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# Oxidoreduction between Cycloalkanols and the Corresponding Cycloalkanones in the Cultured Cells of *Nicotiana tabacum*. Simulation of the Time Courses in the Oxidoreduction

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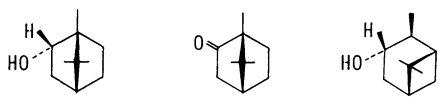
(Received December 16, 1987)

A method for the simulation of the time courses in the oxidation of cycloalkanols and the reduction of cycloalkanones in the cultured cells of *Nicotiana tabacum* was developed on the basis of the permeability of these compounds into the cultured cells and the $^{13}C$ NMR chemical shift of the carbonyl carbon of the cycloalkanones. Further, the method has been applied to the simulation of the time courses in the oxidation of (+)-borneol and (+)-isopinocampheol and the reduction of (−)-carvomenthone in the cultured cells of *N. tabacum*.

Recently, we reported that the balance in the equilibrium of the oxidoreduction between 5- to 7- membered cycloalkanols and their corresponding cycloalkanones in the cultured cells of *Nicotiana tabacum* depends on the carbon number in the carbocyclic ring of the cyclic compounds.[1] It was further found that the oxidoreduction between the cycloalkanols and their corresponding cycloalkanones in the cultured cells is governed by an $NAD^+$-dependent alcohol dehydrogenase which is similar to the dehydrogenase from tea seeds and horse liver. We have developed a method for the simulation of the time courses in the oxidation of cycloalkanols and the reduction of cycloalkanones in the cultured cells on the basis of the permeability of these compounds into the cultured cells and the $^{13}C$ NMR chemical shift of the carbonyl carbon of the cycloalkanones.[2] Furthermore, the method has been applied to the oxidation of (+)-borneol (**1**) to (+)-camphor (**2**) and (+)-isopinocamphenol (**3**) to (−)-isopinocamphone (**4**) and the reduction of (−)-carvomenthone (**5**) to (+)-carvomenthol (**6**) in the cultured cells of *N. tabacum*.[3,4] The results have been partly outlined in the preliminary communications,[1-4] but we wish to describe *en bloc* details of the results together with newly obtained results.



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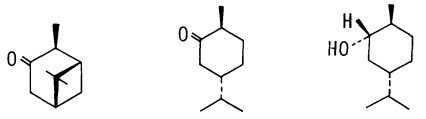

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## Results and Discussion

**Reaction Rates of the Oxidoreduction of Cycloalkanols and Cycloalkanones in the Enzyme System of *N. tabacum*.** Firstly, the enzyme system responsible for the oxidoreduction of cycloalkanols and their corresponding cycloalkanones in the cultured cells of *N. tabacum* was characterized. An enzyme system was partially purified prepared from the cultured cells of *N. tabacum* by treating a cell-free extract of the cells with cold acetone and then ammonium sulfate as described in the experimental section. The substrate specificity of the enzyme system was tested for the oxidation of alkanols. The alkanols used as substrate were ethanol, 1-propanol, 1-butanol, 1-pentanol, 1-hexanol, cyclopentanol, and cyclohexanol. Relative rates observed in the oxidation of these substrates are given in Table 1; ethanol was the best substrate for this enzyme system. The substrate specificity was quite similar to the specificity of alcohol dehydrogenase isolated from tea seeds[5] and horse liver.[6,7]

The reaction rates of the oxidation of cycloalkanols with this enzyme system were determined in 0.1 M† glycine–NaOH buffer (pH 9.0) by tracing the decrease in the UV absorption at 340 nm due to the reduced form of nicotinamide–adenine dinucleotide (NADH).[8] The cycloalkanols used as substrate were cyclopentanol (**7**), cyclohexanol (**8**), 2-methylcyclohexanol (**9**),

Table 1. Substrate Specificity[a]

| | Relative $V_{max}$ |
|---|---|
| Ethanol | 1.0 |
| 1-Propanol | 0.49 |
| 1-Butanol | 0.36 |
| 1-Pentanol | 0.20 |
| 1-Hexanol | 0.07 |

a) Determined in 0.1 M glycine–NaOH buffer, pH 9.0, at 25 °C.

