TABLE III: Molecular Weight, Number of Polypeptide Chains, Iron and Acid-Labile Sulfides of Azoferredoxin.

| | |
|---|---|
| Molecular weight | 55,000 |
| Polypeptide chains | 2 (27,500 each) |
| Fe atoms/molecule (dimer) | 4.05[a] |
| $S^{2-}$ atoms/molecule (dimer) | 4.0[b] |

[a] A sample of azoferredoxin containing 1.5 mg/ml of protein (27.8 mμmoles/ml, mol wt 55,000) was found to have 112.5 mμmoles of iron per ml. [b] A sample of azoferredoxin (850 mμmoles/ml) contained 3400 mμmoles/ml of acid-labile sulfides when compared to ferredoxin (7 sulfide groups per molecule) as a standard.

amino acids (K. M. Dus and L. E. Mortenson, unpublished) and cyanogen bromide peptides (J. Chen and L. E. Mortenson, unpublished). Therefore, it is likely that the monomers are also chemically identical.

The iron and sulfide of azoferredoxin are removed by sodium dodecyl sulfate treatment followed by dialysis (Table II). Whether in sodium dodecyl sulfate azoferredoxin dissociates into monomers because of the removal of the iron and sulfide or whether the iron and sulfide are released for the same reason the monomers dissociate is not yet known. We are presently trying to establish whether azoferredoxin can dissociate into monomers that still contain iron and sulfide.

The number of iron atoms and sulfide groups per azoferredoxin dimer (mol wt 55,000) is four each (Table III). If the subunits are chemically identical each subunit would contain two iron atoms and two sulfide groups.

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# Effect of Alcohols on the Rate of Autoxidation of Ferrocytochrome *c**

Laurence S. Kaminsky, Rodney L. Wright, and Allan J. Davison†

ABSTRACT: The effects of a homologous series of alcohols on the rate of autoxidation of ferrocytochrome *c* was studied in aqueous solution at pH 5 with the following results. The reaction was invariably first order with respect to ferrocytochrome *c*. Low concentrations of alcohol decreased the first-order rate constant, while higher concentrations produced a marked rate enhancement. Increasing the hydrocarbon chain length of the alcohols increased their effectiveness in enhancing autoxidation, while branching in the chain decreased their effectiveness. The order of effectiveness of the various alcohols in enhancing autoxidation was the same as their order of effectiveness in producing a conformational change exposing the heme of ferricytochrome *c*, suggesting that it is their ability to produce a similar conformational change in ferrocytochrome *c* which produces the enhanced reactivity toward oxygen. Greater concentrations of alcohol are required to produce an effect on ferrocytochrome *c*, no doubt because its native conformation is more stable. At higher temperatures, autoxidation was initiated by lower alcohol concentrations. The Arrhenius activation energy was calculated for autoxidation at high and at low alcohol concentrations and was found to be increased from 15 kcal/mole to 48 kcal/mole by increasing solute concentration sufficiently to induce rapid autoxidation. Evidently the autoxidation becomes permissible because the entropy of activation of some rate-determining process becomes more favorable to the extent of over 100 eu. This may reflect the fact that in alcohol mixtures exposure of hydrophobic groups in the heme crevice is less opposed by entropy considerations than in pure aqueous solution.

Knowledge of the various factors contributing to the stability of proteins is important for the determination of their structures in solution. In earlier papers we described the investigation of the effects of a series of organic solvents on ferricytochrome *c* (Kaminsky and Davison, 1969a,b). These studies provided qualitative evidence for a conforma-

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tional change involving the rupture of hydrophobic, hydrogen, and ionic bonds.

The conformations of the oxidized and reduced forms of cytochrome *c* show marked differences. Ferrocytochrome *c* is more stable to thermally induced conformational changes (Butt and Keilin, 1962), undergoes 9–10% less hydrogen–deuterium exchange (Ulmer and Kägi, 1968) and shows greater resistance to proteolytic digestion (Nozaki *et al.*, 1957, 1958) than does ferricytochrome *c*. High concentrations of ethylene glycol, moreover, alter the conformation of ferricytochrome *c*, but not that of the reduced form of the protein (Stellwagen, 1967). It seems pertinent to compare the conformational stability and reactivity of reduced and oxidized cytochrome *c* in the hydrophobic environment of organic solvents, in view of the presumed hydrophobic nature of the mitochondrial membrane where the protein normally functions.

