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Inhibition of Erythrocyte Acetylcholinesterase by Peroxides*

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ABSTRACT: The present *in vitro* studies were undertaken to examine potential mechanisms for the *in vivo* decrease of red cell acetylcholinesterase (AChE) activity noted during *in vivo* exposure to oxygen under high pressure (OHP). The effects of (1) oxygen at normal and increased pressures, (2) gaseous diffusion of hydrogen peroxide, and (3) lipid peroxide on AChE activity of bovine red cell extracts and intact human erythrocytes were investigated. Oxygen *per se* failed to inhibit enzyme activity of extracts or intact red cells. AChE activity of red cell extracts and intact red cells was inhibited by gaseous diffusion of H_2O_2 and by lipid peroxides. It is concluded that *in vivo* inhibition of AChE by OHP is probably not an effect of O_2 *per se* but may result from the formation of lipid (and possibly other) peroxides.

Previous studies in this laboratory (Mengel *et al.*, 1964b; Kann *et al.*, 1964) showed that hemolysis occurring in tocopherol-deficient mice during exposure to oxygen under high pressure (OHP)[1] was associated with peroxidation of erythrocyte lipid. Subsequently we followed the course of a patient whose red cells *in vitro* were similar to those of tocopherol-deficient mice (increased lytic sensitivity and lipid peroxide formation during exposure to H_2O_2). He developed hemolytic anemia and a fall of red cell acetylcholinesterase (AChE) after a brief exposure to OHP (Mengel *et al.*, 1965). The recent observation that red cell AChE was consistently decreased in dogs after exposure to OHP (Zirkle *et al.*, 1965) prompted us to carry out *in vitro* studies of potential mechanisms for AChE inhibition that might be relevant to our *in vivo* observations.

Three major possibilities existed. First, since AChE is an SH-bearing enzyme, inhibition could result from the action of oxygen *per se*. Stadie *et al.* (1945), using purified enzyme but not intact red cells, had shown that this did not occur *in vitro*. Second, enzyme inhibition might have resulted from formation of H_2O_2 within the red cells. This compound may be generated in cells during exposure to ionizing radiation and various oxidant agents, and conceivably this might also occur in cells during *in vivo* exposure to unusually high tensions of oxygen. Finally, it was possible that inhibition resulted from the formation of lipid peroxides since their damaging effects on proteins, amino acids, and enzymes have been well documented (Desai and Tappel, 1963). In addition, Wills (1961) showed that lipid peroxides did inhibit purified enzyme extracts of AChE, but no studies of intact erythrocytes were carried out.

In this report we present the results of *in vitro* studies in which the effects of oxygen *per se*, gaseous diffusion of H_2O_2, and lipid peroxides on AChE activity of purified bovine red cell extracts and intact human erythrocytes were determined. The results support the hypothesis that inhibition of AChE activity during *in vivo*

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[1] Abbreviations used in this work: AChE, acetylcholinesterase; OHP, high oxygen pressure; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); pc, packed cells.

hyperoxia results from peroxide formation rather than a direct effect of oxygen *per se*.

Experimental Procedures

For experiments involving intact erythrocytes fresh human blood was defibrinated using glass beads. Erythrocytes were washed three times in six volumes of physiologic saline after removing the buffy coat and were then resuspended at a hematocrit of 40% in physiologic saline.

Bovine erythrocyte AChE[2] was prepared as follows: 20,000 units was dissolved in 20 ml of a 1% gelatin in water solution. One milliliter of this AChE–gelatin mixture was diluted to 200 ml with demineralized water and used as the standard AChE solution for study.

Acetylcholinesterase activity was determined by a modification of the method of Ellman *et al.* (1961) and Mengel *et al.* (1964a) utilizing acetylthiocholine as a substrate. The progressive formation of yellow pigment by the reaction of released thiocholine with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was followed at 412 mμ in a Beckman Model DB recording spectrophotometer. A change of absorbance of 0.001 was taken as 1 enzyme unit (Eu). Values for bovine AChE extracts were expressed as Eu/min and those for intact erythrocytes as Eu/μl of packed cells/min (Eu/μl pc/min).

