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A PEROXIDE-DEPENDENT REDUCTION OF CYTOCHROME *c* BY NADH

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SUMMARY

(a) Ethyl hydrogen peroxide, fatty acid peroxides, hydrogen peroxide or *p*-dioxane peroxide caused the reduction of ferricytochrome *c* by NADH. Benzoyl peroxide was much less effective in causing this reduction.

(b) O_2 had no effect on the rates of oxidation of NADH or reduction of cytochrome *c*; but the presence of O_2 did change the mechanism of the reaction. This was demonstrated through the use of superoxide dismutase which inhibited the reduction of cytochrome *c* in the presence of O_2 but had no effect in the absence of O_2.

(c) Ethyl hydrogen peroxide was converted to acetaldehyde in the course of this reaction. Indeed, ethyl hydrogen peroxide was converted to acetaldehyde by reaction with ferricytochrome *c*, methemoglobin or hemin but not with ferrocytochrome *c* or with ferricyanide.

(d) A mechanism has been proposed which satisfactorily accounts for these observations.

INTRODUCTION

Superoxide dismutase[1] has proven itself useful in analyzing complex reactions for the involvement of oxygen radicals[2–11]. The aerobic oxidations of NADH and NADPH by peroxidases from horse radish[12–15] and from uterine[16–17] tissues, both in the presence of Mn^{2+} *plus* phenols[12–17] and in their absence[18], are complex reactions for which free-radical mechanisms have been proposed[11,12,17]. This peroxidase–oxidase reaction has been the subject of numerous studies[19] and the mechanisms which have been proposed usually involve the production of radicals of the substrates by univalent oxidation, followed by reactions of these substrate radicals with oxygen[18,20].

We have observed a reaction involving NADH, ferricytochrome *c* and ethyl hydrogen peroxide, in which NADH was oxidized, cytochrome *c* was reduced and acetaldehyde accumulated. In the presence of O_2, this reduction of cytochrome *c* was inhibited by superoxide dismutase whereas, under anaerobic conditions, the reduction of cytochrome *c* was insensitive to this enzyme. It appeared worthwhile to investigate the mechanism of this novel reaction both for its intrinsic interest and for the light it might shed on the mechanism of the peroxidase–oxidase reactions. The results of these studies and a mechanism, which is consistent with these results, form the body of this report.

MATERIALS AND METHODS

Superoxide dismutase was prepared from bovine erythrocytes and was assayed as previously described[1]. Cytochrome *c*, type III, was obtained from Sigma and NADH from P–L Biochemical Corp. Ethyl hydrogen peroxide was purchased from Ferrosan Malme, Sweden through the Gallard-Schlesinger Chem. Mfg. Corp. Linoleic acid was obtained from the Hormel Institute Lipids Preparation Laboratory, Austin, Minn. H_2O_2 was purchased from J. T. Baker and benzoyl peroxide from Matheson, Coleman and Bell. Ethyl hydrogen peroxide was quantitated on the basis of $\varepsilon_{230\,nm}=43\ M^{-1}\cdot cm^{-1}$ (ref. 21). *p*-Dioxane obtained from Eastman Organic Chemicals was freed of peroxides by treatment with metallic sodium or by filtration over basic-activated alumina as described in technical bulletin No. 1 prepared by N. Woelm, 344 Eschwege, West Germany. Woelm basic Alumina I was also used to isolate the peroxides from 100 ml *p*-dioxane[22]. Thus, 100 ml *p*-dioxane were filtered through a column containing 25 g alumina. The alumina was removed from the column, air dried, packed onto another column and eluted with water. This eluate contained *p*-dioxane peroxide which was titrated by the starch–iodide method[23,24]. Lipoxygenase, which was obtained from the Worthington Biochemical Corp., was used to convert linoleic acid to the corresponding conjugated diene peroxide which was recovered from the reaction mixture by extraction into cyclohexane and which was then quantitated on the basis of $\varepsilon_{232.5\,nm}=27.3\cdot10^3\ M^{-1}\cdot cm^{-1}$ (ref. 25).

