Ribonucleinsäure statt, wenn nicht eine genügende Menge von Stickstoffverbindung zugesetzt wird. Aber bei der anaeroben Behandlung ist eine kleine Menge Zucker als Energiequelle notwendig.

Die Ribonucleinsäure des widerstandsfähigen Stammes ist im Beisein von NaN_3 oder 2,4-dinitrophenol, das die Synthese der energiereichen Phosphatverbindung hemmen könnte, inaktiv.

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