α-Tocopherol was prepared by mixing 4.5 mg of *dl*-α-tocopherol with 0.5 ml of Tween-80[3] (polyoxyethylene sorbitan monooleate) and 5 ml of physiologic saline. An aliquot of this mixture was diluted in physiologic saline to a final concentration of 10 μg/ml (23 mμmoles/ml).

Effect of Oxygen. Bovine erythrocyte AChE extracts and suspensions of intact human erythrocytes were exposed to 100% oxygen at normal and increased atmospheric pressures. Commercially available oxygen[4] was used in all studies. For exposure to oxygen at atmospheric pressure, 4 ml of the standard AChE solution or a 40% suspension of red cells was placed in a graduated centrifuge tube and covered tightly with parafilm. A pasteur pipet was inserted to the bottom of the tube and a 2-mm exhaust outlet made in the parafilm top. Oxygen was bubbled into the mixtures at a slow constant rate through the pipet for 24 hr at 37°, after which corrections for volume changes were made. Controls using 100% nitrogen without bubbling were carried throughout each study. Acetylcholinesterase activity was determined on 0.5-ml aliquots of the incubation mixtures.

The chamber used for exposure of extracts and red cell suspensions to oxygen under high pressures (OHP) had a volume of 424 in.[3] and was kept at 37°. Inflow and outflow valves allowed a constant flush of gas throughout the study period.

Bovine AChE was exposed to OHP by incubating 4 ml of the standard solution in open 50-ml beakers for 24 hr at 75 psi absolute (psia). Enzyme activity was determined on 0.5-ml aliquots of the incubate. Suspensions (20%) of red cells in physiologic saline (5 ml) were placed in 50-ml beakers and exposed to 100% oxygen at 60 psia for 8 hr, and 0.5-ml aliquots were used to assay enzyme activity.

Effect of Gaseous Diffusion of Hydrogen Peroxide. In these studies 2 ml of the standard bovine AChE solution or 2 ml of the 40% suspension of red cells in physiologic saline was placed in a 50-ml beaker with either 2-ml of α-tocopherol (20 mμmoles/ml) or physiologic saline. Sodium azide (1 mg/0.5 ml of saline) was added to a duplicate set of beakers containing red cells. The beakers were placed in an air-tight enclosed water bath at 37° at points equidistant from another 50-ml beaker containing 20 ml of 30% H_2O_2 and shaken at 60 cpm. Control preparations not exposed to H_2O_2 were also maintained at 37° while shaking throughout the experiment. Acetylcholinesterase activity was determined before and after 8 hr and 24 hr of diffusion using 1 ml of the bovine AChE mixture added to 5.0 ml of buffer or 0.02 ml of the erythrocyte suspension added to 6.0 ml of buffer.

Effect of Lipid Peroxides. Human erythrocyte lipids were extracted by a modification of the method of Bligh and Dyer (1959). Exactly 4 ml of packed red cells (thrice washed in physiologic saline) was hemolyzed with demineralized H_2O_2 to a total volume of 25 ml and 30 ml of chloroform was added. Methanol (60 ml) (anhydrous, absolute) was added slowly while stirring. The mixture was centrifuged at 4000 rpm for 15 min and the supernatant was decanted and filtered through fat-free filter paper. The residue was washed twice with 58 ml of a methyl alcohol–chloroform–water solution (60:30:25, v/v). After the addition of 30 ml of chloroform and 30 ml of demineralized H_2O to the combined extract and washing solvents, the solution was centrifuged at 4000 rpm for 10 min. The lower chloroform layer was evaporated to dryness under vacuum at 22° and the residue was taken up in 4 ml of physiologic saline.

