appears that, in lymphoid cells from mice or guinea-pigs, increase in the rigidity of surface membrane by free cholesterol is accompanied by increase of free fatty acids. Free fatty acids from various sources, including lymphocytes, have long been known to be highly cytotoxic to many mammalian cells[10,15–19]. TURNELL et al.[20] have also indicated that, in corticosteroid-sensitive lymphocytes, accumulation of free fatty acids is involved in corticosteroid-induced lymphocytolysis. Therefore it is conceivable that free cholesterol, which is believed to be present almost exclusively in the cell surface membrane[3], regulates the levels of cytotoxic free fatty acids in lymphoid cells by changing the rigidity of cell membrane. Concerning this, it is of special interesting that the facile exchange of free cholesterol occurs in vitro between the surface membrane of lymphocytes and the surrounding medium (liposomes) containing free cholesterol and lecithin[5], and unsaturated free fatty acids promote the membrane fluidity of lymphocytes; namely, they decrease the viscosity of lymphocyte membrane[21]. Free fatty acids in lymphocytes have been shown to consist of 65–70% unsaturated fatty acids[22].

In splenic lymphoid cells, no significant difference was found in the regression coefficient of fatty acid to cholesterol between mice and guinea-pigs, as described above. The regression coefficient of phospholipid to cholesterol for mouse splenic lymphoid cells was also similar to that for the corresponding cells from guinea-pigs (1.35 for mice and 1.20 for guinea-pigs, $p < 0.01$). In contrast, the regression coefficient of fatty acid to cholesterol as well as that of phospholipid to cholesterol in lymphoid cells from animals markedly differed from the original tissues. These differences in the regression coefficient among various tissues suggest that each of lymphoid cells from thymus, spleen, mesenteric lymph node or other lymph nodes shows a characteristic change in the composition of membrane lipid which is closely connected with the properties and functions of cell membrane.

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Regulation of Enzymes of Ethanol Metabolism in Yeast (*Rhodotorula gracilis*)[1]

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Summary. The three enzymes of ethanol metabolism alcohol dehydrogenase, aldehyde dehydrogenase and acetyl-CoA synthetase in the obligate aerobic yeast *Rhodotorula gracilis* are repressed by glucose and induced by C_2 metabolic fuels with a regulatory pattern indicating a correlation in the control mechanisms. To try an identification of the molecular signals involved in the transmission of the inducing stimulus, experiments were carried out by blocking with 2 m*M* pyrazole the ethanol $\rightarrow$ acetaldehyde metabolic step. Results indicate that ethanol is not specifically required as a molecular signal for induction.

An interesting aspect of control of cellular level of enzymes is the multiple response of a linked group of enzyme proteins to the same environmental change. In the present paper, results are reported concerning the response to the same inductive or repressive stimulus of three enzymes of ethanol metabolism in the yeast *Rhodotorula gracilis*. The three enzymes are alcohol dehydrogenase (EC 1.1.1.1), aldehyde dehydrogenase (EC 1.2.1.5) and acetyl-CoA synthetase (EC 6.2.1.1). Alcohol dehydrogenase in *Rhodotorula gracilis* – an obligate aerobic organism – is presumably not concerned with alcoholic fermentation, but with oxidative utilization of external alcohols or with internal metabolism of alcoholic compounds. Its level is under epigenetic control, being dependent on the following kinds of environmental effects: induction by ethanol, repression-inactivation by glucose, in vivo stabilization by Zn[2,3]. In addition to ethanol, a high inducing capacity is shown specifically by other C_2 compounds, especially by acetaldehyde[3]. In conditions of Zn deficiency, the action of glucose is not only that of a repressor: glucose also promotes an evident inactivation of enzyme protein in vivo[4], and addition of Zn prevents this inactivation. The nature of the agents that directly contribute to the transfer of the inductive or repressive stimulus is not yet understood. A large number of metabolic intermediates are among the suspected substances that can change their level and act as a regulatory signal. The results presented here show that not only alcohol dehydrogenase, but two other enzymes of the pathway ethanol $\rightarrow$ acetyl-CoA are under epigenetic control in the cell of *Rhodotorula gracilis*. The data indicate a correlated response and point to the existence of a common regulatory metabolic signal.

