EVIDENCE THAT SUPEROXIDE DISMUTASE PLAYS A ROLE IN PROTECTING RED BLOOD CELLS AGAINST PEROXIDATIVE HEMOLYSIS

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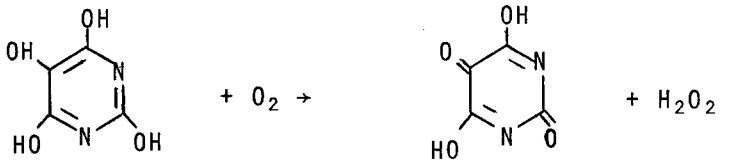
ABSTRACT. We have carried out kinetic studies of the dialuric acid induced hemolysis of vitamin E deficient rat erythrocytes, and have shown that with a careful choice of conditions and in the presence of catalase a very substantial protective effect of externally added superoxide dismutase can be observed.

INTRODUCTION

It has recently been proposed (1) that the function of superoxide dismutase is to rapidly scavange the O_2^- free radical and thus provide a mechanism whereby an organism can avoid possible deleterious effects of this radical or other free radicals which might be produced by its further reaction with cellular components. While there are sufficient indications that O_2^- can be formed within cells (2-4), there is no evidence that O_2^- plays a deleterious role. The purpose of this communication is to report some preliminary results which suggest that O_2^- may be important in the general phenomenon of lipid peroxidation and particularly in the peroxidative hemolysis of red blood cells.

The system chosen for study was the dialuric acid induced hemolysis of the vitamin E deficient rat erythrocyte. This phenomenon was first described by Rose and Gyorgy (5) in 1950 who concluded that the

hemolysis was "linked with the reversible oxidation-reduction system of dialuric acid and alloxan..." Later, these same authors (6) showed that hydrogen



peroxide alone, at concentrations comparable to those produced by added dialuric acid, was not the hemolyzing agent, but at higher concentrations, 0.25 M, would rapidly induce hemolysis of E-deficient cells. Nevertheless, it was found that catalase added externally to 0.5 μM offered complete protection against hemolysis by dialuric acid, and Rose and György (6) suggested that "some intermediate compound formed during oxidation is the actual hemolyzing agent." We have repeated some of the experiments of Rose and György, finding conditions where only a partial protection was afforded by catalase and that this could be increased by added superoxide dismutase.

MATERIALS AND METHODS

Heparin, liver catalase, and alloxan monohydrate were Sigma products. Dialuric acid and thiobarbituric acid were obtained from K and K Laboratories. Draper E-deficient test diet in pellet form was obtained from General Biochemicals. Superoxide dismutase was prepared by the method of McCord and Fridovich (7). All other

materials were of commercial quality, and water was glass distilled.

Male Spraque-Dawley rats weighing about 150 g were housed in individual cages and maintained exclusively on the test diet with free access to water for 4-5 weeks. Blood (2-5 ml) was taken in a heparinized syringe by heart puncture of an ether anesthetized animal (8).

Hemolysis was followed by the decrease in scattering of 740 nm light which occurs upon cell disruption. The exact procedure was as follows: 0.15 ml freshly drawn whole blood from a single animal was diluted to 10 ml in a sodium phosphate buffer osmotically equivalent to 0.85% NaCl (9) and having pH 7.4. 2.5 ml of this solution was transferred to a 10 mm OD test tube which would conveniently fit into the cell compartment of the Coleman Model 124 spectrophotometer, and the experiment was initiated by adding 200 µl of a 16 m<u>M</u> solution of dialuric acid with vigorous aeration. The dialuric acid solution was prepared immediately before use in the buffer described above. The cell suspension was then aerated vigorously for 30 sec on a rotary mixer and placed in the photometer. After approximately 1 min the suspension was aerated again for 15 sec then put back in the photometer. This process was repeated every 1-4 min before and during hemolysis. The enzymes catalase and superoxide dismutase were added prior to the dialuric acid solution. Complete hemolysis was determined by adding red cells to distilled water.