Lipid extract (1 ml) was immediately frozen (under nitrogen) and stored away from light for use as a control sample. The remaining 3 ml of extract was exposed to two Westinghouse Sterilamp (No. G1 T8) ultraviolet lights for 36 hr in round-bottom (6-cm diameter) quartz erlenmeyer flasks at room temperature on a platform shaker (100 cpm) 42 cm below the ultraviolet light source. Formation of lipid peroxides during ultraviolet radiation has been previously reported (Bernheim *et al.*, 1952; Ottolenghi *et al.*, 1955; Baker and Wilson, 1963). The presence of lipid peroxides was determined by measuring the pink chromagen formed by the reaction of 2-thiobarbituric acid with malonaldehyde (Yu and Sinnhuber, 1957; Sinnhuber and Yu, 1958). The term "lipid peroxide" has been used in this report with the understanding that products of lipid peroxidation and not lipid peroxides themselves have been measured (Wilbur *et al.*, 1949; Maier and Tappel, 1959; Yu *et al.*, 1961). Lipid extract (1 ml) exposed to ultraviolet radiation was mixed with 1 ml of 10% tri-

[2] Nutritional Biochemical Corp., Cleveland, Ohio.
[3] Tween-80, Atlas Powder Co., Wilmington, Del.
[4] National Welders Supply Co., Inc., Charlotte, N.C.

chloroacetic acid and filtered through Whatman No. 1 paper. Filtrate (1 ml) was added to 1.2 ml of 0.67% 2-thiobarbituric acid, and the mixture was thoroughly agitated and heated in a boiling water bath for 15 min. After cooling to room temperature absorbance spectra were obtained and final readings taken at 535 mμ against a water blank. A standard curve was prepared using 1,1,3,3-tetraethoxypropane since 1 mole of TEP yields 1 mole of malonaldehyde during acid hydrolysis. Quantities of lipid peroxides were thus expressed as micromoles of malonaldehyde.

To study the effect of lipid peroxides on bovine AChE activity, 0.5 ml of bovine AChE was mixed with 0.5 ml of lipid extract that had been exposed to ultraviolet light along with varying amounts of α-tocopherol and physiologic saline to a total volume of 2 ml, and the solution was incubated at 37° for 1 hr.

To study the effect of lipid peroxides on human erythrocytes, 0.5 ml of peroxidized lipid extract was added to 0.01 ml of red cells (suspended in physiologic saline to a hematocrit of 40%) along with varying amounts of tocopherol and physiologic saline to a total volume of 1.5 ml, and the solution was incubated for 1 hr at 37°. Then 4.5 ml of cholinesterase buffer was added to the incubation mixtures, and AChE activity was determined.

Results

The effects of oxygen per se on bovine erythrocyte AChE extracts are summarized in Table I. Oxygen alone at normal or increased pressures did not lower enzyme activity. Similarly, no effect of oxygen *per se* was noted in studies of intact cells. In addition, using the 2-thiobarbituric acid method, no lipid peroxides were demonstrated in intact red cells after the exposure to oxygen.

TABLE I: Effect of Oxygen on Bovine Acetylcholinesterase Extract Activity.[a]

	Cholinesterase Activity (Eu)				
				Bubbled Gas	
Time (hr)	Control	OHP	Control	100% N_2	100% O_2
0	133	131	112	110	110
12	122	130	106	101	101
24	115	116	102	88	88

[a] The results are the average of duplicate determinations in a representative experiment.

Exposure to diffused hydrogen peroxide resulted in a decrease of AChE activity in bovine red cell extracts and intact human erythrocytes compared to controls (Table II). No further decline or recovery of enzyme activity occurred when duplicate samples were removed from the H_2O_2 exposure after 8 and 24 hr and tested again after 6 and 12 hr. No bleaching of the DTNB–yellow pigment occurred when control samples of red cell extracts and intact cells were exposed to H_2O_2 after the enzyme assay was completed. The addition of tocopherol (10 mμmoles/ml incubate) before exposure to H_2O_2 partially prevented enzyme inhibition of both bovine extracts and intact red cells. Addition of tocopherol to control samples not exposed to H_2O_2 had no effect on enzyme activity.