† 1 M=1 mol $dm^{-3}$.

Table 2. The Rate Constant and the Equilibrium Constant of the Oxidoreduction between Cycloalkanols and the Corresponding Cycloalkanones with the Enzyme System

| Compound | $k_{+1}$[a]/s$^{-1}$ | $k_{-1}$[b]/s$^{-1}$ | $K$ |
|---|---|---|---|
| Cyclopentanol | $2.53\times10^{-4}$ | $7.09\times10^{-5}$ | 3.57 |
| Cyclohexanol | $9.52\times10^{-4}$ | $1.81\times10^{-3}$ | 0.53 |
| 2-Methylcyclohexanol | $1.22\times10^{-3}$ | $5.01\times10^{-3}$ | 0.24 |
| 3-Methylcyclohexanol | $1.03\times10^{-3}$ | $3.16\times10^{-3}$ | 0.33 |
| Cycloheptanol | $3.69\times10^{-4}$ | $1.20\times10^{-4}$ | 3.08 |

a) Determined in 0.1 M glycine–NaOH buffer, pH 9.0, at 25 °C. b) Determined in 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C.

3-methylcyclohexanol (**10**), and cycloheptanol (**11**). The reaction rates of the reduction of cycloalkanones with the enzyme system were determined in 0.1 M potassium phosphate buffer (pH 7.0) by tracing the increase in the UV absorption at 340 nm due to NADH.[8] The cycloalkanones used were cyclopentanone (**12**), cyclohexanone (**13**), 2-methylcyclohexanone (**14**), 3-methylcyclohexanone (**15**), and cycloheptanone (**16**). Table 2 gives the rate constant of the oxidation of the cycloalkanols ($k_{+1}$), the rate constant of the reduction of the cycloalkanones ($k_{-1}$), and the equilibrium constant in the oxidoreduction between the cycloalkanols and the cycloalkanones ($K$). The equilibrium constants indicate that the equilibrium of the oxidoreduction of the 6-membered cyclic compounds shifts toward the side of the reduction, while the equilibrium of the oxidoreduction of the 5- and 7-membered cyclic compounds does toward the side of the oxidation. Such a ring-size effect on the equilibrium was quite similar to the effect on the equilibrium in the bioconversion of the cycloalkanols and the cycloalkanones in the cultured cells of *N. tabacum*.

**Simulation of the Time Courses in the Oxidoreduction between the Cycloalkanols and the Corresponding Cycloalkanones in the Cultured Cells of *N. tabacum*.** Figures 1 and 2 show the time courses in the oxidation of cyclohexanol to cyclohexanone and the reduction of cyclohexanone to cyclohexanol, respectively. The half lives of these substrates in the oxidation and the reduction in the cultured cells were 3 days, whereas those of the substrates in the oxidation and the reduction with the enzyme system were 10 and 45 min, respectively, as deduced from the rate constants given in Table 2. The reaction rate of the oxidoreduction in the enzyme system was much faster than that of the oxidoreduction in the cultured cells. Such a time lag is probably due to a delay arising from permeation of the cycloalkanols and the cycloalkanones into the cells. The rate of permeation is known to depend on a gradient between intra- and extracellular concentrations of the cycloalkanols;[9–12] the permeability constant can be estimated by use of Hill's equation,[11]

$$[S] = [S]_0 \cdot e^{-p \cdot t}, \quad (1)$$

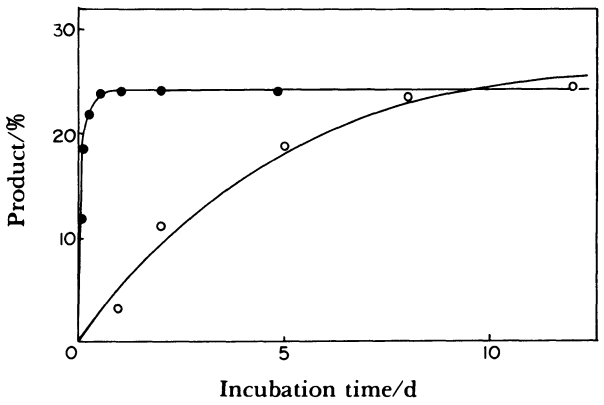


Fig. 1. Time courses in the oxidation of cyclohexanol into cyclohexanone with the enzyme system (—●—) and in the cultured cells (—○—).