The heme of cytochrome *c* is buried in the interior of the protein (Theorell and Åkesson, 1941; Stellwagen, 1967) and this is a major factor in the resistance of ferrocytochrome *c* to autoxidation at physiological pH (Butt and Keilin, 1962).

Conformational changes which render the heme more accessible to the oxygen of the solution enhance the susceptibility of ferrocytochrome *c* to autoxidation. Enhancement of autoxidation by added organic solvent is accordingly interpreted as indicating such conformational changes. In this paper the effects of five alcohols on the rates of autoxidation of ferrocytochrome *c* are reported. Thermodynamic parameters for the resultant conformational changes are calculated and the results compared to our previous results on ferricytochrome *c*.

## Experimental Section

*Materials.* Horse heart cytochrome *c*, grade I, 95% purity, was obtained from Seravac Laboratories, Maidenhead, England (batch 418).

Alcohols were British Drug Houses Analar grade and were used without further purification. Water was redistilled and deionized.

*Methods.* Ferricytochrome *c* was reduced by a modified technique of Yonetani and Ray (1965). Cytochrome *c*, 110 mg, was dissolved in 2 ml of 0.1 M sodium acetate buffer, pH 6.0, saturated with nitrogen. The minimum amount of sodium dithionite sufficient to reduce the cytochrome *c* was added to the solution. The reduced cytochrome *c* was purified by passing through a column (0.9 × 30 cm) of Sephadex G-25 previously equilibrated with the same nitrogen-saturated buffer. The concentration of cytochrome *c* was determined spectrophotometrically using extinction coefficients of Margoliash and Frohwirt (1959).

All solvent mixtures were made up in 50-ml volumetric flasks as follows: 10 ml of sodium acetate buffer, pH 5, to give a final concentration of 0.1 M and ionic strength 0.1, organic solvents and deionized water in varying ratios to give organic solvent contributions of 0–60% (v/v) in steps of 5 and 10%. The solutions were well mixed and allowed to reach thermal equilibrium overnight. The solutions were then made up to the 50-ml mark with a recorded volume of water to compensate for the volume shrinkage due to solvent–water interactions. The pH values of all the solutions, which had shifted due to the effect of the organic solvent on the pH of the buffer salts, were adjusted by the addition of glacial acetic acid back to a reading of pH 5 using a combination glass–calomel electrode. The concept of pH in organic solvents is complex, and this method was chosen to obtain an arbitrary reference point giving roughly comparable conditions of acidity in the various alcohols. In view of the satisfactory behavior of glass electrodes in quite high concentrations of alcohols (Bates, 1964), it is, in any event, likely that the pH value given is fairly accurate.

Ferrocytochrome *c* was added to 1.67 ml of each of the above solutions in the cuvet to give a final concentration of 35 μM cytochrome *c*.

The rates of autoxidation of ferrocytochrome *c* were determined by following the disappearance of the ferrocytochrome *c* by the change of absorbance at 550 mμ using a Beckman DU2 spectrophotometer fitted with a temperature-controlled cell compartment and Beckman 10-in. logarithmic recorder. Oxidation was initiated by the addition of the ferrocytochrome *c* to the solvent mixture in the cuvet. The pH of each reaction mixture was determined in the cuvet with a Radiometer 22 pH meter and temperatures with a Yellow Springs Instrument Company Tele-Thermometer, readings being made with the probe inserted into the cuvet in the cell compartment. Fully oxidized readings for the slower reactions were determined by adding very small quantities of potassium ferricyanide to the reaction mixtures in the cuvet and determining the absorbance of the resultant solution.

For the reactions containing ascorbic acid the solid ascorbic acid was added to the organic solvent mixture to a concentration of 0.01 M just prior to initiation of the reaction.

The orders of the faster reactions were determined, using the differential plot method (Laidler, 1965) by plotting log $(\Delta A/\Delta t)$ against log $(A - A_{ox})$, where $A$ is the absorbance at time, $t$ (the midpoint of the time interval $\Delta t$), $\Delta A$ is the absorbance change in this time interval, and $A_{ox}$ is the absorbance after complete oxidation at infinite time or after addition of a trace of potassium ferricyanide. The first-order rate constants were determined by linear least-squares regression through a plot of log $(A - A_{ox})$ against $t$.