TABLE II: Effect of Hydrogen Peroxide Diffusion on Bovine Extract and Intact Human Erythrocyte Cholinesterase Activity.[a]

	Bovine Extract (Eu/min)			Intact Red Cells (Eu/μl/min)		
Time (hr)	Control	Saline	Tocopherol	Control	Saline	Tocopherol
0	130	130	130	38	38	38
8	127	54	98	37	33	36
24	120	23	58	37	18	27

[a] A 2-ml sample of standard bovine cholinesterase solution or 40% suspension of erythrocytes (in physiologic saline) was incubated with either 2 ml of tocopherol (20 μg) or 2 ml of physiologic saline. The results are the average of duplicate determinations in a representative experiment.

The addition of sodium azide (an inhibitor of catalase) to red cell mixtures did not significantly alter the effect of peroxide diffusion. Concomitant with depression of AChE activity of intact erythrocytes during the 24-hr period of H_2O_2 diffusion, progressive hemolysis occurred when compared to control samples without H_2O_2 (Table III). When red cells were completely lysed by hypotonic saline solutions no decrease of AChE activity was noted before or after 12- or 24-hr incubation at 37°.

TABLE III: Lysis of Human Erythrocytes during Exposure to Diffused Hydrogen Peroxide.

	Hemolysis (%)	
Incubation Time (hr)	H_2O_2 Diffusion (37°)	Control (37°)
0	0.8	0.9
8	8.5	3.0
12	31.0	7.5
24	77.0	10.0

TABLE IV: Inhibition of Bovine Erythrocyte Cholinesterase by Lipid Peroxides.[a]

Final Concn of Lipid Peroxide (mμmoles malonaldehyde/ml)	Cholinesterase Activity (Eu)
0.4	136
0.6	134
1.4	35
2.0	10
2.6	0

[a] The amounts of lipid peroxide indicated were added to 0.5 ml of the standard bovine cholinesterase solution. The total volume was brought to 1.5 ml with the addition of physiologic saline and incubated at 37° for 1 hr. Values are the results of duplicate determinations in a representative experiment performed on three separate occasions.

Incubation with preformed lipid peroxides resulted in decreased enzyme activity of bovine erythrocyte AChE extracts (Table IV). At a final concentration of 2.6 mμmoles/ml of lipid peroxide, no enzyme activity was detected after 1 hr of incubation. Using various incubation times and enzyme concentrations the inhibition consistently increased in parallel with the increase in peroxide content of the lipid extract. When similar amounts of lipid peroxide were added to samples after DTNB–pigment formation in the enzyme assay, no bleaching effect was noted. The addition of tocopherol before incubation protected enzyme activity (Table V). Addition of tocopherol after incubation did not result in return of enzyme activity, and no spontaneous recovery of activity was noted over a 24-hr period after completion of incubation. When lipid extracts that had been frozen under nitrogen and stored away from light exposure were used, no inhibition of enzyme activity occurred (these extracts also contained no 2-thiobarbituric acid reacting pigments). If the lipid extracts were exposed to light for 24 hr at room temperature, a small amount of 2-thiobarbituric acid reacting material was detected, and these extracts were found to be minimally inhibitory to AChE activity.

Intact human erythrocyte AChE activity was also decreased by addition of small amounts of lipid peroxides (Table VI). As with bovine red cell extracts, larger quantities of lipid peroxides produced greater decreases of enzyme activity which occurred more quickly. A partial protective effect was observed when tocopherol was added to the initial incubation mixture. In contrast to the studies of bovine red cell extracts, increasing tocopherol concentrations did not give added protection. Addition of α-tocopherol after 1 hr of incubation did not result in return of enzyme activity to normal, and no spontaneous return of activity occurred over a 24-hr period.

TABLE V: Effect of Varying Concentrations of Tocopherol on Lipid Peroxide Inhibition of Bovine Cholinesterase Activity.[a]

Final Concn of Lipid Peroxide (mμmoles malonaldehyde/ml)	Final Concn of α-Tocopherol (mμmoles/ml)	Cholinesterase Activity (Eu)
0	0	130
0	16	130
2	0	40
2	1	54
2	2	59
2	4	93
2	8	130
2	16	130

[a] A 0.5-ml aliquot of bovine cholinesterase was mixed with a 0.5-ml aliquot of ultraviolet-exposed lipid extract, varying specific amounts of *dl*-tocopherol and saline (as needed), to a total volume of 2 ml and incubated for 1 hr at 37°. Values are an average of duplicate determinations in a representative experiment performed on three separate occasions.

