Some Studies of Tocopherol Deficiency in Infants and Children

III. RELATION OF BLOOD CATALASE ACTIVITY AND OTHER FACTORS TO HEMOLYSIS OF ERYTHROCYTES IN HYDROGEN PEROXIDE

By HAROLD M. NITOWSKY, M.D.* AND J. TYSON TILDON, B.S.†

IN A previous paper, an inverse relation was reported between plasma tocopherol and hemolysis of erythrocytes in hydrogen peroxide for infants and children with steatorrhea and for thriving young premature and full-term infants.[1] The absence of this expected relationship in unfed newborn infants led to the consideration that factors other than plasma tocopherol, e.g. differences in structure or composition of the neonatal erythrocyte, might affect its susceptibility to hydrogen peroxide. Since *in vitro* addition of purified catalase had been shown by Rose and György[2] to inhibit dialuric acid hemolysis of erythrocytes of vitamin E-deficient rats, and since some variation had been reported in the catalase activity of the erythyrocytes of newborn infants,[3] we studied the relationship between catalase activity and hydrogen peroxide hemolysis. In addition to these investigations, the *in vitro* effects of substances other than tocopherol on hemolysis have been determined.

From the Departments of Pediatrics of Sinai Hospital of Baltimore and The Johns Hopkins Medical Institutions.

* Research Associate in Pediatrics, Sinai Hospital of Baltimore and Instructor of Pediatrics, Johns Hopkins University School of Medicine.

† Research Assistant in Pediatrics, Sinai Hospital of Baltimore.

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CATALASE

In preliminary studies, adult red cells, resistant to the hemolytic action of hydrogen peroxide, were incubated for 60 minutes with buffer solutions containing 10^{-3} to 10^{-5} molar 2,4-dichlorophenol, a specific inhibitor of catalase.[4] The cells were then washed and resuspended in saline, and hemolysis in hydrogen peroxide was determined in the usual way.[5,6] A typical experiment is presented in Table I. It is seen that inhibition of catalase by 2,4-dichlorophenol made the previously resistant cells susceptible to hemolysis in hydrogen peroxide.

TABLE I

Effect of Catalase Inhibitor on Hemolysis of Red Cells in Hydrogen Peroxide

Agent	Concentration	Hemolysis
	Molar	%
2,4-dichlorophenol	5×10^{-7}	2
"	5×10^{-6}	5
"	5×10^{-5}	45
"	5×10^{-4}	24
"	5×10^{-3}	94
Buffer control		2

Measurements of blood catalase activity in newborn infants were made by titrating with potassium permanganate the residual amount of hydrogen peroxide left after addition of a blood lysate. The lysate (1:1000) was added to 0.2 *N* H_2O_2 in 0.01 *M* phosphate buffer, pH 6.8 at 2–4° C, and the destruction of peroxide stopped by addition of 2 *N* H_2SO_4. The destroyed peroxide was determined at 0, 1, 3, and 5 minutes after addition of the

lysate and the pseudomonomolecular reaction rate constants were calculated. The initial catalase activity, K_0, was determined by extrapolation on a semilogarithmic plot of the reaction rate constants as a function of time. The exponential decline in the rate constants

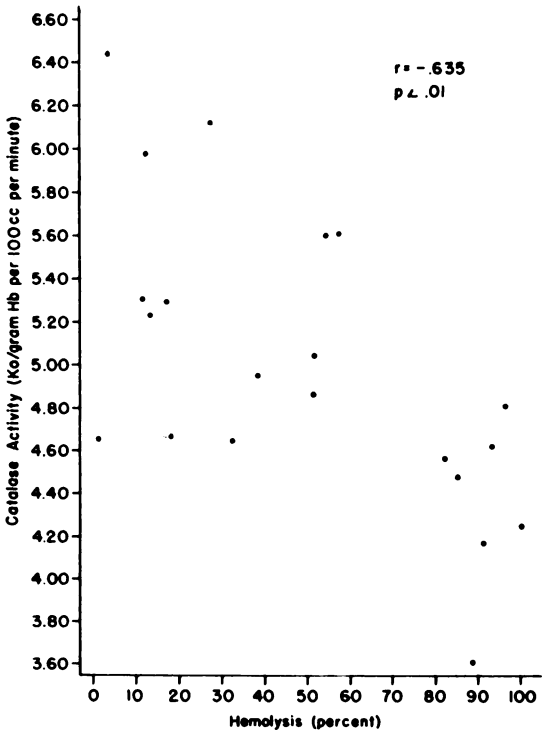

Fig. 1. Relation in unfed newborn infants between blood catalase activity and erythrocyte hemolysis in hydrogen peroxide.

resulted in a linearity which permitted easy extrapolation. The initial rate constant, K_0, was proportional to the hemoglobin concentration and was expressed in units per gram of hemoglobin per 100 ml per minute. The proportionality between K_0 and number of erythrocytes was not as satisfactory, perhaps because of errors in erythrocyte enumeration and differences in hemoglobin content of red cells.