Assays

Spectrophotometric assays were performed at 25 °C through the use of a Gilford Model 200 spectrophotometer. Reactions under controlled atmospheres were executed in cuvettes which allowed purging the reaction mixture with the desired gas prior to sealing the cuvette. These cuvettes were fashioned after the design of Lazarow and Cooperstein[26] and were fabricated by Pyrocell. The oxidation of NADH was followed at 340 nm using $\varepsilon_{340\,nm}=6.22\cdot10^3\ M^{-1}\cdot cm^{-1}$ (ref. 27) and the reduction of cytochrome *c* was followed at 550 nm using $\varepsilon_{550\,nm}=2.1\cdot10^4\ M^{-1}\cdot cm^{-1}$ (ref. 28). When NADH oxidation was followed at 340 nm, in solutions containing cytochrome *c*, it was necessary to correct for that change at 340 nm, which was due to the reduction of cytochrome *c*. Since the magnitude of this correction would depend upon the change in molar extinction at 340 nm which accompanies the reduction of cytochrome *c* and upon the ratio of cytochrome *c* reduced per NADH oxidized; an empirical method was used in arriving at the magnitude of this correction. Thus, a reaction mixture containing NADH, ethyl hydrogen peroxide and ferricytochrome *c*, under the conditions given in Table II, was monitored at both 340 nm and at 550 nm. The rate of reduction of cytochrome *c* which was observed was then duplicated in a reaction which had no associated absorbance changes at 340 nm, with the exception of that due to the reduction of cytochrome *c*. This was done by using xanthine oxidase *plus* xanthine as the cytochrome *c* reductant and by adjusting the concentration of xanthine oxidase until the desired rate of cytochrome *c* reduction was achieved. The rate of change of absorbance at 340 nm which accompanied this rate of reduction of cytochrome *c* was then measured and was used to correct the rate which had been measured in the non-enzymatic peroxidation–oxidation reaction. The correction was small. Thus, when $\Delta A_{550\,nm}$/min was 0.018 and $\Delta A_{340\,nm}$/min was 0.018, as in Table IV, the change at 340 nm which was due to the reduction of cytochrome *c* was 0.0018.

Gas chromatography

Reactions were performed in 25-ml Erlenmeyer flasks sealed with serum caps and agitated at 25 °C. The gas phase in these flasks was sampled with a Hamilton gas-tight syringe and the gas sample (0.3 ml) was analyzed on a Varian Aerograph, series 1200 equipped with a flame-ionization detector and using a column of 50/80 Parapak R maintained at 70 °C. The detector temperature was 250 °C. The following compounds were used to standardize the aerograph; ethylene, ethanol, ethane, ethyl hydrogen peroxide, acetaldehyde and acetic acid. Individual compounds eluted in reproduceable times and peak area correlated directly with sample size.

Microdiffusion

The central well of Conway microdiffusion dishes contained 2.5 ml of 0.01 M semicarbazide hydrochloride while the outer well contained the reaction mixtures suspected of producing acetaldehyde. At intervals 0.1-ml aliquots of the center well solution were diluted into 2.5 ml of 0.05 M potassium phosphate, pH 7.8, and the absorbance at 224 nm[29] was recorded against a reagent blank. Solutions of acetaldehyde of known concentration were used to calibrate this method. Absorbance at 224 nm was a linear function of the amount of acetaldehyde in the sample and at short intervals of time (less than 30 min at 25 °C) was also a linear function of the time allowed for diffusion. The molar extinction coefficient for the acetaldehyde semicarbazone may be calculated to be $1.57 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 224 nm, from the data of Burbridge *et al.*[30]. Under our conditions of assay, which included the diffusion of acetaldehyde from the original sample into a semicarbazide trap, the corresponding extinction coefficient was $1.3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ if 60 min were allowed for the diffusion. The process of diffusion was nearly complete in 60 min. We actually allowed only 30 min for diffusion: under which condition only 60% of the acetaldehyde was recovered.