TABLE VI: Effect of Lipid Peroxides and α-Tocopherol on Intact Erythrocyte Cholinesterase Activity.[a]

Final Concn of Lipid Peroxide (mμmoles/malonaldehyde/ml)	Final Concn of α-Tocopherol (mμmoles/ml)	Cholinesterase Activity (Eu/μl pc/min)
0	0	35.3
0	8	34.0
4	0	17.0
4	2	24.2
4	4	25.3
4	8	25.3

[a] Peroxidized lipid extract (1 ml) was added to 0.01 ml of human erythrocytes, suspended to a hematocrit of 40%. Varying amounts of *dl*-tocopherol were added, and incubation was carried out at 37° for 1 hr. Values are the average of duplicate determinations in a representative experiment performed on three separate occasions.

Discussion

Although the clinical features of oxygen toxicity have

been recognized for many years, the underlying mechanisms of cell damage have not been elucidated. Previous studies in this laboratory showed that *in vivo* hemolysis during exposure to oxygen under high pressure was associated with peroxidation of erythrocyte lipids (Mengel *et al.*, 1964b; Kann *et al.*, 1964).

We subsequently observed both hemolysis and a fall of erythrocyte AChE activity after *in vivo* exposure to OHP in one patient (Mengel *et al.*, 1965) and a consistent decrease of erythrocyte AChE activity in dogs exposed to OHP (Zirkle *et al.*, 1965).

The present *in vitro* studies were undertaken to examine potential mechanisms of AChE inhibition that could be relevant to our *in vivo* studies. Although Stadie *et al.* (1945) had previously shown that purified AChE extracts were not inhibited by OHP, no studies of intact cells had been carried out. The present studies showed that oxygen *per se* at normal or increased atmospheric pressures did not significantly decrease enzymatic activity of red cell extracts or intact red cells. It was therefore unlikely that *in vivo* inhibition of red cell AChE activity during OHP resulted from the effects of high oxygen tensions alone.

In contrast, low-level steady-state exposure to diffused hydrogen peroxide resulted in a decrease of enzyme activity in bovine red cell extracts and intact human erythrocytes. The observation that the inhibition of catalase activity of intact red cells did not increase enzyme inhibition was in agreement with the observations of Cohen and Hochstein (1963), who demonstrated that glutathione peroxidase was the primary mechanism for detoxifying H_2O_2 under low level, steady-state conditions. Catalase itself is not inhibited by fatty acids or their peroxides (Wills, 1961). No studies to the present time have demonstrated hydrogen peroxide formation in cells during *in vivo* exposure to OHP. It is not inconceivable, however, that this might occur.

That lipid peroxide formation during *in vivo* OHP might account for inhibition of AChE activity was suggested by the observation of Wills (1961), who demonstrated inhibition of AChE extracts by lipid peroxides. In the present studies low concentrations of lipid peroxides were shown to inhibt enzyme activity of bovine red cell extracts and also intact erythrocytes. Although inhibition of AChE may result from interaction with fatty acids, *per se* (Putnam, 1948; Wills, 1961), this effect would not be applicable to our studies since addition of equal amounts of unperoxidized lipid did not exert any inhibitory effect.

Tocopherol, a known antioxidant and inhibitor of lipid peroxidation, prevented inhibition of bovine extract activity when added before incubation. Complete protection occurred only when the amounts of tocopherol added were 4–5 times greater than the amounts of lipid peroxide, as determined by the 2-thiobarbituric acid method. However, Hochstein and Ernster (1964) showed that during *in vitro* peroxidation of rat liver microsomes the malonaldehyde formation in the 2-thiobarbituric acid test accounted for only a fraction of the oxygen consumed. Hence it is likely that greater quantities of lipid peroxides were added in our studies than were indicated by the 2-thiobarbituric acid values. Tocopherol gave only partial protection of intact red cell enzyme activity, suggesting the possibility that it was either not taken into the cell or bound in some inactive form.