Materials and methods. The strain of *Rhodotorula gracilis*[4] was maintained on nutrient malt-agar slopes. Cells were grown on synthetic medium with the following composition: 15 m*M* $(NH_4)_2SO_4$; 8.6 m*M* NaCl; 5.7 m*M* K_2HPO_4; 4 m*M* $MgSO_4$; 2.3 m*M* $CaCl_2$; 0.018 m*M*

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Effect of glucose on the formation of alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and acetyl-CoA synthetase (ACS) stimulated by C_2 compounds in the yeast *Rhodotorula gracilis*

Condition	Enzyme specific activity (nmoles/min/mg protein) ADH	ALDH	ACS
Start	0	11	12
Mineral medium	7	57	21
2 m*M* ethanol	123	123	51
,, ,, + 20 m*M* glucose	5	11	17
2 m*M* acetaldehyde	137	141	71
,, ,, + 20 m*M* glucose	2	13	12
2 m*M* acetyl-phosphate	87	151	48
,, ,, + 20 m*M* glucose	0	9	10

Cells grown 24 h at 30 °C on 200 m*M* glucose, without Zn addition, washed and transferred for 4 h into the media indicated

$FeCl_3$; 5 mg/l Ca-pantothenate; 5 mg/l thiamine-HCl. Initial pH was brought to 4.5. Zn was added as 0.007 m*M* $ZnSO_4$, and carbon sources were added as indicated in the Table and Figures. Growth was carried our aerobically at 30 °C in flasks with rotative eccentrical agitation. It was followed by measurements of optical density at 623 nm or by cell counts.

For preparation of cell extracts, cells were suspended (0.2 g/ml) in 100 m*M* potassium phosphate buffer, pH 7.5, and disrupted in a Braun apparatus[5] with glass beads 0.23–0.25 mm diameter. For the assay of enzyme activities, the cell homogenate was centrifuged for 30 min at 100,000 *g* and the pellet and the upper lipid layer were discarded. Total proteins were determined by a biuret method[6] on whole homogenates. Enzyme activities were assayed at 30 °C by optical tests, in a 2400 Gilford spectrophotometer. Alcohol dehydrogenase was determined according to MAEHLY and BONNICHSEN[7]. Aldehyde dehydrogenase was determined in a mixture having the following composition[2]: 100 m*M* *Tris*-HCl buffer, pH 8.5; 5 m*M* Na_2EDTA; 2 m*M* pyrazole; 1.5 m*M* NAD^+; 0.06 m*M* acetaldehyde; 50 or 100 µl/ml enzyme preparation. Acetyl-CoA synthetase was determined according to JONES and LIPMANN[8].

Results. In the cells of *Rhodotorula gracilis*, ethanol and other C_2 metabolites are very effective inducers of the three enzymes of the ethanol → acetyl-CoA pathway, namely alcohol dehydrogenase, aldehyde dehydrogenase and acetyl-CoA synthetase. Results showing the stimulation of the formation of the three enzymes by C_2 compounds ethanol, acetaldehyde and acetylphosphate are given in Figure 1. Acetylphosphate was preferentially used in place of acetate: acetate is also an inducer of alcohol dehydrogenase, but acetylphosphate is a better inducer and a better carbon source for the yeast. In order to determine quantitatively the inducing capacity, the final specific activity of each enzyme after a 4-hours exposition of glucose-grown cells to each agent was measured. The resulting values of the induction ratio, that is the ratio between the induced enzyme level and the enzyme level in mineral basal medium (RICHMOND[9]), are indicated in the same Figure. These data show a concurrent response of all three enzymes to the C_2 inducers.

The inductive response is lost in presence of the inhibitor of protein synthesis cycloheximide and of the amino acid analogue *p*-fluorophenylalanine. It is, however, only partially inhibited in presence of an inhibitor that acts specifically on mitochondrial protein synthesis, that is chloramphenicol.

Glucose determines also a pattern of regulatory response similar for the three enzymes: it consistently blocks the inductive formation of alcohol dehydrogenase, aldehyde dehydrogenase and acetyl-CoA synthetase even in presence of inducers (Table).