Catalase activity in a group of 21 newborn infants was found to be inversely related to red cell hemolysis in H_2O_2 ($r = -0.635$, $P < 0.01$). No correlation was found in these infants between plasma tocopherol levels and catalase activity ($r = -0.072$), or between these levels and red cell hemolysis ($r = 0.032$). With a slight modification of method, observations have been made in an additional 44 unfed newborn infants. The inverse correlation (r) for the whole group of 65 infants is -0.474 ($P < 0.001$).

These results suggest that in newborn infants with low plasma tocopherol, high catalase activity may protect against hydrogen peroxide. Studies are being extended to older infants and children with low plasma tocopherols in whom the expected inverse relationship of the latter to hemolysis was found. Since this was already present in the young full-term infants at average age 7 weeks, and in the premature infants over 1 month of age, the relationship of catalase activity to fetal hemoglobin, which disappears during the early months of life, seems worthy of investigation.

EFFECT OF SUBSTANCES OTHER THAN ALPHA-TOCOPHEROL ON HEMOLYSIS

A variety of substituted hydroquinones and estrogens, in addition to several tocopherols, are capable of preventing *in vitro* hemolysis of the red cells of vitamin E-deficient rats by dialuric acid.[2] While fat-soluble antioxidants other than the tocopherols are inactive *in vivo*, the oral administration of methylene blue to tocopherol-deficient rats confers resistance to the *in vitro* hemolytic action of dialuric acid.[7]

We have determined the protective effects of a variety of substances added *in vitro* to newborn infants' red cells known to be hemolyzed by hydrogen peroxide. A tabulation of agents employed and the range of effective and ineffective concentrations are shown in Tables II and III. The method for testing these substances involved the addition of the desired amounts of the agent in 2 ml of phosphate buffer to 2-ml portions of a 5 per cent saline suspension of red cells. The tubes were incubated at 37° C for one hour and centrifuged; the supernatant was withdrawn and discarded and the cells were washed with 2 ml saline. A 5 per cent erythrocyte suspension in saline was then prepared, and the peroxide hemolysis test made in the usual manner. *d*-Alpha-tocopherol, α-tocopherol hydroqui-

TABLE II

Prevention by Various Substances of Hemolysis of Washed Erythrocytes by Hydrogen Peroxide

Effective agents	Range of effective concentrations
	μg/ml
Vitamins and derivatives:	
d-Alpha tocopherol	0.5– 10
Alpha-tocopherol hydroquinone	50 – 500
Thiamine hydrochloride	500 – 5,000
Sodium ascorbate	2500 –12,500
Miscellaneous agent	
Methylene blue	5 – 50
Quinones:	
2-Methyl-1,4-naphthoquinone	0.5– 50
1,4-Naphthoquinone	2.5– 25
1,2-Naphthoquinone	2.5– 25
p-Xyloquinone	0.5– 25
Tolu-*p*-quinone	50
2,5-Dichloroquinone	25
Quinone	2.5– 25
Hydroquinones:	
Hydroquinone	2.5– 25
3,4-Dihydroxyphenylalanine	25 – 250
1-Epinephrine bitartrate	25 – 250
Catechol	50

TABLE III

Failure of Various Substances to Prevent *in vitro* Hemolysis of Washed Erythrocytes by Hydrogen Peroxide

Ineffective agents	Range of ineffective concentrations
	μg/ml
Vitamin B Complex:	
Pyridoxine hydrochloride	5 – 500
Nicotinamide	5 –5,000
Vitamin B_{12}	0.05– 5
Riboflavin	5 – 500
Sodium folate	25 –2,500
Miscellaneous agents:	
Cortisone acetate	2.5 –2,500
Adenosine	5 – 500
Glutathione	0.5 – 500
Cysteine hydrochloride	0.5 – 500
DL-Methionine	0.5 – 500
2,3-Dimercapto-1-propanol	5 – 50
Resorcinol	0.5 – 500
Ephedrine sulfate	0.5 – 500
d-Amphetamine sulfate	0.5 – 500
Alpha-tocopherol esters	0.5 – 500
(Acetate, phosphate, carbosuccinate)	0.5 – 12.5

none, thiamine hydrochloride, and sodium ascorbate were found effective *in vitro* in preventing hemolysis. Methylene blue, and quinone, hydroquinone, and some of their substituted derivatives were also effective.

In preliminary studies, we have found no *in vivo* effects following oral administration of ascorbic acid or intramuscular administration of thiamine hydrochloride or the bisulfite salt of 2-methyl-1,4-naphthoquinone (water-soluble vitamin K); on the other hand, the positive *in vitro* effects of these metabolically useful substances suggest that they may condition the degree of correlation between plasma tocopherol of infants and hemolysis of their erythrocytes in hydrogen peroxide.