The exact mechanism of AChE inhibition by peroxides cannot be stated with certainty. At present, its complete structure is not known. Studies have revealed two adjacent "active" sites, one anionic which binds the cationic quaternary nitrogen of acetylcholine and the other an "esteratic" site (Whittaker, 1951; Wilson, 1959).

Inactivation of enzymes by peroxides may reflect alterations at active sites of the enzyme. It was of interest that serine, a vital amino acid at the active site of the AChE molecule, has been shown to be one of the most susceptible of amino acids to peroxidative and free radical damage (Ambe *et al.*, 1961).

Acetylcholinesterase is presumed to contain sulfhydryl groups, and all enzymes known to possess SH groups tested by Wills (1961) were shown to be inactivated by emulsions of unsaturated fatty acid, presumably on the basis of their peroxide content. Linoleic acid and other unsaturated fatty acids caused rapid destruction of SH groups in proportion to the amount of peroxide present (Lewis and Wills, 1962), and compounds containing SH groups (*i.e.*, gluathione or cysteine) exerted a protective effect. In addition the SH groups of sulfhydryl proteins have been shown to be oxidized to S–S and further oxidation products by fatty acid peroxides (Dubouloz and Fondarae, 1953). Alterations of S–S bonds adjacent to active sites of enzymes may also inactivate enzymes (Melzer and Epstein, 1963).

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Electron-Transport Systems of Yeast. II. Purification and Properties of a Soluble Reduced Diphosphopyridine Nucleotide Dehydrogenase*

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ABSTRACT: A soluble highly purified reduced diphosphopyridine nucleotide (DPNH) dehydrogenase has been prepared from yeast. The enzyme catalyzed the oxidation of DPNH by ferricyanide, 2,6-dichlorophenolindophenol (indophenol), and cytochrome *c*, and contained 0.6 mole of flavin–adenine dinucleotide, (FAD) and 0.4 g.-atom of iron per mole of enzyme. Both FAD and iron were removed from the enzyme by acid treatment, but complete reactivation of the apoenzyme was effected by addition of FAD alone.

Two *p*-mercuriphenylsulfonic acid (PCMS) sensitive sulfhydryl groups, active in enzymatic catalysis, appear to be located after the flavin in the sequence of electron transport and in close spatial approximation to the site of binding of DPNH by the enzyme.

Previously, we have described the properties of an electron-transport particle (ETP)[1] isolated from *Saccaromyces cerevisiae* (Mackler *et al.*, 1962). The ETP differs from similar preparations from beef heart (Mackler and Green, 1956; Crane *et al.*, 1956) in several properties, although the enzymes catalyze the same reactions. Preparations of ETP from yeast contain only flavin–adenine dinucleotide (FAD), in contrast to heart ETP which contains both flavin mononucleotide (FMN) and FAD and much higher amounts of nonheme iron and coenzyme Q (ubiquinine) than does the yeast ETP. In addition, amytal and seconal inhibit the reduced diphosphopyridine nucleotide (DPNH) oxidase activity of the heart enzyme, but do not affect the activity of the yeast preparation.

The present report describes the preparation and properties of a soluble DPNH dehydrogenase from preparations of yeast ETP. Differences in structure, composition, and kinetic properties between this enzyme and the previously reported DPNH dehydrogenase (Mackler, 1961) prepared from beef heart will be discussed.

Materials and Methods

Assays of enzymatic activity were performed spectrophotometrically as described previously (Mackler, 1961; Rao *et al.*, 1963) at 38° with the exception that the assays contained either 0.2 ml of 0.2 M potassium phosphate buffer, pH 7.5, or 0.2 ml of 0.2 M sodium

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[1] Abbreviations: ETP, electron-transport particle; DPNH, reduced diphosphopyridine nucleotide; FAD, flavin–adenine dinucleotide; FMN, flavin mononucleotide; indophenol, 2,6-dichlorophenolindophenol; PCMS, *p*-mercuriphenylsulfonic acid.