The logarithm of the first-order rate constant was plotted as a function of mole per cent solvent (the mole fraction of organic solvent expressed as a percentage). Activation energies were determined from Arrhenius plots.

## Results

Addition of the alcohols, methanol, ethanol, and 2-propanol to buffered solutions of ferrocytochrome *c* at pH 5 resulted in retardation of autoxidation at low levels of alcohol. This effect was not observed with 1-propanol and *tert*-butyl alcohol. As the alcohol content was raised to higher levels the rate of autoxidation suddenly increased markedly until a level was reached at which autoxidation was too rapid to be followed with the standard spectrophotometer. The mole per cent of an alcohol required to initiate rapid autoxidation was a function of the chain length of the alcohol in the series methanol, ethanol, and propanol. The longer the chain the less alcohol needed to effect autoxidation (Figure 1). A greater mole per cent of 2-propanol was however required to initiate rapid autoxidation than in the case of 1-propanol and approximately the same quantity of *tert*-butyl alcohol was required to initiate rapid autoxidation (Figure 1). The mole per cents of alcohols required for acceleration of autoxidation to 100 times the rate of 0 mole % alcohol at 25° were: methanol 28%, ethanol 17%, 1-propanol 7%, 2-propanol 10%, and *tert*-butyl alcohol 9%.

At elevated temperatures the concentration of any particular alcohol required to produce rapid autoxidation of ferrocytochrome *c* was decreased. Thus, in the case of ethanol, for

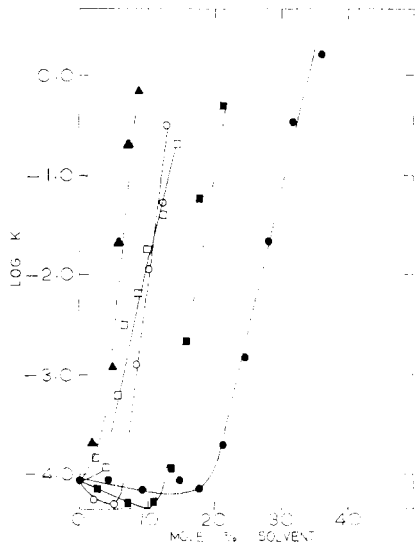

FIGURE 1: Effect of alcohols on the rate of autoxidation of ferrocytochrome *c*. The logarithm of the first-order rate constant is plotted as a function of alcohol concentration at 25°: (●), methanol; (■), ethanol; (▲) 1-propanol; (○), 2-propanol; (□) *tert*-butyl alcohol. Final concentration of cytochrome *c* in cuvet was 35 μM. Final concentration of acetate buffer was 0.1 M, pH 5.

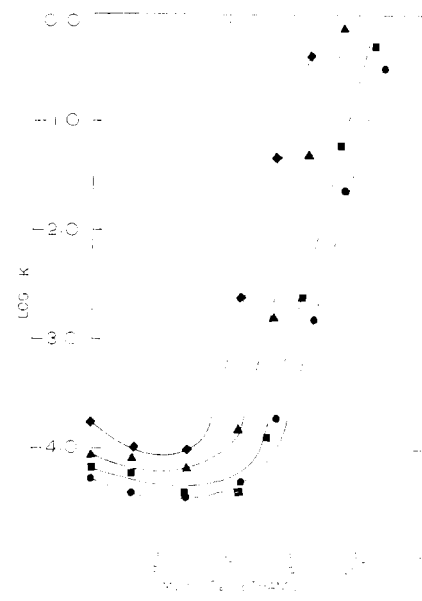

FIGURE 3: Effect of ethanol on the rate of autoxidation of ferrocytochrome *c*. The logarithm of the first-order rate constant is plotted as a function of ethanol concentration: (●) at 20°; (■) at 25°; (▲) at 30°; (◆) at 35°. Final concentration of cytochrome *c* in cuvet was 35 μM. Final concentration of acetate buffer was 0.1 M, pH 5.

example, at 20° autoxidation was initiated at 13 mole % ethanol, at 25° at 12 mole %, at 30° at 10 mole %, and at 35° at 9 mole %. The effects of temperature on the alcohol induced autoxidation rates of ferrocytochrome *c* are shown in Figures 2, 3, 4, 5, and 6.