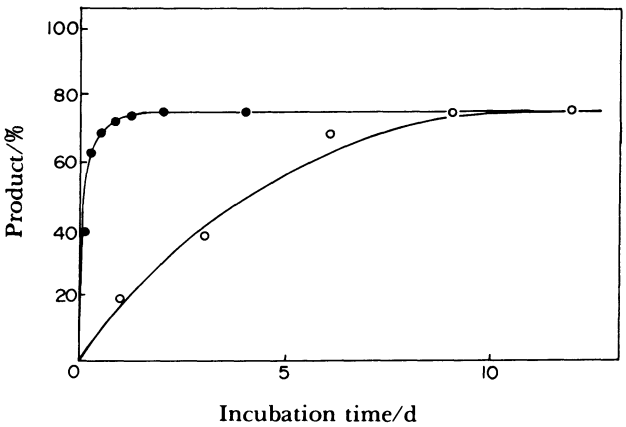


Fig. 2. Time courses in the reduction of cyclohexanone into cyclohexanol with the enzyme system (—●—) and in the cultured cells (—○—).

where $[S]$,$[S]_0$, and $p$ denote the concentration of the foreign substrate (cycloalkanol) at the time "$t$", the initial concentration of the foreign substrate, and the permeability constant, respectively. The permeability constants for permeation of the cycloalkanols and the corresponding cycloalkanones into the cultured cells were determined by substituting the data of the time courses in the bioconversion of 5- to 7-membered cycloalkanols to their corresponding cycloalkanones in the cultured cells[4] for Hill's equation; the average

of the permeability constants in the oxidation of 5- to 7-membered cycloalkanols to their corresponding cycloalkanones was $4.82\times10^{-6}$ s$^{-1}$.

On the basis of the permeability constants and the rate constants, the time courses in the oxidation of the cycloalkanols in the cultured cells were simulated as follows. Temporal infinitesimal change in the concentration of the product [P] at the time "$t$" is given by

$$d[P] = \{[S]_0 \cdot p \cdot k_{+1}/(k_{+1} + k_{-1}) - [P] \cdot p\} \cdot dt, \quad (2)$$

where $k_{+1}$ and $k_{-1}$ denote the rate constant of the oxidation of the cycloalkanols and the rate constant of the reduction of the cycloalkanones, respectively. The initial term shows the infinitesimal change in the concentration of the product which is formed by oxidation of the foreign substrate and the last term shows the infinitesimal change in the concentration of the product which is permeated into the cells. The solution of this differential Eq. 2 is

$$[P] = \{[S]_0 \cdot k_{+1}/(k_{+1} + k_{-1})\}(1 - e^{-p \cdot t}). \quad (3)$$

Thus, it was established that the concentration of the product can be simulated by the permeability constant, the rate constant of the oxidation of the cycloalkanols, and the rate constant of the reduction of the cycloalkanones.

**Correlation between the Reaction Rate of Enzymatic Oxidoreduction and the $^{13}$C NMR Chemical Shift.** The rate constants of the oxidation of the cycloalkanols ($k_{+1}$) and the reduction of the cycloalkanones ($k_{-1}$) and the equilibrium constants of the oxidoreduction between the cycloalkanols and the corresponding cycloalkanones ($K$) were correlated with $^{13}$C NMR chemical shifts of the carbon atom bearing a hydroxyl group in the cycloalkanols and the carbonyl carbon in the cycloalkanones.[2] The correlations of ln $k_{+1}$, ln $k_{-1}$, and ln $K$ against the $^{13}$C NMR chemical shifts of the cycloalkanols are represented by the following empirical formulas (4)—(6) with $r$ (the correlation coefficient)=0.75, 0.71, and 0.68, respectively.

Rate constants;

$$k_{+1} = \exp(-0.195\delta_{c-o} + 6.4) \quad (4)$$

$$k_{-1} = \exp(-0.515\delta_{c-o} + 29.2) \quad (5)$$

Equilibrium constant;

$$K = (k_{+1}/k_{-1}) = \exp(0.318\delta_{c-o} - 22.6) \quad (6)$$

where $\delta_{c-o}$ denotes the $^{13}$C NMR chemical shift of the carbon atom bearing the hydroxyl group. However, these $^{13}$C NMR chemical shifts were not well-correlated with the rate constants and the equilibrium constants.