A parallel in the regulated changes of the three enzymes results also from experiments with pyrazole. Pyrazole is a well-known specific inhibitor of the first enzyme of ethanol oxidation, alcohol dehydrogenase. Its inhibitory effect on alcohol dehydrogenase of *Rhodotorula gracilis* has been established both on enzyme preparations and in vivo[3]. The inhibition kinetics is competitive versus the substrate ethanol and the value of the inhibition constant is 0.3 µ*M*. At 1 m*M* or higher concentrations, the inhibition is practically complete, even on the ethanol metabolism of living

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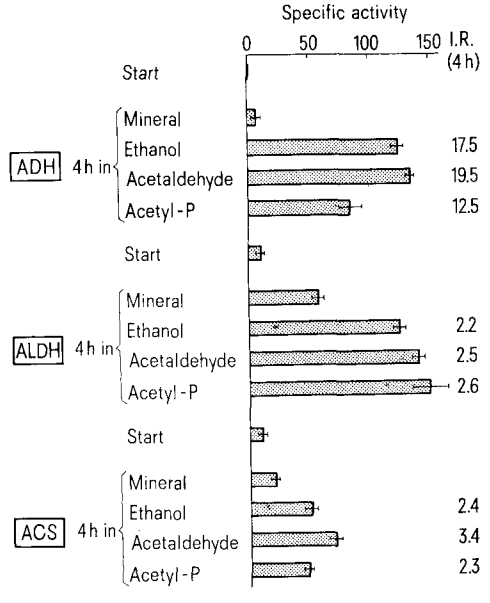

Fig. 1. Induction profile for alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and acetyl-CoA synthetase (ACS) in the yeast *Rhodotorula gracilis*. Cells grown 24 h on 200 m*M* glucose, without Zn addition, washed and transferred for 4 h into the media indicated: mineral=basal medium with no added carbon source; ethanol= basal medium + 2 m*M* ethanol; acetaldehyde=basal medium + 2 m*M* acetaldehyde; acetyl-P=basal medium + 2 m*M* acetyl-phosphate. Temperature 30 °C. Results are expressed as increase of specific activity (nmoles/min/mg protein) in 4 h. The values indicated are means of 5 experiments and the bars show ± SEM. Induction ratio (I.R.)=enzyme level in (basal medium + tested compound)/ enzyme level in mineral.

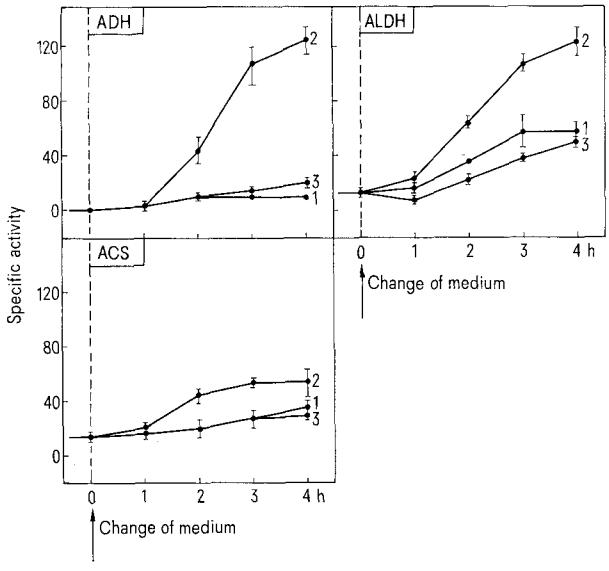

Fig. 2. Effect of pyrazole on the formation of alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and acetyl-CoA synthetase (ACS) stimulated by ethanol in the yeast *Rhodotorula gracilis*. Cells grown 24 h on 200 m*M* glucose, without Zn addition, washed and transferred for the time indicated into the following media: 1: basal medium with no added carbon source; 2: basal medium + 2 m*M* ethanol; 3: basal medium + 2 m*M* ethanol + 2 m*M* pyrazole. Temperature 30 °C. Results are expressed as enzyme specific activity (nmoles/min/mg protein). Each point represents the mean of 5 experiments and the vertical bar shows ± SEM.