Plots of log $(\Delta A/\Delta t)$ against log $(A - A_{ox})$ for the reactions had slopes equal to one within experimental error. No systematic deviations were observed and the reactions are thus accurately first order throughout their observable range. The logarithms of the first-order rate constants of the above reactions were plotted against $1/T$ over the temperature range of 20–35°, both at a low alcohol mole per cent (corresponding to the region of retardation of autoxidation) and at a high alcohol mole per cent (corresponding to a region of moderately rapid autoxidation) linear relationships were obtained (Figure 7). The slopes of these plots are given in Table I. These slopes were used to calculate the Arrhenius activation energies at the two alcohol concentrations. Assuming that the first step of the denaturation process is rate determining or at

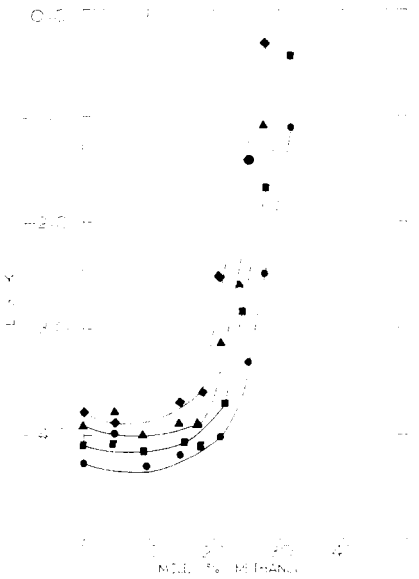

FIGURE 2: Effect of methanol on the rate of autoxidation of ferrocytochrome *c*. The logarithm of the first-order rate constant is plotted as a function of methanol concentration: (●) at 20°; (■) at 25°; (▲) at 30°; (◆) at 35°. Final concentration of cytochrome *c* in cuvet was 35 μM. Final concentration of acetate buffer was 0.1 M, pH 5.

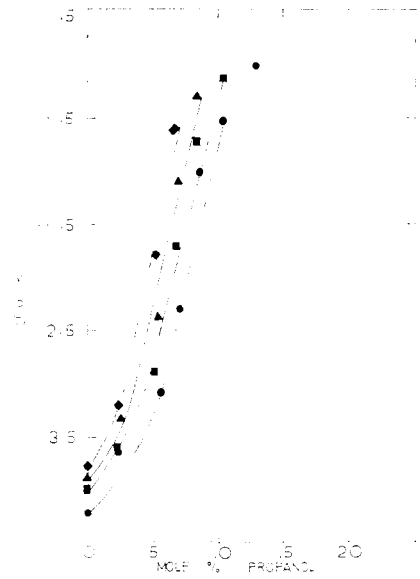

FIGURE 4: Effect of 1-propanol on the rate of autoxidation of fertocytochrome *c*. The logarithm of the first-order rate constant is plotted as a function of 1-propanol concentration: (●), at 20°; (■) at 25°; (▲) at 30°; (◆) at 35°. Final concentration of cytochrome *c* in cuvet was 35 μM. Final concentration of acetate buffer was 0.1 M, pH 5.

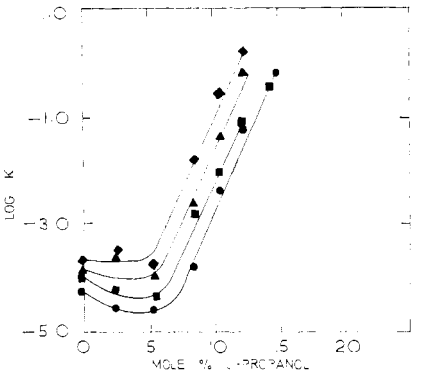


FIGURE 5: Effect of 2-propanol on the rate of autoxidation of ferrocytochrome *c*. The logarithm of the first-order rate constant is plotted as a function of 2-propanol concentration: (●) at 20°; (■), at 25°; (▲) at 30°; (◆) at 35°. Final concentration of cytochrome *c* in cuvet was 35 μM. Final concentration of acetate buffer was 0.1 M, pH 5.