On the other hand, a plot of ln $k_{+1}$, ln $k_{-1}$, and ln $K$ against the $^{13}$C NMR chemical shift of the carbonyl carbon yielded a straight line, as shown in Figs. 3 and 4. These correlations are represented by the following empirical formulas (7)—(9) with $r$ (the correlation coefficient)=0.97, 0.95, and 0.94, respectively.

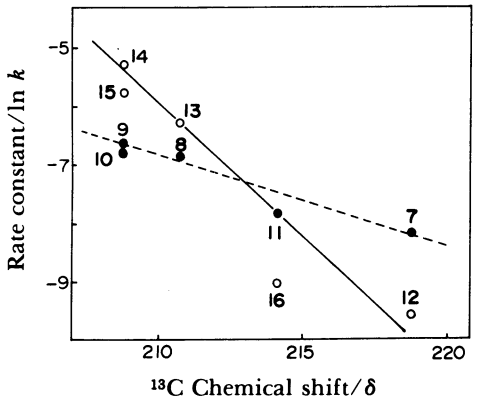


Fig. 3. Correlation of the rate constants of the oxidation of cycloalkanols ($k_{+1}$; --●--; cyclopentanol (**7**), cyclohexanol (**8**), 2-methylcyclohexanol (**9**), 3-methylcyclohexanol (**10**), and cycloheptanol (**11**)) and the reduction of cycloalkanones ($k_{-1}$; —○— cyclopentanone (**12**), cyclohexanone (**13**), 2-methylcyclohexanone (**14**), 3-methylcyclohexanone (**15**), and cycloheptanone (**16**)) with the $^{13}$C NMR chemical shift of the carbonyl carbon of the cycloalkanones involved in the reaction.

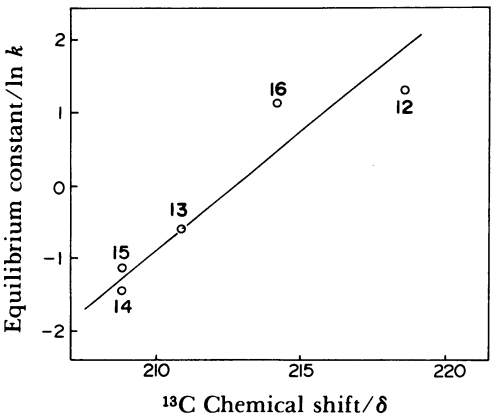


Fig. 4. Correlation of the equilibrium constant of the oxidoreduction between cycloalkanols and corresponding cycloalkanones with the $^{13}$C NMR chemical shift of the carbonyl carbon of the cycloalkanones (cyclopentanone (**12**), cyclohexanone (**13**), 2-methylcyclohexanone (**14**), 3-methylcyclohexanone (**15**), and cycloheptanone (**16**)).

Rate constants;

$$k_{+1} = \exp(-0.162\delta_{c=o} + 27.1) \quad (7)$$

$$k_{-1} = \exp(-0.447\delta_{c=o} + 87.7) \quad (8)$$

Equilibrium constant;

$$K = (k_{+1}/k_{-1}) = \exp(0.284\delta_{c=o} - 60.5) \quad (9)$$

where $\delta_{c=o}$ denotes the $^{13}$C NMR chemical shift of the carbonyl carbon of the cycloalkanones. The rate

constants and the equilibrium constants were predicted by the $^{13}C$ NMR chemical shifts of the carbonyl carbon of the cycloalkanones. This indicates a dependence of the reaction rate of the enzymatic oxidoreduction on the electron density of the carbonyl carbon of the cycloalkanones.

