# Effects of Alcohols on the Autoxidation of Cytochrome $c_1$†

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ABSTRACT: Methanol, ethanol, propanols, and butanol increased the autoxidation of cytochrome $c_1$ measured spectrophotometrically. The effectiveness of alcohol was found to be dependent upon the length of the carbon chain of the alcohol. From the thermodynamic constants for the activation of the autoxidation and other evidence, it is concluded that the alcohol reaction leads to the denaturation of the cytochrome. Denatured cytochrome $c_1$ was not reducible by ascorbate and showed distinctly different absorption spectra from the native cytochrome. The multiple spectral splittings of reduced cytochrome $c_1$ at liquid nitrogen temperatures were abolished by alcohol.

In mitochondria, cytochrome *c* accepts electrons from cytochrome $c_1$ of reductase and transfers them to cytochrome *a* of cytochrome oxidase and eventually to molecular oxygen. This sequential transfer of electrons is the basis of the concept of the respiratory chain first formulated by Keilin (1966). Since the initial report on the existence of cytochrome *c*, numerous techniques have been used in an attempt to elucidate the mechanisms of the actions of this respiratory component during the last half century (*cf.* Keilin, 1966; Margoliash and Schejter, 1966; Kamen *et al.*, 1971). Kaminsky and coworkers (Kaminsky and Davison, 1969a,b; Kaminsky *et al.*, 1971, 1972) have taken an approach in studying cytochrome *c* by examination of its reaction with various organic solvents, especially ethers and alcohols, hoping to gain some insight into the mode of its enzymic action. However, all these investigations, including the alcohol studies, did not elucidate the mechanisms of electron transport of cytochrome *c* even after the three-dimensional structures of both oxidized and reduced cytochromes *c* have been elegantly worked out (Dickerson *et al.*, 1971; Takano *et al.*, 1973). Indeed, the mechanisms are still at the stage of speculation without a general agreement (for example, Takano *et al.*, 1973; Salemme *et al.*, 1973).[1] Evidently this is due partly to the dearth of knowledge about the natural electron donor (cytochrome $c_1$) and acceptor (cytochrome oxidase) for cytochrome *c*. These considerations make the studies of cytochrome $c_1$ by every conceivable technique more pressing. But, unfortunately, in contrast to the ready supply of cytochrome *c* in practically kilogram quantities, even a reproducible preparation of cytochrome $c_1$, at least in our hands, has not been a reality until recently (Yu *et al.*, 1972a). With the availability of purified cytochrome $c_1$ we have recently examined the effects of alcohols on the autoxidation of this cytochrome.

## Experimental Section

*Materials.* Cytochrome $c_1$ at the purity level of 25 nmol/mg of protein was prepared from bovine heart as described previously (Yu *et al.*, 1972a). Water used was treated by ion exchangers and doubly redistilled. Alcohols were obtained from Fisher Co. and all other chemicals at the highest available purity from various commercial sources.

*Methods.* Cytochrome $c_1$, as prepared, was in reduced form. The small amount of oxidized form present in the sample was reduced by a slight excess of sodium dithionite and the excess dithionite was removed by passage over a Sephadex G-50 column equilibrated with 50 mM phosphate buffer (pH 7.4) anaerobically. The concentration of cytochrome $c_1$ was determined spectrophotometrically with a millimolar extinction coefficient of 17.5 for $A_{red}(552.5) - A_{red}(540)$ (Yu *et al.*, 1972a). The spectrophotometric measurements were carried out in Cary spectrophotometers, Model 14 or 16, at approximately 23° unless otherwise specified. The low-temperature spectroscopy was done as previously reported (Yu *et al.*, 1972a).

For the determination of the rate of autoxidation of cytochrome $c_1$ induced by alcohol, generally 26 μM of ferrocytochrome $c_1$ in 50 mM phosphate buffer (pH 7.4) was mixed with an equal volume of an appropriate concentration of alcohol in a small tube. Both solutions were prior equilibrated at a desirable temperature. After mixing, the solution was transferred to a cuvet and the spectrum between 600 and 500 nm was recorded. The rate of autoxidation of ferrocytochrome $c_1$ was determined by following the absorbance decrease at 552.5 with 540 nm as a reference point.