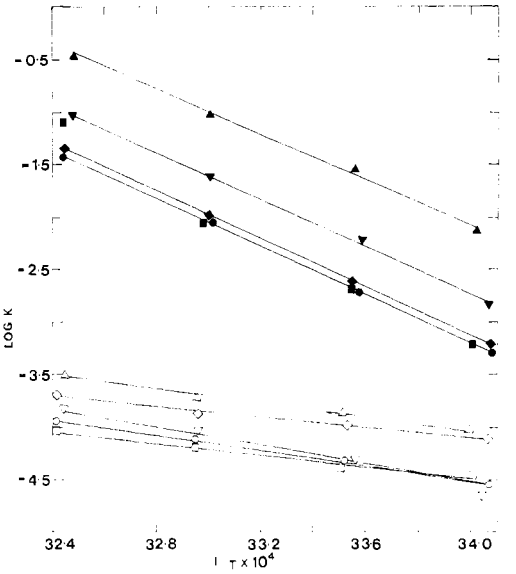


FIGURE 7: Effect of temperature on the first-order rate constants for the alcohol-induced autoxidation of cytochrome *c* at low alcohol concentrations (○□△▽◇) and high concentrations (●■▲▼◆). The logarithms of the first-order rate constants are plotted as a function of the inverse of the absolute temperature: (○●) methanol; (□■), ethanol; (△▲), 1-propanol; (▽▼) 2-propanol; (◆◇) *tert*-butyl alcohol. Final concentration of cytochrome *c* in cuvet was 35 μM. Final concentration of acetate buffer was 0.1 M, pH 5.

least that the reactants are in equilibrium or pseudoequilibrium with the transition state of the rate-determining step, activation enthalpy, entropy, and free-energy changes can be calculated. Similar calculations have been carried out in analogous systems and a comparison of the results is of interest. These calculations were carried out at alcohol concentrations, causing both slowing and acceleration of autoxidation. The energies of activation, at high concentrations of all the alcohols, were constant at 48 kcal/mole. At 25° this corresponds to $\Delta H^{\ddagger} = 47.4$ kcal/mole, $\Delta S^{\ddagger} = 88$ cal/deg mole, and $\Delta G^{\ddagger} = 21.1$ kcal/mole. At low alcohol concentrations the mean energy of activation was 15 kcal/mole, which corresponds to $\Delta H^{\ddagger} = 14.4$ kcal/mole, $\Delta S^{\ddagger} = -28.6$ cal/deg mole and $\Delta G^{\ddagger} = 22.9$ kcal/mole.

When ascorbic acid was added to the cuvet to a final concentration of 0.01 M at zero and low levels of alcohol, the cytochrome *c* was entirely in the reduced form in the steady state. When the concentration of alcohol was sufficiently increased, a fully oxidized steady state for the cytochrome *c* was rapidly approached.

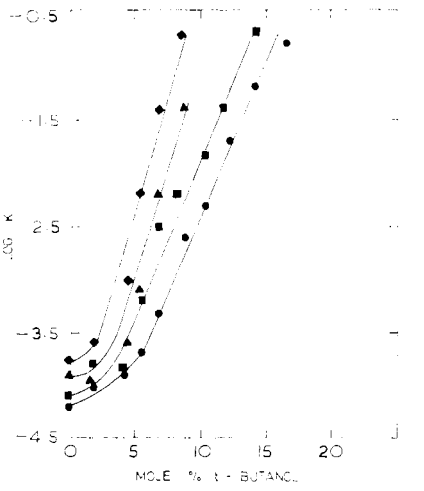


FIGURE 6: Effect of *tert*-butyl alcohol on the rate of autoxidation of ferrocytochrome *c*. The logarithm of the first-order rate constant is plotted as a function of *tert*-butyl alcohol concentration: (●) at 20°; (■) at 25°; (▲), at 30°; (◆) at 35°. Final concentration of cytochrome *c* in cuvet was 35 μM. Final concentration of acetate buffer was 0.1 M, pH 5.

## Discussion

The fact that low concentrations of three of the five alcohols used in this study retarded the rate of autoxidation of cytochrome *c*, while higher concentrations produced a marked acceleration of autoxidation, indicates the existence of at least two modes of cytochrome *c*–alcohol interaction.

The decreased reactivity toward oxygen in the presence of low alcohol concentrations might result either from the known creation of charge in the reduction of oxygen by ferrocytochrome *c* (Davison, 1970) or from tighter closing of the heme crevice due to effects on the conformation of the protein, while the increased accessibility of the heme to oxygen at high alcohol concentrations indicates a conformational change involving opening of the heme crevice.