RESULTS

A peroxide-dependent reduction of cytochrome c by NADH

The addition of *p*-dioxane to buffered solutions of NADH *plus* cytochrome *c* caused a rapid increase in absorbance at 550 nm and a concomitant decrease at 340 nm. Repetitive scans of the absorption spectrum of the reaction mixture demonstrated that the decrease at 340 nm was due to the oxidation of NADH while the increase at 550 nm was due to the reduction of cytochrome *c*. When the *p*-dioxane was freed of peroxides, by treatment with metallic sodium, it lost most of its ability to foster this reduction of cytochrome *c* by NADH and when the peroxide-free *p*-dioxane was subsequently bubbled with O_2, it regained this activity. Table I documents these effects and also presents the observations that superoxide dismutase at 5 μg/ml powerfully inhibited this reaction. It appeared likely that *p*-dioxane peroxide was responsible for the reaction, which generated the O_2^-, which in turn was the reductant of cytochrome *c*, under the conditions used. Iodometric assays for peroxides[21] demonstrated that the activity of *p*-dioxane, in promoting the reduction of cytochrome *c* by NADH, was proportional to its content of peroxides. In addition, removal of peroxides from the *p*-dioxane, by treatment with Woelm alumina[22], also eliminated its ability to cause this reaction.

The response of the rate of the reaction to changes in the concentrations of the

TABLE I

THE ROLE OF PEROXIDES IN THE REDUCTION OF CYTOCHROME *c* BY NADH

Reaction mixtures contained $1 \cdot 10^{-4}$ M NADH, $1 \cdot 10^{-5}$ M ferricytochrome *c*, $2.4 \cdot 10^{-4}$ M O_2, $1 \cdot 10^{-4}$ M EDTA, 0.05 M potassium phosphate and the components indicated in the table. The pH was 7.8 and the temperature was 25 °C. Reactions were initiated by the addition of 25 μl of *p*-dioxane to 2.5 ml of reaction volume. The *p*-dioxane had been treated with sodium, filtered and then used directly or after bubbling with O_2 for the indicated periods of time. Rates are given in terms of the change in molarity of ferrocytochrome *c* per minute. These rates were calculated from changes in absorbance at 550 nm by use of $\Delta\varepsilon = 2.1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ (ref. 28).

No.	*Additions*	*ΔFerrocytochrome c (M) $\times 10^6$ per min*
1	None	0.000
2	*p*-Dioxane	0.238
3	*p*-Dioxane+15 min O_2	0.286
4	*p*-Dioxane+30 min O_2	0.357
5	*p*-Dioxane+45 min O_2	0.476
6	*p*-Dioxane+60 min O_2	0.690
7	*p*-Dioxane+90 min O_2	0.738
8	No. 7 *plus* 5 μg/ml of superoxide dismutase	0.038

reactants was investigated. Fig. 1 illustrates the effect of NADH on the rate of reduction of cytochrome *c*. This reaction rate was saturable with NADH and half of the maximal rate of reduction of cytochrome *c* was achieved at $8.6 \cdot 10^{-6}$ M NADH. Fig. 2 illustrates the effect of *p*-dioxane peroxide on the rate of reduction of cytochrome *c* by NADH. Once again, saturation behavior was seen and half of the maximal rate was reached with $6.7 \cdot 10^{-5}$ M *p*-dioxane peroxide.

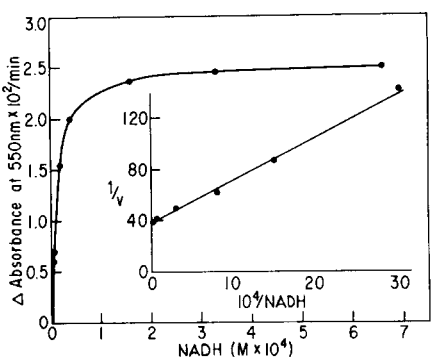

Fig. 1. The effect of NADH on the rate of reduction of cytochrome *c*. Reaction mixtures contained $3 \cdot 10^{-5}$ M ferricytochrome *c*, $2.4 \cdot 10^{-4}$ M *p*-dioxane peroxide, 0.05 M potassium phosphate, $2.4 \cdot 10^{-4}$ M O_2, $1 \cdot 10^{-4}$ M EDTA and the indicated concentrations of NADH at pH 7.8 and 25 °C. Initial rates of reduction of cytochrome *c* were followed at 550 nm and reactions were initiated by the addition of the *p*-dioxane peroxide.