Alcohol concentrations were presented in mole per cent which is mole fraction expressed as a percentage.

## Results

*Autoxidizability of Cytochrome* $c_1$. Cytochrome $c_1$ showed practically no autoxidation in the neutral pH region at 0°. When the pH of the solution was higher than 10 or lower than 5, the autoxidation rate became discernible and eventually the cytochrome denatured; *i.e.*, the cytochrome was no longer ascorbate reducible. In the neutral pH region, even at about 23°, the autoxidation rate was still low; only about 9% of ferrocyto-

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[1] The Dickerson mechanism of the reduction of cytochrome *c* is, in a way, actually an elaborate extension and great refinement of the general proposal originated by Winfield (1965) for electron transfer. However, other workers argue against this mechanism because of (1) the difficulty of imposing one more electron on a tyrosine residue (see, for example, Castro (1971) and references cited therein; and personal communication with Dr. C. E. Castro, who described to us his numerous unpublished results); (2) the recent discovery of the replacement of tyrosine-74, which was considered as an invariable amino acid residue, by phenylalanine in cytochrome *c* isolated from a fungus (Morgan *et al.*, 1972) and a protozoan (Pettigrew, 1972, 1973); and (3) the extensive comparison of cytochromes *c* and $c_2$ from all structural and functional facets (Salemme *et al.*, 1973). These as well as other (*e.g.*, Yandell *et al.*, 1973; Hodges *et al.*, 1974) workers have proposed the electron transport involving cytochrome *c* by direct transfer to the heme iron or by the so-called peripheral attack through the edge of the porphoryrin to the iron. However, it is the consensus of opinion that the electron-transfer mechanism of cytochrome *c* is far from clear.

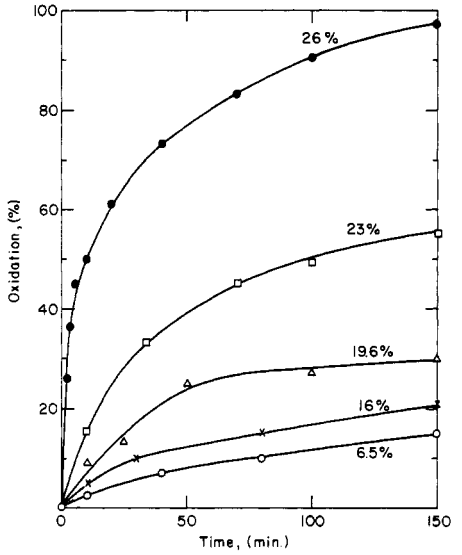


FIGURE 1: Effect of methanol on the autoxidation rate of cytochrome $c_1$. Cytochrome $c_1$, 13 $\mu$M, in 50 mM phosphate buffer (pH 7.4) was incubated with various concentrations of methanol at room temperature (23°). The autoxidation of the cytochrome was followed by measuring the absorption difference between 552.5 and 540 nm as a function of time. 100% oxidation was obtained by the addition of ferricyanide at the end of incubation.

chrome $c_1$ became oxidized in 2 hr at the concentration of approximately 13 $\mu$M. Under the same conditions, cytochrome $c$ showed a higher autoxidation rate than that of cytochrome $c_1$.

*Effect of Methanol on the Autoxidation Rate of Cytochrome $c_1$.* As shown in Figure 1, the autoxidation rate of cytochrome $c_1$ at 23° was higher in the presence of methanol than in its absence. The rate was approximately directly proportional to the concentration of methanol present in the system. When the concentration of methanol was increased to higher than 16%, the rate was greatly increased. Methanol at 27% caused complete oxidation of ferrocytochrome $c_1$ within 1 hr at 23°. Lowering the temperature decreased the rate of autoxidation.