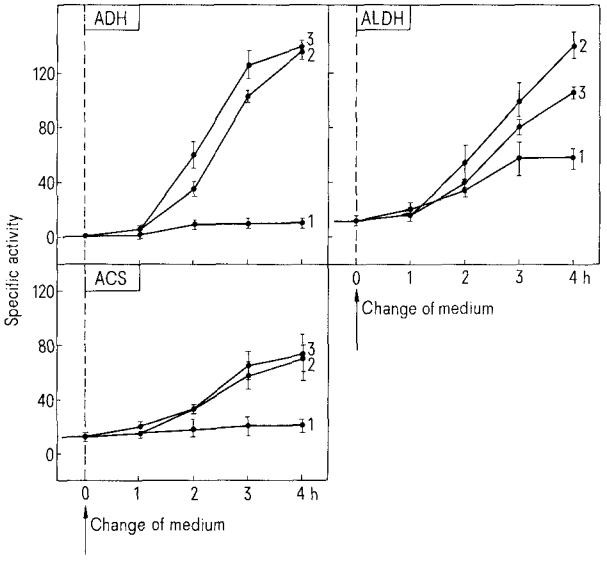

Fig. 3. Effect of pyrazole on the formation of alcohol dehydrogenase (ADH); aldehyde dehydrogenase (ALDH) and acetyl-CoA synthetase (ACS) stimulated by acetaldehyde in the yeast *Rhodotorula gracilis*. Cells grown 24 h on 200 m*M* glucose, without Zn addition, washed and transferred for the time indicated into the following media: 1: basal medium with no added carbon source; 2: basal medium + 2 m*M* acetaldehyde; 3: basal medium + 2 m*M* acetaldehyde + 2 m*M* pyrazole. Temperature 30 °C. Results are expressed as enzyme specific activity (nmoles/min/mg protein). Each point represents the mean of 5 experiments and the vertical bar shows ± SEM.

yeast cells. The use of pyrazole therefore selectively blocks the ethanol ⇄ acetaldehyde step in vivo. The effects of this metabolic interruption in experiments of induction by ethanol or by acetaldehyde of alcohol dehydrogenase, aldehyde dehydrogenase and acetyl-CoA synthetase are shown in Figures 2 and 3. It is evident that blocking the ethanol ⇄ acetaldehyde step abolishes – for all three enzymes – the inductive response to ethanol, but leaves undisturbed the inductive response to acetaldehyde.

Discussion. In the obligate aerobic organism *Rhodotorula gracilis*, the most evident function of alcohol dehydrogenase, aldehyde dehydrogenase and acetyl-CoA synthetase is to catalyze in a coordinate metabolical sequence the oxidation of external alcohols.

In other yeasts, in addition to an alcohol dehydrogenase type with oxidative function, other isoenzymes are present, namely a fermentative and a mitochondrial type[10–13]. The regulation patterns of the fermentative and mitochondrial isoenzymes appear, however, to be different from the oxidative isoenzyme, as only the latter is completely repressed by glucose [13,14]. It is interesting to note that in *Rhodotorula gracilis* only one isoenzyme of alcohol dehydrogenase is largely predominant and shows a regulatory pattern similar to that described for the oxidative type in other yeasts[2].

Results presented in the present paper indicate that the pattern of regulatory response is common to alcohol dehydrogenase, aldehyde dehydrogenase and acetyl-CoA synthetase. All three enzymes appear to be repressed by glucose and induced by C_2 metabolic fuels.

Some more direct indication on the molecular signals involved in the transmission of the inducing stimulus comes from the experiments with pyrazole. This inhibitor causes a specific interruption of the metabolism at the ethanol ⇄ acetaldehyde step.