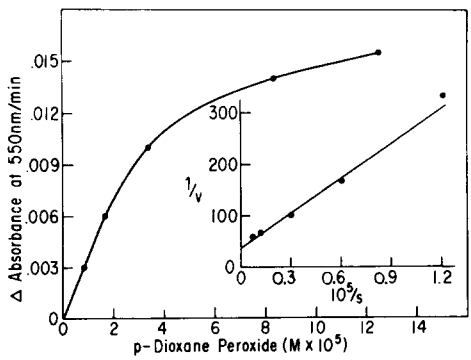

Fig. 2. The effect of *p*-dioxane peroxide on the rate of reduction of cytochrome *c*. Reaction mixtures contained $2 \cdot 10^{-5}$ M ferricytochrome *c*, $3 \cdot 10^{-4}$ M NADH, $2.4 \cdot 10^{-4}$ M O_2, $1 \cdot 10^{-4}$ M EDTA, 0.05 M potassium phosphate and the indicated concentrations of *p*-dioxane peroxide. Reactions were initiated and followed as in Fig. 1.

p-Dioxane peroxide could be replaced by ethyl hydrogen peroxide and by the fatty acid peroxides which were generated by the action of soybean lipoxygenase on linoleic acid but benzoyl peroxide in the range $8 \cdot 10^{-5}$–$2.4 \cdot 10^{-3}$ M was ineffective. Fig. 3 illustrates the effect of ethyl hydrogen peroxide on the rate of reduction of cytochrome *c* by NADH. Half of the maximal rate was reached with $2 \cdot 10^{-2}$ M ethyl hydrogen peroxide. It is apparent that *p*-dioxane peroxide was effective at much lower concentrations than was ethyl hydrogen peroxide but that the maximal rate achieved with ethyl hydrogen peroxide was approximately twice as great as that which was reached at saturation with *p*-dioxane peroxide. H_2O_2 was also able to facilitate this reaction and half of the maximal rate of reduction of cytochrome *c* was achieved at $5.3 \cdot 10^{-4}$ M H_2O_2.

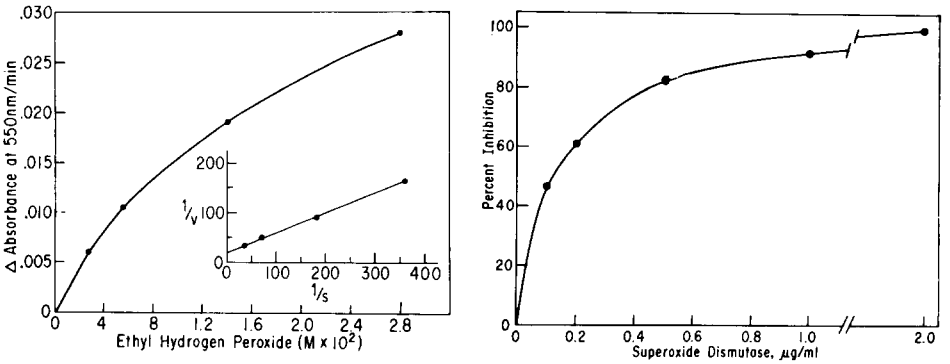

Fig. 3. The effect of ethyl hydrogen peroxide on the rate of reduction of cytochrome *c*. Reaction conditions as described in the legend of Fig. 2 except that ethyl hydrogen peroxide was used in the place of *p*-dioxane peroxide.