*Effect of Ethanol on the Autoxidation Rate of Cytochrome $c_1$.* Figure 2 shows the effect of the concentration of ethanol on the autoxidation rate of cytochrome $c_1$. Similar to the case of

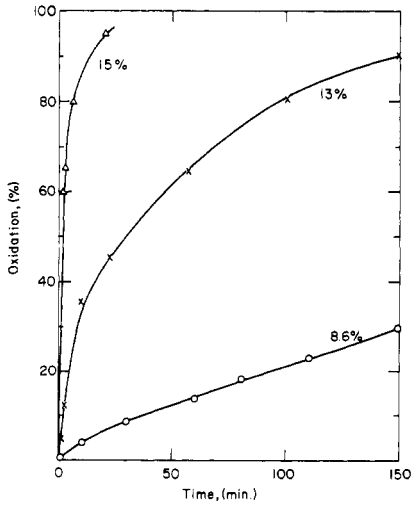


FIGURE 2: Effect of ethanol on the autoxidation rate of cytochrome $c_1$. The conditions were the same as those described in the legend to Figure 1.

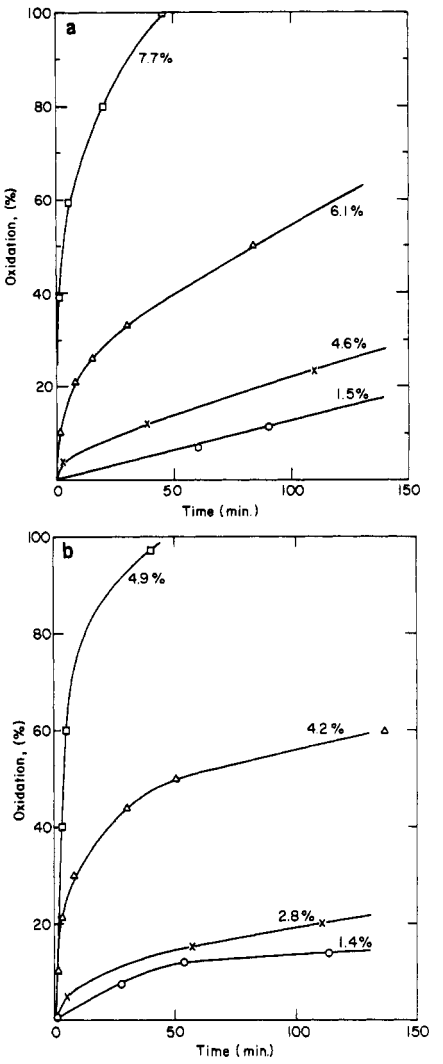


FIGURE 3: Effect of 2-propanol and 1-propanol on the autoxidation rate of cytochrome $c_1$: (a) 2-propanol, (b) 1-propanol. See the legend to Figure 1 for the conditions.

methanol, the autoxidation rate induced by ethanol exhibited two phases; a slower rate was found at an ethanol concentration lower than 7% and a faster rate at a concentration higher than 12%. Reaction of cytochrome $c_1$ with 14.4% ethanol at 23° for 10 min resulted in complete oxidation of the ferrocytochrome.

*Effect of Propanols on Autoxidation Rate of Cytochrome $c_1$.* 2-Propanol had less effect on the autoxidation of cytochrome $c_1$ than that of 1-propanol, as shown in Figure 3. The rate of autoxidation of cytochrome $c_1$ at 6.4% 1-propanol was the same as that caused by 7.4% 2-propanol. However, the general behavior of these two isomers toward the induction of autoxidation was quite similar to that of methanol or ethanol; that is, lower concentrations caused a slowly increased autoxidation rate, while higher concentrations (higher than 4% of 1-propanol or 5.6% of 2-propanol) resulted in a rapidly increased autoxidation.