When we use this indication, Eq. 3 can be related with the $^{13}C$ NMR chemical shift of the carbonyl carbon of the cycloalkanones given in Eqs. 7 and 8. For the time courses in the oxidation of the cycloalkanols and their related compounds, the proportion of the concentration of product [P] (cycloalkanone) to the initial concentration of substrate $[S]_0$ (cycloalkanol) is found to be

$$P = [P]/[S]_0 = \{1 - \exp(-4.82 \times 10^{-6} \cdot t)\}/ \{1 + \exp(-0.284\delta_{c=o} + 60.5)\}. \quad (10)$$

The time courses in the reduction of the cycloalkanones and their related compounds were simulated in a manner similar to that described above. The simulated equation is as follows:

$$P = \{1 - \exp(-4.82 \times 10^{-6} \cdot t)\}/ \{1 + \exp(0.284\delta_{c=o} - 60.5)\}. \quad (11)$$

Therefore, the time courses in the oxidation of the cycloalkanols and the reduction of the cycloalkanones in the cultured cells were found to be simulated on the basis of the permeability of the cycloalkanols and their corresponding cycloalkanones into the cultured cells and the $^{13}C$ NMR chemical shift of the carbonyl carbon of the cycloalkanones or the corresponding carbonyl compounds.

**Application of the Simulation to the Oxidation of (+)-Borneol and (+)-Isopinocampheol and the Reduction of (−)-Carvomenthol.** The equation for the simulation of the time courses in the oxidation of the cycloalkanols was applied to the oxidation of (+)-borneol (**1**) to (+)-camphor (**2**) in the cultured cells. Figure 5 shows the time course in the bioconversion of borneol to camphor in the cultured cells[4] and the time course simulated by use of Eq. 10 with the $^{13}C$ NMR chemical shift of the carbonyl carbon of camphor ($\delta_{c=o}$ 218.9). The simulation curve well fits with the bioconversion curve. Furthermore, the time course in the oxidation of (+)-isopinocampheol (**3**) to (−)-isopinocamphone (**4**) was simulated by use of Eq. 10 with the $^{13}C$ NMR chemical shift of the carbonyl carbon of isopinocamphone ($\delta_{c=o}$ 214.3) and is shown in Fig. 6. This simulation curve well fits with the bioconversion curve of isopinocampheol in the cultured cells, as seen in Fig. 6.

On the other hand, the time course in the reduction of (−)-carvomenthone (**5**) to (+)-carvomenthol (**6**) was simulated by use of Eq. 11 with the $^{13}C$ NMR chemical shift of the carbonyl carbon of carvomenthone ($\delta_{c=o}$ 211.8), as shown in Fig. 7. The simulation curve also well fits with the time course in the bioconversion of carvomenthone to carvomenthol in the cultured cells.

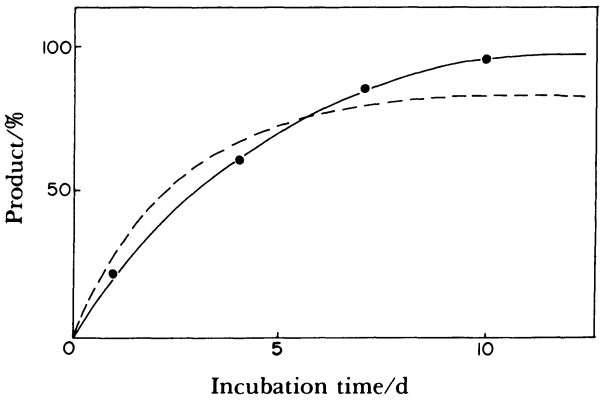


Fig. 5. Comparison of the bioconversion of (+)-borneol (**1**) into (+)-camphor (**2**) in the cultured cells (—●—) with its simulation (-----).

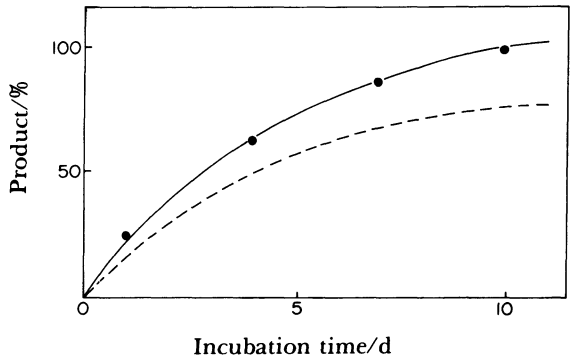


Fig. 6. Comparison of the bioconversion of (+)-isopinocampheol (**3**) into (−)-isopinocamphone (**4**) in the cultured cells (—●—) with its simulation (-----).