Fig. 4. The inhibition of the reduction of cytocrome *c* by superoxide dismutase. Reaction mixtures contained $3 \cdot 10^{-5}$ M cytochrome *c*, $3 \cdot 10^{-4}$ M NADH, $1.4 \cdot 10^{-2}$ M ethyl hydrogen peroxide, $2.4 \cdot 10^{-4}$ M O_2, $1 \cdot 10^{-4}$ M EDTA, 0.05 M potassium phosphate and the indicated concentrations of superoxide dismutase at pH 7.8 and 25 °C. Reactions were initiated by the addition of ethyl hydrogen peroxide.

When no attempts had been made to eliminate O_2 from the reaction mixtures, the reduction of cytochrome *c* was inhibited by superoxide dismutase. This effect is illustrated in Fig. 4. It is clear that 0.1 μg/ml of superoxide dismutase caused nearly 50% inhibition while 2.0 μg/ml inhibited completely. Superoxide dismutase inhibited equally well whether added before the reaction had been started or after the reduction of cytochrome *c* was well under way. Brief exposure of the superoxide dismutase to 100 °C completely eliminated its ability to interfere with the reduction of cytochrome *c* in these reaction mixtures. It is clear that O_2^- was the reductant of cytochrome *c* under these aerobic conditions. The oxidation of NADH, which was a concomitant of the reduction of cytochrome *c*, was not inhibited by superoxide dismutase. This indicates that the reduction of cytochrome *c* was not an essential component of the process responsible for the oxidation of NADH.

The role of oxygen

The reduction of cytochrome *c* and the oxidation of NADH could be observed

in the absence of O_2 as well as in its presence. This is illustrated by the data in Table II.

The primary difference imposed by the absence of O_2 was that the reduction of cytochrome *c* was then insensitive to the presence of superoxide dismutase. It is apparent that whereas O^{2-} was the reductant of cytochrome *c*, in the presence of O_2, some other reducing species served this function in its absence.

TABLE II

EFFECT OF O_2 ON RATES

Reaction mixture containing $3 \cdot 10^{-4}$ M NADH, $1.4 \cdot 10^{-2}$ M ethyl hydrogen peroxide, $3 \cdot 10^{-5}$ M ferricytochrome *c*, $1 \cdot 10^{-4}$ M EDTA, and 0.05 M potassium phosphate (pH 7.8) and 25 °C were equilibrated with the indicated mixtures of N_2 and O_2.

Equilibrated with	$\Delta A_{340\,nm}$ *per min*	$\Delta NADH\,(M) \times 10^6$ *per min*	$\Delta A_{550\,nm}$ *per min*	Δ*Ferrocytochrome c* $(M) \times 10^6$ *per min*
0% O_2	0.0175	2.54	0.0175	0.83
20% O_2	0.018	2.6	0.018	0.86
100% O_2	0.018	2.6	0.0175	0.83

TABLE III

SUBSTITIONS

NADH, $3 \cdot 10^{-4}$ M; NADPH, $3 \cdot 10^{-4}$ M; β-mercaptoethanol (R-SH), $1 \cdot 10^{-3}$ M; NAD^+, $3 \cdot 10^{-4}$ M; Na_2SO_3, $3 \cdot 10^{-4}$ M; ethyl hydrogen peroxide (EtOOH), $1.4 \cdot 10^{-2}$ M; ferricytochrome *c*, $3 \cdot 10^{-5}$ M; ferrocytochrome *c*, $3 \cdot 10^{-5}$ M; human methemoglobin (MetHb), $4 \cdot 10^{-7}$ M; rabbit microsomal cytochrome P450 (Cyt. P 450), $1.5 \cdot 10^{-6}$ M; ferricyanide, $3 \cdot 10^{-5}$ M; hemin, $1.5 \cdot 10^{-6}$ M. All components in 0.05 M potassium phosphate, $1 \cdot 10^{-4}$ M EDTA, $2.4 \cdot 10^{-4}$ M O_2, pH 7.8, 25 °C.