*Effect of 1-Butanol on Autoxidation Rate of Cytochrome $c_1$.* Similar to other alcohols, incubation of ferrocytochrome $c_1$ with butanol showed rapid oxidation of the cytochrome, as summarized in Figure 4. Cytochrome $c_1$ could only tolerate low concentrations of butanol. When the butanol concentration was higher than 1.0%, a rapid oxidation of cytochrome $c_1$ was observed. Incubation of ferrocytochrome $c_1$ with 1.7% of butanol

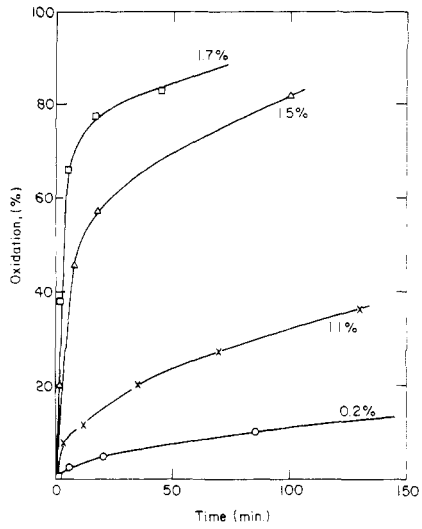


FIGURE 4: Effect of 1-butanol on the autoxidation rate of cytochrome $c_1$. See the legend to Figure 1 for the conditions.

for 20 min at room temperature resulted in more than 80% oxidation of cytochrome $c_1$. Prolonged incubation of cytochrome $c_1$ with butanol solution even at low concentrations invariably resulted in complete, irreversible denaturation of the cytochrome.

The reversible denaturation of cytochrome $c_1$ by the removal of alcohol was observed when the conditions, *viz.*, the concentration of an alcohol and the temperature used, caused not more than 25% of the autoxidation rate (*i.e.*, the ordinates of Figures 1-4). We found that increase of the autoxidation rate over 25% decreased the degree of the reversibility of alcohol denaturation up to finally complete irreversibility. This observation is in contrast to the case of cytochrome *c* whose autoxidation due to the reaction with an alcohol can be completely reverted back to the normal after the removal of the alcohol unless the latter concentrations are so high as to induce aggregations of the cytochrome (Kaminsky *et al.*, 1971).

*Effect of Temperature on Alcohol-Induced Autoxidation of Cytochrome* $c_1$. Alcohol-induced autoxidation rate of ferrocytochrome $c_1$ was found to be greatly dependent upon the temperature. For example, ferrocytochrome $c_1$ in 32.6% methanol at 15° gave an autoxidation rate of 0.35 $\mu$M per min at protein concentration of 0.5 mg/ml. At 23°, the rate increased more than 10 times or to 4 $\mu$M per min. Similar results were also found for other alcohols. The effects of temperature on autoxidation were much less drastic at lower concentrations of alcohols than at higher concentrations. Figure 5 summarizes the temperature effect on the autoxidation rate of various alcohols.

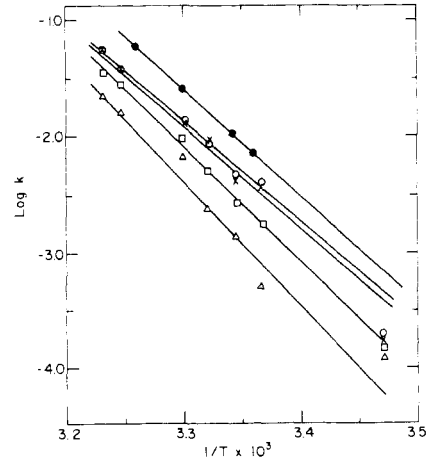


FIGURE 5: Effect of temperature on the first-order rate constant for the alcohol-induced autoxidation of cytochrome $c_1$. Final concentration of cytochrome $c_1$ was 20 $\mu$M in 50 mM phosphate buffer (pH 7.4). The alcohol concentrations were 32.6, 17.3, 9.2, 5.6, and 1.5% for methanol (○), ethanol (x), 2-propanol (△), 1-propanol (□), and 1-butanol (●), respectively.

TABLE I: Comparison of Arrhenius Activation Energies for Autoxidation of Cytochromes $c_1$ and *c* in Alcohol.

| Alcohol | Activation Energies | |
|---|---|---|
| | Cytochrome $c_1$ | Cytochrome *c*[a] |
| Methanol | 41 | 48.5 |
| Ethanol | 41 | 48.5 |
| 2-Propanol | 37 | 48.9 |
| 1-Propanol | 46 | 46.7 |
| 1-Butanol | 41 | |

[a] From Kaminsky and Davison (1969a).