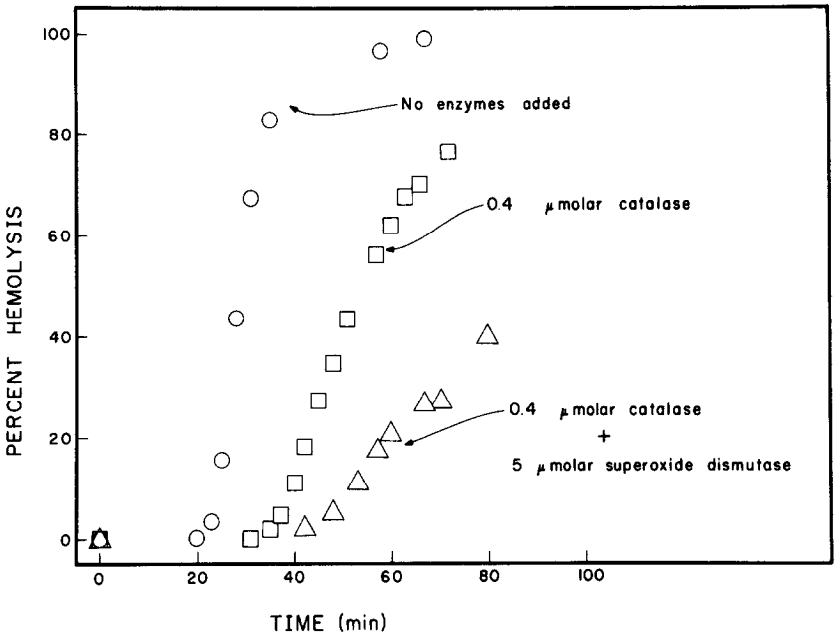
Analysis for lipid peroxidation was carried out essentially the same as described by Kann and Mengel (10):

1.5 ml whole blood was diluted to 10 ml with the buffer described above, 2.5 ml of this solution was used for treatment with dialuric acid as above after which 1 ml 12% trichloroacetic acid (TCA) was added with mixing. The precipitate was removed by centrifugation, and to 2 ml of the supernatant, 2.4 ml of a 0.67% suspension of thiobarbituric acid was added, this solution was heated at 100°C for 15 min. The pink color was quantified by the 535 nm absorption.

RESULTS

Before deciding on the present method of following the hemolysis reaction the procedures developed by other workers were used (6, 11, 12), and it was found that shortly after adding the dialuric acid solution the cell suspension turned a murky brown color due to methemoglobin formation. The hemolysis results obtained with these techniques gave several indications of protection by catalase and dismutase but were not in general reproducible. We found that the methemoglobin formation could be prevented if the dialuric solution was added with stirring and this was followed immediately with extensive aeration on a vortex mixer. Using this technique hemolysis occurred at a time after the beginning of the experiment which was characteristic and repeatable for a sample of blood.

The figure shows the time dependence of hemolysis induced by dialuric acid when no enzymes are added to the system, in the presence of 0.4 μmolar catalase, and with 0.4 μmolar catalase plus 5 μmolar superoxide dismutase. In this experiment 50% hemolysis occured at approx. 29 min



The effect of catalase and catalase plus dismutase on the time dependence of the dialuric acid induced hemolysis of vitamin-E deficient rat erythrocytes. See text for details.

for the system alone, 53 min, and >80 min in the presence of catalase and catalase plus dismutase, respectively. Superoxide dismutase was never found to have a protective effect by itself, perhaps because of peroxide inhibition (13). Both catalase and superoxide dismutase lose their effect when boiled for several minutes.

Since dialuric acid appears to be oxidized to alloxan rather rapidly under our conditions (14) we have tested whether alloxan may also promote hemolysis. These experiments indicated that alloxan alone did not induce hemolysis as was found by other workers (6).

We have carried out a few determinations of lipid peroxide formation and these are shown in the Table.

The Effects of Catalase and Superoxide Dismutase on Lipid Peroxidation in the Dialuric Acid - Vitamin E Deficient Erythrocyte System

Solution*	A_{535}	nmoles malondialdehyde+
No dialuric acid added	0.10	0
System alone without aeration	0.51	33
System alone with aeration	0.48	30
+ 0.4 μM catalase	0.25	12
+ 0.4 μM catalase and 5 μM SD	0.22	9.6

*As described in Materials and Methods. The reaction was started in all tubes at the same time and was stopped at the point where hemolysis occurred in the tube having no added enzymes.

+Reference 15

It is clear from this very limited study that catalase is protecting the system from lipid peroxidation, and the data suggest that the dismutase-catalase mixture has a somewhat greater protective effect. A correlation was not made between lipid peroxidation and hemolysis, but this has been done by other workers (16) who have shown that hemolysis is probably the result of the peroxidation of membrane lipids.