Components	$\Delta A_{340\,nm}$ *per min*	$\Delta NADH\,(M) \times 10^6$ *per min*	$\Delta A_{550\,nm}$ *per min*	Δ*Ferrocyt. c* $(M) \times 10^6$ *per min*
NADH+EtOOH +ferricyt. *c*	0.000			
NADH +ferricyt. *c*	0.000			
NADH+EtOOH +ferricyt. *c*	0.018	2.6		
NADH+EtOOH +ferrocyt. *c*	0.000			
NADH+EtOOH +MetHb	0.012			
NADH+EtOOH +Cyt. P450	0.011			
NADH+EtOOH +ferricyanide	0.007			
NADH +hemin	0.014			
NADH+EtOOH +hemin	0.043			
NADPH+EtOOH+ferricyt. *c*	0.022	3.2	0.022	1.05
R-SH +ferricyt. *c*			0.004	0.19
R-SH+EtOOH +ferricyt. *c*			0.017	0.81
NAD^++EtOOH +ferricyt. c			0.000	
Na_2SO_3 +ferricyt. *c*			0.000	
Na_2SO_3+EtOOH +ferricyt. *c*			0.002	0.095

The role of cytochrome c and of NADH

NADH was not oxidized by ethyl hydrogen peroxide, *per se*. The ferricytochrome *c*, which promoted this reaction, could be replaced by methemoglobin, cytochrome P_{450} or by hemin. Ferricyanide was less effective and ferrocyanide or ferrocytochrome *c* were without any effect. NADH could be replaced by NADPH or by mercaptoethanol but not by sodium sulfite. β-Mercaptoethanol itself did cause a gradual reduction of cytochrome *c* but this was augmented by the presence of ethyl hydrogen peroxide. These results are shown in Table III. It appeared that the reaction under study was related to a heme-catalyzed peroxidation of some reductant, such as NADH or β-mercaptoethanol, to free radicals which could then participate in further reactions. It became important to identify the product formed from the ethyl hydrogen peroxide.

The fate of the oxidant

Reaction mixtures containing NADH, cytochrome *c* and ethyl hydrogen peroxide were found to generate a volatile compound which was identified as acetaldehyde by gas chromatography and by its reaction with semicarbazide. Table IV demonstrates the identical behavior of this volatile product and of acetaldehyde upon gas-chromatographic analysis. Acetaldehyde can conveniently be assayed, after reaction

TABLE IV

GAS-CHROMATOGRAPHIC IDENTIFICATION OF ACETALDEHYDE

The reaction mixtures contained $7.5 \cdot 10^{-5}$ M ferricytochrome *c*, $7.5 \cdot 10^{-4}$ M NADH, $3.5 \cdot 10^{-2}$ M ethyl hydrogen peroxide, $2.4 \cdot 10^{-4}$ M O_2, $1 \cdot 10^{-4}$ M EDTA and 0.05 M potassium phosphate, pH 7.8 and 25 °C and in a total volume of 1.0 ml. The reaction was performed in a sealed flask as described under Materials and Methods and the gas was sampled after the reaction had run for 30 min.

Compound injected	*Retention time (min)*
Ethanol	—
Ethylene	0.8
Acetic acid	9.6
Ethane	2.4
Acetaldehyde	22.4
Volatile component of reaction mixture	22.4

with semicarbazide, in terms of the absorbance of the semicarbazone at 224 nm. This reaction is carried out in Conway microdiffusion vessels in order to eliminate interference by non-volatile components[29]. When applied to reaction mixtures this method demonstrated that ethyl hydrogen peroxide generated acetaldehyde in the presence of ferricytochrome *c*, but not in its absence. There was a short lag in the appearance of the acetaldehyde semicarbazone, but this could have been due to physical processes rather than to chemical ones, *i.e.* it might reflect the lag imposed by the need for diffusion of the acetaldehyde from the reaction mixture into the center well of the microdiffusion chambers. NADH did not affect the rate of production of

acetaldehyde but it did lengthen the lag in its appearance. These results are illustrated in Fig. 5.