The Arrhenius activation energy calculated from Figure 5 was 41 kcal/mol for methanol and ethanol, 47 kcal/mol for 2-propanol, and 46 kcal/mol for 1-propanol. Table I compares the activation energy for the autoxidation of cytochrome $c_1$ with that of cytochrome *c* as a function of the alcohol. In general, cytochrome $c_1$ is slightly more sensitive to temperature in alcohol for autoxidation than is cytochrome *c*. The other thermodynamic constants calculated from the absolute reaction rate theory for the autoxidation of cytochrome $c_1$ caused by alcohol are summarized in Table II. It may be noted that the range, among various alcohols, of the entropy changes in the activation is significantly larger than that of the corresponding free energy change for the autoxidation.

*Effect of Alcohol on Cytochrome* $c_1$ *Spectral Properties.* The ferricytochrome $c_1$ which was a product of the autoxidation of ferrocytochrome $c_1$ in the presence of an alcohol became no longer ascorbate reducible. It was, therefore, of interest to see whether alcohols affected the spectral properties of cytochrome $c_1$. Cytochrome $c_1$, 27 $\mu$M, was treated with 17.3% ethanol at 23° for about 10 min. The difference spectra of the reduced minus oxidized forms in the presence of ethanol were then taken. Sodium dithionite and ferricyanide were used as re-

TABLE II: Some Thermodynamic Values of Alcohol-Induced Autoxidation of Cytochrome $c_1$ at 25°.[a]

| Alcohol | $\Delta H^*$ (kcal/mol) | $\Delta F^*$ (kcal/mol) | $\Delta S^*$ (cal/deg) |
|---|---|---|---|
| Methanol, 32.6% | 41 | 20.6 | 68.5 |
| Ethanol, 17.3% | 41 | 20.7 | 68.2 |
| 2-Propanol, 9.2% | 47 | 21.5 | 85.5 |
| 1-Propanol, 5.6% | 46 | 21.0 | 84.0 |
| 1-Butanol, 1.5% | 41.3 | 19.7 | 72.5 |

[a] $\Delta H^*$ was obtained from Arrhenius plots. $\Delta F^*$ was calculated from first-order rate constants using the absolute reaction rate theory. $\Delta F^* = RT \ln (kT/hk')$, where $k$ was the Boltzmann constant; $h$, Planck constant; $k'$, first-order rate constant; and $R$, the universal gas constant.

ducing and oxidizing agents, respectively. Curves 1 and 2 of Figure 6 show the difference (reduced *minus* oxidized) spectra of the ethanol reacted and unreacted cytochrome $c_1$, respectively. Several features may be noted from these traces: in general, the absorption maxima became somewhat broadened and the absorbance decreased, and the distinct shoulder at 530 nm was practically abolished by ethanol action. Curve 3 of Figure 6 shows the "double difference spectra" of the alcohol untreated and treated cytochrome $c_1$. The spectrum was measured between the $[c_1^{2+}][c_1^{3+}$-alcohol] and $[c_1^{3+}][c_1^{2+}$-alcohol]. Each component was kept in a separate cuvet of 5-mm light path. The decrease in $\alpha$ absorption and change in $\beta$ absorption of the alcohol-reacted sample showed clearly in the spectrum.[2] Carbon monoxide readily reacted with the reduced form of the alcohol-treated cytochrome $c_1$ to a great extent. More than 90% of absorption in the visible ($\alpha$ and $\beta$) region was abolished from the difference spectrum of the dithionite reduced CO complex minus the oxidized form of the alcohol-treated sample.