When cells are exposed to ethanol, the inducing effect could arise from an action of ethanol molecule or from a derived metabolic effect. Among the more evident effects, ethanol is known to change the redox state of the NAD+/NADH couple toward reduction, and to affect also the state of free adenine nucleotide system (MAITRA and ESTABROOK[15,16], see also VEECH et al.[17]). When the cells are exposed to ethanol+pyrazole, it is reasonable to assume that the cytoplasmic level of ethanol is unchanged or increased, that of acetaldehyde, acetate and deriving pools is lowered, and the redox state of the NAD+/NADH couple moves in the direction of oxidation. In this metabolic condition, there is minimal or no induction for all three enzymes investigated. The inducing stimulus is, on the contrary, fully active when the cells are exposed to acetaldehyde+pyrazole. In this condition, one must assume that the cellular level of ethanol is near zero, that of acetaldehyde and deriving intermediates is increased

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and the redox state of NAD+/NADH system is again in the direction of reduction[18]. It must be pointed out that in this case the inductive response excludes the possibility that pyrazole by itself has some irreversible effect on the alcohol dehydrogenase molecule or some other inhibitory effect on the cell systems. The observed pattern of response with pyrazole is reasonably interpreted by assuming that the molecule of ethanol is not specifically required as a signal to induce this enzyme sequence. The change of significant intermediate is therefore to be searched for among the metabolic effects specifically deriving from the utilization of the C_2 fuels. Possibly the state of redox couples and adenine nucleotide systems are primarily involved.

In any case the response is concurrent for all three enzymes. This suggests a correlated regulation of level of enzymes catalyzing the ethanol → acetyl-CoA pathway, under the control of a common metabolic signal.

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Antigenicity of Thermal Denatured Yoshida Glycoprotein

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Summary. The correlation between antigenic functionality and conformation of Yoshida glycoprotein upon heating has been investigated. Heating modifies the conformation of the antigen, but does not abolish the reaction with its specific antibody.

There has been a great deal of work in the past decade on the problem of determining the functionality of small synthetic antigen molecule[1], whereas for natural antigens detailed studies on the relation between structure and function are limited to few examples. Among these, convincing evidence has been reported for serum albumin and Yoshida glycoprotein[2-6]. In the course of extensive investigations on the possibility of substituting haptenic groups in the polypeptide chain of Yoshida glycoprotein, it has been observed that this protein behaves on heating in a very peculiar way and could represent an useful experimental model to study thermal denaturation of proteins. Because literature data concerning reversible thermal denaturation of proteins are scanty[7-11], in this report we give a preliminary account of the experiments undertaken in order to correlate antigenic functionality of Yoshida glycoprotein to conformational changes of the molecule upon heating.

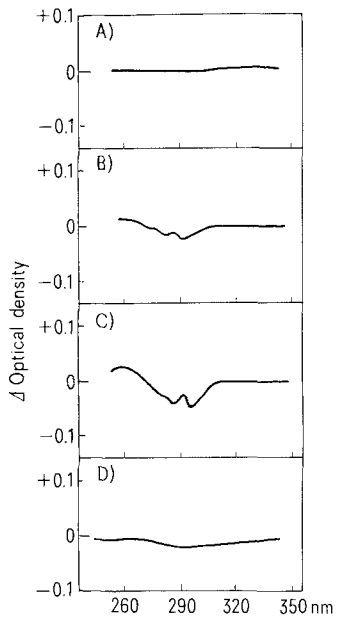

Fig. 1. Temperature difference spectra. A) Native Yoshida glycoprotein against native; B) Yoshida glycoprotein heated at 70 °C against native; C) Yoshida glycoprotein heated at 90 °C against native; D) Yoshida glycoprotein heated at 90 °C and cooled at room temperature against native. The protein concentration both in sample and reference cell was 1 mg/ml. The spectra were recorded 30 min after that temperature was reached. The pH of solutions was 7.0.

Materials and methods. Glycoprotein was isolated from Yoshida ascites tumor fluid as previously reported[12,13]. The purity was ascertained by ultracentrifugation and electrophoresis, during which it behaves as homogeneous monodisperse system as confirmed by the occurrence of one single precipitine arc in immunoelectrophoresis.

Thermal denaturation was carried out in a Colora thermostat and the temperature was measured by thermistored apparatus to an accuracy of ± 0.1 °C. Spectroscopic measurements were performed with a Beckman DK1 recording spectrophotometer equipped with a thermospacer apparatus.

Protein solutions were prepared by diluting 1 ml stock solution (5 mg/ml) to 5 ml in a volumetric flask. The concentration of protein was determined spectrophotometrically using an $E\,{}^{1\ \mathrm{cm}}_{1\ \mathrm{mg/ml}}$ of 0.700. The antigenic activity of the glycoprotein was measured in its capacity

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