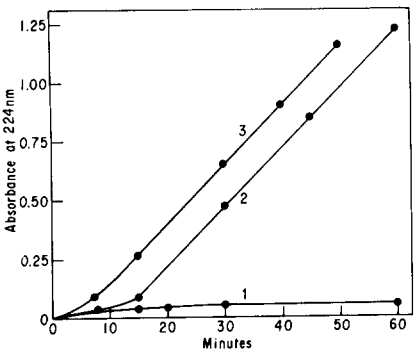

Fig. 5. The effects of ferricytochrome *c* on the decomposition of ethyl hydrogen peroxide to acetaldehyde. Reaction mixtures, which were placed in the outer well of Conway Microdiffusion dishes, contained $2.4 \cdot 10^{-4}$ M O_2, $1 \cdot 10^{-4}$ M EDTA, 0.05 M potassium phosphate and: 1, $1.4 \cdot 10^{-2}$ M ethyl hydrogen peroxide; 2, $1.4 \cdot 10^{-2}$ M ethyl hydrogen peroxide *plus* $3 \cdot 10^{-5}$ M ferricytochrome *c plus* $3 \cdot 10^{-4}$ M NADH; 3, $1.4 \cdot 10^{-2}$ M ethyl hydrogen peroxide *plus* $3 \cdot 10^{-5}$ M ferricytochrome *c*. The pH was 7.8 and the temperature was 25 °C. The center well contained 0.01 M semicarbazide–HCl to trap acetaldehyde and it was sampled and treated as described under Materials and Methods.

DISCUSSION

The following set of reactions provides a basis for understanding the properties of this reaction system. In this scheme Fe^{3+} denotes the iron of ferricytochrome *c*.

(a) $CH_3{-}CH_2{-}OOH + Fe^{3+} \longrightarrow CH_3{-}CH_2{-}O\cdot + Fe^{3+}{-}OH\cdot$

(b) $Fe^{3+}{-}OH\cdot + CH_3{-}CH_2{-}O\cdot \longrightarrow Fe^{3+} + H_2O + CH_3{-}CHO$

(c) $Fe^{3+}{-}OH\cdot + NADH \longrightarrow Fe^{3+} + H_2O + NAD\cdot$

(d) $NAD\cdot + Fe^{3+} \longrightarrow Fe^{2+} + NAD^+$

(e) $CH_3{-}CH_2{-}O\cdot + Fe^{3+} \longrightarrow Fe^{2+} + CH_3CHO + H^+$

(f) $NAD\cdot + O_2 \longrightarrow O^{2-} + NAD^+$

(g) $CH_3{-}CH_2{-}O\cdot + O_2 \longrightarrow CH_3CHO + H^+ + O_2^-$

(h) $Fe^{3+} + O_2^- \longrightarrow Fe^{2+} + O_2$

(i) $NAD\cdot + NAD\cdot + H^+ \longrightarrow NADH + NAD^+$

(j) $CH_3{-}CH_2{-}O\cdot + CH_3{-}CH_2{-}O\cdot \longrightarrow CH_3{-}CHO + CH_3CH_2OH$

(k) $O^{2-} + O^{2-} + 2H^+ \longrightarrow H_2O_2 + O_2$

(l) $CH_3{-}CH_2{-}O\cdot + NAD\cdot \longrightarrow CH_3{-}CHO + NADH$

In Reaction a ethyl hydrogen peroxide reacts with ferricytochrome *c* to yield the ethoxyl radical and what may be considered to be quadrivalent iron. The hydrophobicity of the heme crevice and the ability of cyanide to insert as the sixth ligand of the iron of ferricytochrome *c* (ref. 31) provides a basis for understanding Reaction a.