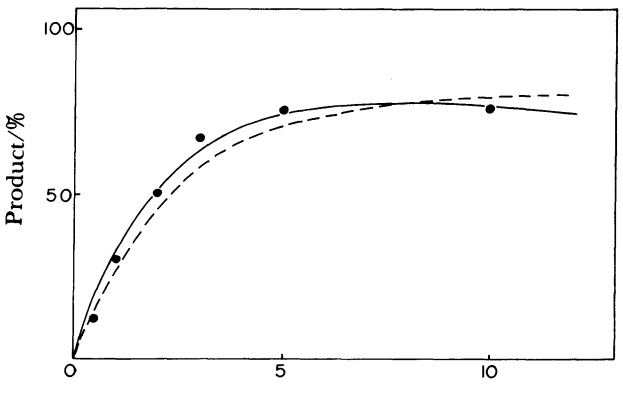


Fig. 7. Comparison of the bioconversion of (−)-carvomenthone (**5**) into (+)-carvomenthol (**6**) in the cultured cells (—●—) with its simulation (-----).

It was thus established that the time courses in the oxidation of the cycloalkanols and the related alcohols and the reduction of the cycloalkanones and the related ketones in the cultured cells can be simulated on

Table 3. The Purification of the Enzyme System

| | Total protein | Total act. | Sp. act. | Purification |
|---|---|---|---|---|
| | mg | U | U mg$^{-1}$ | Fold |
| Cell-free extract | 75.6 | 17.8 | 0.23 | 1.0 |
| Acetone fraction | 5.0 | 9.8 | 1.92 | 8.3 |
| $(NH_4)_2SO_4$ fraction | 0.8 | 8.2 | 10.2 | 44 |

the basis of the permeability of the cycloalkanols, the cycloalkanones, and their related compounds into the cells and the carbonyl carbon $^{13}C$ NMR chemical shifts of the cycloalkanones and the related carbonyl compounds involved in the equilibrium of the oxidoreduction between the cycloalkanols and the cycloalkanones.

## Experimental

GLC analyses were performed on an instrument equipped with an FID and a glass column (3 mm×2 m) packed with 15% DEGS or 2% OV-17 on Chromosorb W (AW-DMCS; 80—100 mesh), varying the column temperature over the range of 80—200 °C at the rate of 3 °C min$^{-1}$. $^{13}C$ NMR spectra were obtained at 22.6 MHz in $CDCl_3$ with tetramethylsilane as an internal reference.

**Materials.** Cyclopentanol, cyclohexanol, 2-methylcyclohexanol, 3-methylcyclohexanol, and cycloheptanol and their corresponding cycloalkanones were of commercial origin from Wako Pure Chemical Ind., Ltd. The chemical shifts of the carbonyl carbon of the cycloalkanones in their $^{13}C$ NMR spectra were 218.6, 210.8, 208.8, 208.8, and 214.2, respectively. (+)-Camphor (**2**) was purchased from Tokyo Kasei Co., Ltd. and purified by recrystallization followed by sublimation; mp 176—178 °C, $[\alpha]_D^{25}$ +42.9° (*c* 1.1, EtOH) (lit,[13] $[\alpha]_D^{25}$ −44.2°); $^{13}C$ NMR ($CDCl_3$) $\delta$=218.9 (C=O). (+)-Borneol (**1**) was prepared from (+)-camphor (**2**) by reduction with $NaBH_4$ in methanol; mp 201—202 °C, $[\alpha]_D^{25}$ +37.6° (*c* 1.0, EtOH) (lit,[13] $[\alpha]_D^{25}$ +37.9°). (+)-Isopinocampheol (**3**) was synthesized from (−)-α-pinene by hydroboration;[14] mp 55—57 °C, $[\alpha]_D^{25}$ +31.6° (*c* 5.1, EtOH) (lit,[3] $[\alpha]_D^{25}$ −31.0° for the (−)-enantiomer). (−)-Isopinocamphone (**4**) was prepared from (+)-isopinocampheol (**3**) by oxidation with sodium dichromate;[15] $[\alpha]_D^{25}$ −9.8° (neat) (lit,[3] $[\alpha]_D^{25}$ −9.9°); $^{13}C$ NMR ($CDCl_3$) $\delta$=214.3 (C=O). All the samples used as substrate were >99% pure on GLC.