Figure 7 shows the difference spectra of alcohol-treated and untreated ferrocytochrome $c_1$ at about $-160°$. The colorful spectral splittings characteristic of the native cytochrome were abolished by alcohol. In the presence of detergents very low concentrations of alcohol, *i.e.*, 0.3 or 0.6% ethanol, did not abolish the spectral splitting at low temperatures, but affected only the absorption intensities of some of the splittings in the $\alpha$ region. The $\alpha - 1$ intensity decreased, whereas the $\alpha - 2$ was relatively unchanged. When the purified cytochrome $c_1$ was made in a detergent-free solution, low concentrations of alcohol did not likewise affect the spectral splitting at low temperatures. Similar results were also observed for cytochrome $c_1$ in its particulate form, *i.e.*, in the succinate–cytochrome *c* reductase or in the soluble cytochrome $b$–$c_1$ complex (*cf.* Figure 3, Yu *et al.*, 1972b).

## Discussion

The results presented clearly show that autoxidation of cytochrome $c_1$ is increased by alcohol. Among the alcohols tested, the ability to enhance autoxidation is dependent upon the length of the carbon chain; the longer the chain the more effective the alcohol. An alcohol with a branched chain exhibits less effect than its corresponding straight chain isomer.

In general, in spite of the greatly divergent amino acid composition of these two cytochromes (unpublished results from this laboratory) the effect of alcohol on the autoxidation of cytochrome $c_1$ resembles the effect in cytochrome *c* (Kaminsky *et al.*, 1971). There is, however, a distinct difference; lower concentrations of alcohols do not retard autoxidation. In cytochrome *c*, as reported by Kaminsky *et al.* (Kaminsky and Davison, 1969a,b; Kaminsky *et al*, 1971), when the concentration of methanol, ethanol, and 2-propanol are lower than 20, 10, and 5%, respectively, the rate of autoxidation of cytochrome *c* is lower in the presence than in the absence of alcohol. One interpretation for such phenomenon was an increase in the helical content of the cytochrome by the formation of hydrogen bonds between the solvent and the protein molecules (Kaminsky *et al.*, 1971). A rather low content of the helical structure in cytochrome *c*, as determined by X-ray diffraction crystallography (Dickerson *et al.*, 1971; Takano *et al.*, 1973), makes this explanation quite likely. The helical content of cytochrome $c_1$, as predicted from the circular dichroism studies (Yu *et al.*, 1971), however, is much higher than that in cytochrome *c*. Thus, the contribution from the increase in the helix by hydrogen bonding between the solvent and protein should be of relatively minor importance in the case of cytochrome $c_1$ and no retarda-

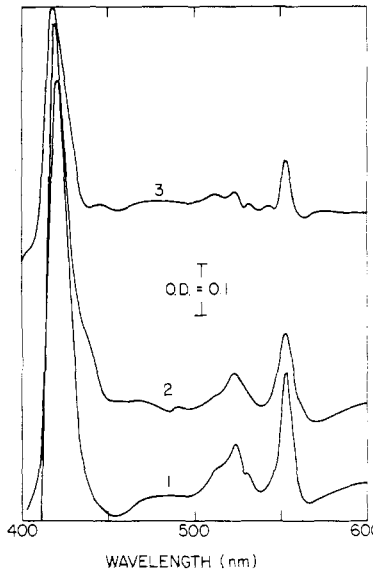


FIGURE 6: Effect of ethanol on the spectral properties of cytochrome $c_1$. Difference spectra of reduced and oxidized form cytochrome $c_1$: (curve 1) intact cytochrome $c_1$; (curve 2) 17.3% ethanol treated cytochrome $c_1$; (curve 3) difference spectra of 1 and 2. Cytochrome $c_1$ used, 27 $\mu$M; cuvet light path, 0.5 cm. The reduced form was effected by dithionite and the oxidized form by ferricyanide.