Of considerable interest were the *in vitro* tests of 2-methyl-1,4-naphthoquinone (menadione) and its salts. These substances were as effective as *d*-α-tocopherol on a molar basis. The addition of these agents to the red cell suspension caused a rapid formation of methemoglobin, as has been previously observed.[8] In spite of this oxidation reaction, a resistance of the red cells during subsequent exposure to hydrogen peroxide was noted. The *in vitro* protective action of water-soluble vitamin K derivatives is paradoxical in the light of a recent report that large doses of vitamin K cause hemolysis in vitamin E-deficient rats, and that kernicterus occurs in premature infants without isoimmunization after administration of large doses of vitamin K.[9]

Since the tocopherols function as reversible biological antioxidants, it has been proposed that a fundamental role of vitamin E is inhibition of fat oxidation.[10–13] Tappel[14] has found that α-tocopherol inhibits the hematin compound catalysis of unsaturated fatty acid oxidation and vitamin A and carotene co-oxidation. Other phenolic antioxidants also inhibit linoleate oxidation catalyzed by hemoglobin and cytochrome C.[15] It is of interest that several antioxidants which we have found to inhibit *in vitro* the hemolysis of susceptible red cells by H_2O_2 are reported to inhibit the activity of plant lipoxidase in catalysis of oxidation of unsaturated fatty acids.[16] Those substances which we found had no effect on hemolysis are similarly reported to have no effect on lipoxidase. A summary of these findings is presented in Table IV. The par-

TABLE IV

Comparison of Effects of Various Agents on Lipoxidase Activity and Erythrocyte Hemolysis

Agent	Inhibition of lipoxidase activity*	Reversal of erythrocyte hemolysis
Alpha tocopherol	+	+
Hydroquinone	+	+
Quinone	+	+
3,4-Dihidroxyphenylalanine	+	+
Epinephrine	+	+
Catechol	+	+
Ascorbic acid	+	+
Resorcinol	−	−
Sodium fluoride	−	−
Alpha tocopherol acetate	−	−

* Adapted from Sumner and Myrback.[16]

allel effects support the recently advanced hypothesis that vitamin E may play a role in maintaining the integrity of the erythrocyte by inhibition of oxidase action of hemoglobin on the unsaturated fatty acids of the cell membrane.[17] This role as a physiologic antioxidant need not preclude a more specific action of tocopherol through other enzyme systems, as has recently been demonstrated by Nason and Lehmann[18] in the enzymatic reduction of cytochrome C by reduced DPN (diphosphopyridine nucleotide).

CONCLUSION

Studies have been made of some factors which may be responsible for the variable susceptibility of the erythrocytes of newborn infants to the *in vitro* hemolytic action of a dilute hydrogen peroxide solution. Measurement of catalase activity in the red cells of these infants revealed a significant inverse correlation between this activity and erythrocyte hemolysis in H_2O_2.

A number of substances, some of physiologic importance, have been shown to be effective *in vitro* in reversing hemolysis of susceptible red cells. While no similar *in vivo* effects have as yet been demonstrated, it is postulated that some of these physiologic agents may influence the resistance of cells to hemolysis by H_2O_2 at low plasma tocopherol levels.

The correlation between the ability of various antioxidants to inhibit both hemolysis and catalysis of unsaturated fatty acid oxidation by lipoxidase or hematin compounds lends support to the hypothesis that vitamin E may play a role in maintaining the integrity of the erythrocyte by inhibition of an oxidase action on the unsaturated fatty acids of the cell membrane.

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DISCUSSION

DR. P. GYÖRGY (Philadelphia, Pa.): The hemolysis test in its detailed sequence is still shrouded in mystery, even after the excellent presentation of Dr. Gordon and Dr. Nitowsky. The hemolysis test *in vivo* is, at least in our hands, much more specific than has been claimed by some authors. This statement covers a large number of powerful antioxidants, given to rats in doses below the toxic level. The lack of real parallelism between the tocopherol level and the hemolysis test is not surprising. The catalase content of the red blood cells may be one interfering factor, as clearly demonstrated by Dr. Gordon and Dr. Nitowsky. The other possibility is that not all the tocopherol present in plasma is biologically active. It is conceivable that tocopherol bound on protein, in analogy to calcium bound on protein, is biologically inactive. Proof for this assumption is furnished by the observation that in the presence of serum more tocopherol is required to reverse a positive hemolysis test *in vitro*. In this connection, it is possible that fetuin, which is one special constituent of the plasma in the newborn, has a special affiinity for tocopherol.

With regard to the specificity of the hemolysis test, it should be mentioned that rats kept on the usual necrogenic yeast diet (with 18 per cent yeast) show a marked positive hemolysis test within a few weeks. By increasing the proportion of yeast in the diet from 18 to 40 per cent, the hemolysis test slowly becomes less intensive and may become negative. Yeast is free of vitamin E. In co-operation with Dr. Forbes and Dr. Zilliken, we have isolated a simple compound which has shown vitamin E-like activity on the hemolysis test *in vitro* and also *in vivo*. The relation of this substance to the vitamin E-like constituent postulated on the basis of studies on chicks by Scott and his associates at Cornell University, or to the Factor III of Schwartz, has to be further investigated.

The activity of alcoholic extracts from various yeasts on the hemolysis test seems to vary inversely with their necrogenic effect when fed as the sole source of protein to rats.