DISCUSSION

Superoxide anion is known to be formed by the reaction of oxygen with certain reduced aromatic systems such as flavin (17), viologens (paraquat) (18), and phenazine methosulfate (19). The structural similarity of dialuric acid to these substances suggest that it too may react with oxygen to yield superoxide. If this is true and superoxide dismutase is exerting its effect by rapidly destroying O_2^- our results can be reasonably explained: The dialuric acid induced hemolysis of E-deficient red blood cells is effected by peroxidation of membrane lipids (16). The fact that both catalase and dismutase together give protection against hemolysis and lipid peroxidation suggests that both H_2O_2 and O_2^- are somehow involved. It is possible that O_2^- and H_2O_2 interact to yield a reactive fragment, perhaps the hydroxyl radical, and that this species is actually

$$H^+ + O_2^- + H_2O_2 \rightarrow O_2 + H_2O + \underline{OH}\cdot$$

responsible for the observed effects. This would also explain why catalase alone can produce a high degree of protection (6, 11).

The results (a) give support to the early contention of Rose and Györgyi (6) that "an intermediate compound" is involved in the dialuric acid induced hemolysis, (b) suggest the above intermediate to be the superoxide anion, and (c) indicate that O_2^- may be involved in the general phenomenon of lipid peroxidation. It is possible that the dialuric acid induced hemolysis of vitamin-E deficient

red blood cells may serve as a model for the study of a general class of hemolytic anemia thought to involve lipid peroxidation as the terminal lesion (15, 20, 21) and in the study of the biological effects of oxygen at higher than normal pressures (16).

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REFERENCES

1. J. M. McCord, B. B. Keele, Jr., and I. Fridovich, Proc. Nat. Acad. Sci., 68, 1024 (1971).

2. V. Massey, S. Strickland, S. G. Mayhew, L. G. Howell, P. C. Engel, R. G. Matthews, M. Schuman, and P. A. Sullivan, Biochem. Biophys. Res. Comm., 36, 891 (1969).

3. W. H. Orme-Johnson and H. Beinert, Biochem. Biophys. Res. Comm., 36, 905 (1969).

4. R. Nilsson, F. M. Pick, and R. C. Bray, Biochim. Biophys. Acta, 192, 145 (1969).

5. C. S. Rose and P. Gyorgy, Blood, 5, 1062 (1950).

6. C. S. Rose and P. Gyorgy, Am. J. Physiol., 168, 414 (1952).

7. J. M. McCord and I. Fridovich, J. Biol. Chem., 244, 6049 (1969).

8. E. J. Farris and J. Q. Griffith, "The Rat in Laboratory Investigation," Hafner Publishing Co., New York, Second Edition, 1949.

9. E. B. Hendry, Edinburgh Med. J., 155, 142 (1948).

10. H. E. Kann, Jr. and C. E. Mengel, in Third International Conference on Hyperbaric Medicine, National Academy of Sciences, Washington, D. C., 1969, p. 65.

11. J. Bunyan, J. Green, E. E. Edwin, and A. T. Diplock, Biochem. J., 77, 47 (1960).

12. R. E. Heikkila, J. A. Mezick, and D. G. Cornwell, Physiol. Chem. and Physics, 3, 93 (1971).

13. G. Rotilio, L. Morpurgo, C. Giovagnoli, L. Calabrese, and B. Mondovi, Biochemistry, 11, 2187 (1972).

14. G. M. Richardson, Biochem. J., 26, 1959 (1932).

15. C. E. Mengel, H. E. Kann, Jr., and W. D. Meriwether, J. Clin. Invest., 46, 1715 (1967).

16. C. E. Mengel, H. E. Kann, Jr., W. W. Smith, and B. D. Horton, Proc. Soc. Exp. Med., 116, 259 (1964).

17. D. P. Ballou, G. Palmer, and V. Massey, Biochem. Biophys. Res. Comm., 36, 898 (1969).

18. T. C. Stancliffe and A. Pire, FEBS Letters, 17, 297 (1971).

19. M. Nishikimi, N. A. Rao, and K. Yagi, Biochem. Biophys. Res. Comm., 46, 849 (1972).

20. J. T. Dodge, G. Cohen, H. J. Kayden, and G. B. Phillips, J. Clin. Invest., 46, 357 (1967).

21. J. W. Harris and R. W. Kellermayer, "The Red Cell," Harvard University Press, 1970, Chap. 9.