**Enzyme System from the Cultured Cells of *N. tabacum*.** The cultured cells of *N. tabacum* (100 g) were homogenized in 50 mM Tris-HCl buffer (pH 7.2, 1000 ml) containing 10 mM 2-mercaptoethanol. The homogenate was stirred for 3 h at 25 °C. After filtration through several layers of cheesecloth, the filtrate was centrifuged at 13000 g for 10 min to give a cell-free extract. Into the cell-free extract, cold acetone (0 °C) was poured with vigorous stirring under ice-cooling. The soln, after standing for 30 min, was centrifuged at 17000 g for 15 min to give a precipitate, which was suspended in 50 mM Tris-HCl buffer (pH 7.2, 300 ml) containing 10 mM 2-mercaptoethanol. The suspension was treated with ammonium sulfate to give a 25—60% ammonium sulfate fraction. The salting-out product was suspended in 0.1 M potassium phosphate buffer (pH 7.0). The suspended material, after dialysis for 15 h against the same buffer solution, was centrifuged at 30000 g for 20 min. The supernatant was used as an enzyme system. The enzyme system was purified 44-fold against the cell-free extract in the activity for the conversion of ethanol to acetaldehyde by oxidation, as given in Table 3. The properties of the enzyme system were characterized as follows.

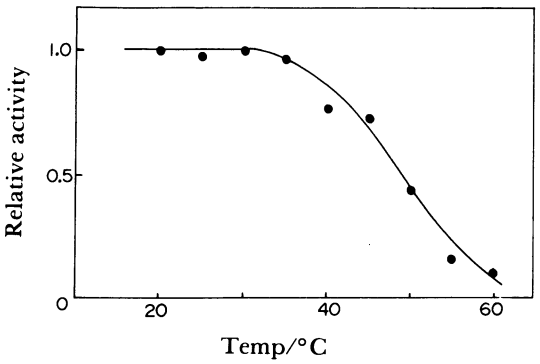


Fig. 8. Thermal stability of the enzyme system partially purified from the cultured cells of *N. tabacum*.

**Substrate Specificity in the Oxidation of Alkanols.** The mixture containing the enzyme system (0.6 μg protein), alkanol (0.1 M), such as ethanol, 1-propanol, 1-butanol, and 1-hexanol, and $NAD^+$ (0.05 mM) was diluted with 3 ml of 0.05 M Tris-HCl buffer (pH 8.5) containing 10 mM 2-mercaptoethanol and then the solution was incubated at 25 °C for 2 h. The reaction was monitored by means of UV absorption measurements. The initial rates in the oxidation of the alkanols were determined by measuring the change in the UV absorption at 340 nm for NADH. Table 1 gives the relative rates in the oxidation of the alkanols.

**Thermal Stability:** The enzyme system was held at various temperatures (20—60 °C) for 15 min prior to incubation. The enzyme activity in the oxidation of ethanol was determined by measuring the change in the UV absorption at 340 nm. Figure 8 shows the thermal stability of the enzyme system.

**Optimum pH:** The enzyme activity in the oxidation of ethanol was determined in three kinds of buffer solutions as follows: 0.1 M potassium phosphate buffer, pH 5.5—7.8; 0.1 M Tris-HCl buffer, pH 7.5—9.0; glycine-NaOH buffer, pH 8.6—11.6. Figure 9 shows the effect of pH on the enzyme activity.