The fact that effectiveness in enhancing autoxidation

TABLE I: Comparison of Arrhenius Activation Energies for Various Alcohols.

| Alcohol | $E_A$ at Low Alcohol Concentration (kcal/mole) | $E_A$ at High Alcohol Concentration (kcal/mole) |
|---|---|---|
| Methanol | 17.5 | 48.5 |
| Ethanol | 12.5 | 48.5 |
| 1-Propanol | 14.6 | 46.7 |
| 2-Propanol | 20.0 | 48.9 |
| *tert*-Butyl alcohol | 11.0 | 48.5 |

increases with increasing length of the hydrocarbon chain in the series methanol, ethanol, and 1-propanol indicates that, as in the case of ferricytochrome *c*, the heme crevice of ferrocytochrome *c* is more effectively opened by more hydrophobic agents. This may reflect the importance of hydrophobic interactions between amino acids in the crevice and the surface of the heme.

It is known moreover that ethanol is capable of causing helix formation in nonhelical proteins because of enhanced hydrogen bond interactions (Weber and Tanford, 1959; Tanford and De, 1961), and the absence of $\alpha$-helical structure in cytochrome *c* (Dickerson *et al.*, 1967), makes it likely that marked changes would occur if helix formation was induced.

The branched-chain alcohols 2-propanol and *tert*-butyl alcohol do not follow the trend of the straight-chain alcohols, with respect to carbon number, being less effective in initiating rapid autoxidation of cytochrome *c*. A similar trend was observed in the effect of alcohols on the Soret peak of ferricytochrome *c* (Kaminsky and Davison, 1969). Evidently steric factors such as accessibility of the interior of the protein molecule to the solvent are important.

The Arrhenius activation energies calculated in the presence of a fixed amount of alcohol sufficient to produce autoxidation at a rate 100 times greater than the control, were independent of the type of alcohol. This suggests that the energy of activation is a function of the induced conformational change of the protein rather than of the alcohol involved. The energies of activation at the lower alcohol concentrations show some variation which, because of the extreme slowness of the reactions, is within experimental error. In view of the constancy of activation energy values at high alcohol concentrations, an average activation energy has been used for all the alcohols at lower concentrations.

Thermodynamic values at both high and low alcohol concentrations were calculated from the activation energies using absolute reaction rate theory. The standard free energy of activation at the higher alcohol concentrations lies within the range of values (22 $\pm$ 5 kcal/mole), which has been found for the denaturation of a wide variety of proteins under many conditions (Joly, 1965). The positive standard entropy of activation is also of the magnitude expected for a protein denaturation. The estimate of the entropy of activation of $-29$ eu can be compared with the value of $-48$ eu for the overall entropy change of crevice opening found from the stability constant of the crevice closed form of ferricytochrome *c* (George *et al.*, 1967).

In earlier studies on ferricytochrome *c* (Kaminsky and Davison, 1969) we examined the effects of organic solvents on the Soret absorbance and related this to conformational changes of the protein. The agreement of the trends produced by the various alcohols on rates of autoxidation, with these effects on spectra, support the view that the changes in autoxidation rate reflect conformational changes of ferrocytochrome *c*. The degree of unfolding of the polypeptide chain, necessary for autoxidation cannot be estimated however, since the environment of the heme may apparently be partially destroyed prior to general unfolding of the native protein (Stellwagen, 1968). The molar concentrations of alcohol required to produce half the maximum effect in the present study were always larger than the corresponding values in the ferricytochrome *c*. This suggests that the ferrocytochrome *c* molecule is more resistant to alcohol-induced conformational changes than is ferricytochrome *c*. These findings provide further evidence that the reduced molecule of cytochrome *c* is more stable than the oxidized form (Butt and Keilin, 1962; Nozaki *et al.*, 1957, 1958). No quantitative comparison is possible as the methods of probing the conformational changes of the two forms differ so greatly.

Skov and Williams (1967) have shown that the ascorbate reduction of cytochrome *c* is conformation dependent. The altered conformation of the cytochrome *c* in high concentrations of alcohol may thus partly explain the inability of ascorbate to effect reduction of cytochrome *c* in the steady state. Enhanced autoxidation under these conditions undoubtedly contributes also.

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