Ferrocytochrome *c* has a tighter structure than does ferricytochrome *c* and does not react with cyanide[31] so its inability to react with ethyl hydrogen peroxide is not surprising. Our ability to saturate the reaction system with ethyl hydrogen peroxide, as was shown in Fig. 3, or with NADH, as shown in Fig. 1, indicates that either Reaction a or c could be saturated and therefore that the limitation on the rate of reduction of cytochrome *c* lay in one of the other reactions of this sequence. It is interesting that ethyl hydrogen peroxide, H_2O_2 and *p*-dioxane peroxide could gain entry to the heme crevice of ferricytochrome *c* but that benzoyl peroxide seemed to be excluded. The products of Reaction a can participate in a second Reaction b and Reactions a+b account for the conversion of ethyl hydrogen peroxide into acetaldehyde, in the presence of ferricytochrome *c*.

The quadrivalent iron generated by Reaction a causes the univalent oxidation of NADH as shown in Reaction c. In non-enzymatic oxidations, NADH readily participates in univalent oxidation-reduction reactions[32]. The NAD· radical generated by Reaction c could directly reduce cytochrome *c* as in Reaction d or it could dismute as in Reaction i or it could react with an ethoxyl radical as in Reaction l or with O_2 as in Reaction f. Similarly, the ethoxyl radical generated by Reaction a could directly reduce cytochrome *c* as in Reaction e or it could dismute as in Reaction j or it could react with an NAD· radical as in l or with O_2 as in g. In the absence of O_2 all of the reduction of cytochrome *c* was caused by NAD· and by ethoxyl radicals as in d and e and was therefore insensitive to superoxide dismutase. When O_2 is present it reacts with NAD· and with CH_3–CH_2–O· more rapidly than does cytochrome *c*· that is, Reactions f and g must be faster than d and e. Under these circumstances the reduction of cytochrome *c* in the presence of O_2 will be due to O_2^- as in Reaction h and superoxide dismutase will inhibit by eliminating O_2^- *via* Reaction k. Under the conditions described in Table II 1 mole of cytochrome *c* was reduced per 3 moles of NADH oxidized and this ratio, which becomes 1:6 when considered in terms of electron equivalents, was unaffected by O_2. This suggests that reactions which resulted in the oxidation of NADH, without a concomitant reduction of cytochrome *c*, were predominant. Thus we would propose that Reactions a, b, c, i, j and l were (*in toto*) faster than Reactions d, e, f, g and h. From the data in Fig. 5 we can calculate that the rate of change of molarity of acetaldehyde was $6.9 \cdot 10^{-5}$ M/min under the conditions described in Fig. 5 and in Table II. This calculation takes into account the molar extinction coefficient for the acetaldehyde semicarbazone, $1.57 \cdot 10^4\ M^{-1} \cdot cm^{-1}$, as well as the 60% recovery in the 30-min microdiffusion assay and a 26-fold dilution of the semicarbazone prior to recording of its absorbance. It follows that 27 moles of acetaldehyde were produced per mole NADH oxidized. Reactions a and b were evidently more rapid than any of the other reactions considered. The ability of ferricytochrome *c* to catalyze the decomposition of ethyl hydrogen peroxide to acetaldehyde as shown in Fig. 5 and to do so equally well whether or not NADH was present, is not surprising when considered in this light. Indeed the lag in acetaldehyde production, which was caused by NADH (Fig. 5), may be related to Reaction c which consumed Fe^{3+}–OH· and thus slowed Reaction b. This would have the effect of decreasing the rate of production of acetaldehyde until larger amounts of CH_3–CH_2–O· and NAD· had accumulated so that acetaldehyde production by Reaction l could then occur. The fact that NADH did not affect the post-lag rate of acetaldehyde production suggests that Reaction j was not quantitatively significant.

There are similarities between the reactions discussed above and those proposed[18,11] in explanation of the peroxidase–oxidase systems. The ability of ferricytochrome *c* to act both as a catalyst of the peroxidation reaction and as the reagent which serves to detect the production of O_2^- is certainly a novel element of the reactions under consideration. It is clear that superoxide dismutase played an important role in clarifying these reactions.

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