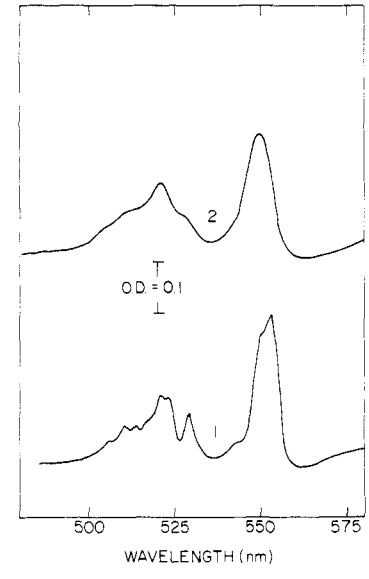


FIGURE 7: Effect of ethanol on the spectral properties of cytochrome $c_1$ at $-150°$. Cytochrome $c_1$ used was 27 $\mu$M, cuvet light path, 0.2 cm. (Curve 1) intact cytochrome $c_1$; (curve 2) 17.3% ethanol treated sample.

[2] In answering the comments of a referee, it should be pointed out that these changes in the spectral behavior, from the reaction of alcohol, in the $\alpha$ and $\beta$ regions, such as decreased absorbance at 552.5 nm, is not due to the autoxidation because the system in the reduced state contained excess dithionite. Likewise, they are not due to the "destruction" or loss of the heme by the reaction with an alcohol because the pyridine hemochromogen spectra of the system were found to be exactly the same, qualitatively and quantitatively, before and after the alcohol reaction. Consequently, the loss of iron is an impossible event. It is also not likely that mesoheme is dissociated from the protein moiety by alcohol treatment. This explanation is not based on *a priori* consideration that alcohol cannot cleave the heme-protein linkages of the covalent nature but on the observation of the absorption spectra which did not show the slightest indication of free mesoheme or of any free heme.

tion in autoxidation could thus be observed.

The activation energies calculated from the Arrhenius plots are in a very narrow range among the alcohols tested, 41–48 kcal/mol, which are comparable to the values for cytochrome *c* (Kaminsky *et al.*, 1971). It is known that the standard free energy change and the entropy change for the activation can be deduced directly from results of temperature studies by taking advantage of the absolute reaction rate theory. The values for the standard free energy change of the activation as shown in Table II are within the values for the denaturation of a wide variety of proteins under divergent conditions (Tanford, 1961; Joly, 1965). The relatively large positive entropy change as expected is also in the magnitude of the reported value for cytochrome *c*. At any rate, all these thermodynamic data suggest that the induced autoxidation by alcohols is probably by a process which directly causes protein denaturation or induces a conformational change of the protein leading eventually to denaturation. That the solubility in aqueous media and the reducibility toward ascorbate for the alcohol-treated cytochrome $c_1$ are greatly reduced supports the theory of denaturation.

More specifically, in the native state, these data as well as others to be reported elsewhere would suggest the heme in cytochrome $c_1$ is as equally, if not more so, deeply buried in protein millieu as is the heme in cytochrome *c*. Alcohol evidently alters the relative configuration between the prosthetic group and the protein moiety of the cytochrome and the denaturation must also involve the exposure of the heme crevice of cytochrome $c_1$. The latter process likely precedes the gross alteration of the conformation of the cytochrome and consequently its solubility, spectra, and ascorbate-reducibility change. If the heme of cytochrome $c_1$ is indeed deeply buried in the crevice, then one must think how proximate the heme is to cytochrome *c* for the purpose of the donation of electrons and, equally important, to cytochrome *b* for the acceptance of electrons. Whether similar to the "limited semiconductor hypothesis" of Dickerson and coworkers (Dickerson *et al.*, 1972; Takano *et al.*, 1973) for the electron transfer of cytochrome *c* through tyrosine-74 → tryptophane-59 → tyrosine-67 → porphyrin → iron on one hand or more or less direct transfer (*e.g.*, Salemme *et al.*, 1973) between heme groups of $c_1$ and *c* on the other, we fear, cannot be definitely answered, but the readers may conjecture. In our opinion, our results of the effect of alcohol on cytochrome $c_1$ may give a slight weight to the Dickerson–Winfield mechanism.[1]

Finally it is clear for the practitioner that any effort would be futile to rescue residue rich in cytochromes from submitochrondrial particles after the solubilization of succinate dehydrogenase by butanol methods (King, 1967). These cytochromes, at least cytochrome $c_1$ as shown in this paper, have opened heme crevices and then also denatured to various degrees.

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