**Effect of 2-Mercaptoethanol:** The enzyme system (5 ml, 3.0 μg protein) was dialysed for 4 days at 5 °C against (i) Tris-HCl buffer (pH 8.5) containing 10 mM 2-mercaptoethanol and (ii) Tris-HCl buffer (pH 8.5) lacking 2-mercaptoethanol. A part of each enzyme system was pipetted out at a regular time interval and the enzyme activity in the oxidation of ethanol was determined in the same manner as above. Figure 10 shows the effect of 2-

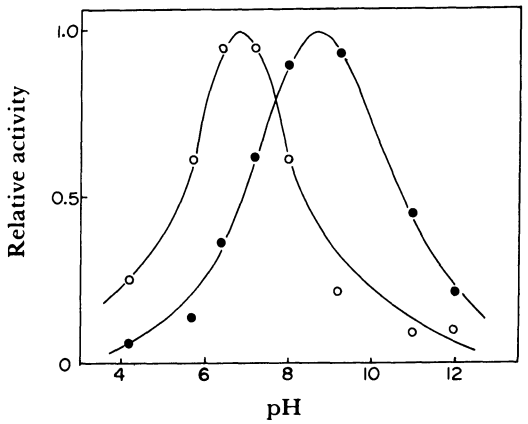


Fig. 9. Effect of pH on the activity of the enzyme system partialy purified from the cultured cells of *N. tabacum*. —○—; NADH-dependent reaction, —●—; NAD-dependent reaction.

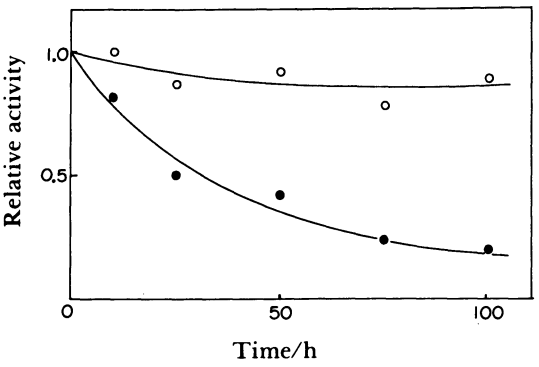


Fig. 10. Effect of dialysis on the activity of the enzyme system partially purified from the cultured cells of *N. tabacum*. —○—; Containing 10 mM 2-mercapto-ethanol, —●—; Lacking 2-mercaptoethanol.

mercaptoethanol on the enzyme activity.

**Oxidoreduction between Cycloalkanols and the Corresponding Cycloalkanones with the Enzyme System.** The initial rates of the oxidoreduction were determined by measuring the change in the UV absorption at 340 nm for NADH and by calculating on the least square method with the Lineweaver-Burk's equation.[16]

**Oxidation:** To a solution of cycloalkanol (0.01—4.0 mM) and $NAD^+$ (0.5 mM) in 0.1 M glycine-NaOH buffer (3 ml, pH 9.0), the enzyme system (0.6 μg protein) was added. The mixture was incubated at 25 °C for 2 h. During the incubation, the reaction was monitored by means of the UV absorption measurements. Table 2 gives the rate constants of the oxidation of the cycloalkanols.

**Reduction:** To a solution of cycloalkanone (0.5—10 mM) and NADH (0.2 mM) in 0.1 M potassium phosphate buffer (3 ml, pH 7.0), the enzyme system (0.6 μg protein) was added. The incubation was carried out at 25 °C for 2 h under monitoring by the UV spectrometry as above. The rate constants of the reduction of the cycloalkanones are given in Table 2.

**Time Courses in the Bioconversion of the Cycloalkanols in the Cultured Cells of *N. tabacum*.** A suspension of the cultured cells of *N. tabacum* "Bright Yellow" was prepared as described in Refs. 2 and 17; the cells were cultured in a 300-ml conical flask containing 100 ml of Murashige and Skoog's medium[18] in each case. Each substrate (10 mg) was administered to the flask containing the precultured suspension cells (50 g fresh wt/100 ml Murashing-Skoog's medium). The culture was incubated at 25 °C for 10 days in the dark with shaking at 70 rpm. A part (10 ml) of the incubated mixture was pipetted out under sterile conditions at a regular time interval, ground in a mortar, and extracted with ether. Products in each ether extract were identified, after removal of the solvent, by GLC and co-GLC with authentic samples. Amounts of the products and the unchanged substrate were determined on the basis of the peak areas on GLC and are expressed as the relative percentage for the total amount of a whole reaction mixture obtained.

This work was in part supported by Grant-in-Aids for Developmental Scientific Research Nos. 57840030 (1982) and 59840013 (1984) from the Ministry of Education, Science and Culture.

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