BBA 75540

ERYTHROCYTE MEMBRANE INTERACTIONS WITH MENADIONE AND THE MECHANISM OF MENADIONE-INDUCED HEMOLYSIS

JAMES A. MEZICK, C. THOMAS SETTLEMIRE, G. P. BRIERLEY, KAYE P. BAREFIELD, WALLACE N. JENSEN AND DAVID G. CORNWELL

Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio 43210 (U.S.A.)

(Received June 9th, 1970)
(Revised manuscript received August 24th, 1970)

SUMMARY

Menadione-induced hemolysis was preceded by increased K^+ permeability and swelling. Swelling and hemolysis were prevented by the non-penetrating solutes sucrose and phosphate. Thiodione, the 3-glutathionyl ether of menadione, did not affect K^+ permeability and did not cause hemolysis. Labeled menadione was irreversibly bound to erythrocyte ghosts and binding was greatly reduced by pre-incubation with *p*-chloromercuribenzoate (PCMB). Hemolysis was not related to the oxidation of glutathione, hemoglobin or membrane lipids by menadione or thiodione. These studies suggest that menadione is a specific membrane sulfhydryl inhibitor which alters cation permeability and induces colloid-osmotic hemolysis.

INTRODUCTION

Menadione hemolyzes normal erythrocytes and oxidizes both hemoglobin and reduced glutathione (GSH). The mechanism for menadione-hemolysis is unknown although several hypotheses have been proposed which relate hemolysis to various oxidation reactions. BROBERGER *et al.*[1] suggested that menadione oxidized hemoglobin to methemoglobin and the subsequent reduction of methemoglobin with NADPH, a reaction catalyzed by methemoglobin reductase, lowered the concentration of NADPH so that sufficient NADPH was not available to maintain GSH in the reduced state. This hypothesis was supported by HARLEY AND ROBIN[2] who suggested that low levels of menadione activated methemoglobin reductase. However, BEUTLER[3] showed that the formation of methemoglobin was not required for hemolysis and he proposed that another oxidation product of hemoglobin could be involved in hemolysis. COHEN AND HOCHSTEIN[4] later found that menadione generated H_2O_2 which oxidized GSH and they proposed that insufficient NADPH was available to reduce the oxidized glutathione in individuals who were deficient in erythrocyte glucose-6-phosphate dehydrogenase and sensitive to menadione-hemolysis.

Since menadione generates H_2O_2, a second hypothesis involving lipid peroxidation may be proposed. TSEN AND COLLIER[5] and JACOB AND LUX[6] have shown that

Abbreviation: PCMB, *p*-chloromercuribenzoate.

oxidizing agents yield lipid peroxides which accompany the hemolysis of erythrocytes from animals which are deficient in vitamin E. Thus the hydrogen peroxide generated by menadione may oxidize membrane lipids as well as hemoglobin and GSH and the oxidation of membrane lipids may be the primary event in the hemolytic process.

Menadione forms 3-thioethers with sulfhydryl groups[7]. This chemical reaction is the basis for a third hypothesis that may be proposed for menadione-hemolysis since a number of investigators[8-12] have found that hemolysis is induced by specific sulfhydryl inhibitors. In the present investigation, we show that menadione-hemolysis is not related to the oxidation of hemoglobin, GSH or membrane lipids. Menadione-hemolysis is preceded by an increased K^+ permeability and the change in permeability is associated with the binding of membrane sulfhydryl groups by menadione.

MATERIALS AND METHODS

Experimental animals and blood collection

Male Wistar rats were obtained as weanlings and separated into two groups. Group I rats received Purina laboratory chow. Group II rats were fed a vitamin E-deficient diet[13] purchased from General Biochemicals (Chagrin Falls, Ohio). After 12 weeks the plasma tocopherol levels[14] were below 100 μg % in the Group II rats. Rats were anesthetized with ether and blood was obtained from the abdominal aorta with a 10-ml heparinized syringe. The blood was centrifuged at 1000 $\times$ g for 15 min to separate the cells from the plasma. The plasma and buffy coat were removed by aspiration and the cells were washed three times with 3 vol. of 0.15 M NaCl. Packed cells were used throughout the study.

Reagents

Menadione was purchased from Mann Research Laboratories (New York, N.Y.) and sublimed, m.p. 106–107°. Labeled menadione, [2-*Me*-^{14}C]1,4-naphthoquinone, was purchased from Nuclear-Chicago Corp. (Chicago, Ill.) and radiochemical purity was confirmed by reverse isotope dilution studies. Thiodione, the 3-glutathionyl ether derivative of menadione, was prepared by the method of NICKERSON *et al.*[15] in 86 % yield, m.p. 198–200°. Anal. calc. for $C_{21}H_{23}N_3O_8S$: C, 52.83; H, 4.86; N, 8.81; S, 6.72. Found: C, 52.44; H, 4.86; N, 9.03; S, 6.67 (Midwest Microlab Inc., Indianapolis, Ind.). The separation of menadione (R_F 0.01) and thiodione (R_F 0.64) was accomplished on layers of silica gel G impregnated with 5 % silicone in ether; the developing solvent was water–acetone (9:1, v/v). Dialuric acid was purchased from K and K Laboratories Inc. (Plainview, N.Y.). *p*-Chloromercuribenzoate (PCMB) was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Hemolysis studies

Incubation media included saline–ethanol which contained 9 vol. 0.15 M NaCl and 1 vol. absolute ethanol, saline–phosphate–ethanol which contained 9 vol. saline–phosphate (pH 7.4) (see ref. 16) and 1 vol. absolute ethanol, and saline–sucrose–ethanol which contained 9 vol. saline–sucrose (equal volumes of 0.15 M NaCl and 0.30 M sucrose) and 1 vol. absolute ethanol. Menadione and dialuric acid were first dissolved in absolute ethanol while thiodione was first dissolved in 0.15 M NaCl. All solutions were prepared immediately before use.

Unless stated otherwise, 0.3 ml of packed cells was incubated with 5.4 ml of a 1.58 mM solution of the test compound in a specified medium. The suspension was incubated at 37° in a Dubnoff shaker and all aliquots were removed at stated time intervals for various analytical determinations.

Analytical methods

The degree of hemolysis was determined by the method of SHEETS *et al.*[17]. Hemoglobin was determined as cyanmethemoglobin[18]. Methemoglobin was measured by the EVELYN–MALLOY method[19]. The free GSH content of the erythrocytes was measured on a protein-free filtrate by the method of BEUTLER *et al.*[20]. Menadione in saline–ethanol had no effect on the GSH determination.

A modification of the 2-thiobarbituric acid test[21] was used to measure lipid peroxides. To 1.0 ml of the cell suspension or hemolysate was added 1.0 ml of 10% trichloroacetic acid and 2.0 ml of 0.67% 2-thiobarbituric acid. The mixture was heated at 50–60° for 15 min, cooled and centrifuged. The absorbance of the supernatant was measured at 534 mμ in a Beckman DU spectrophotometer and the relative amounts of lipid peroxides were expressed in absorbance units, $A_{534\,m\mu}$.

K^+ was determined in the following manner. The cell suspension, 0.7 ml, was removed, diluted with 1.0 ml of the solution used in the incubation, and centrifuged at 23000 × *g* for 1 min. The supernatant was removed and the pellet was extracted with 8 ml 0.5 M $HClO_4$. The mixture was centrifuged at 23000 × *g* for 1 min. A Perkin–Elmer atomic absorption spectrophotometer Model 303 was then used to determine the K^+ concentration in the $HClO_4$ extract.

Prelytic swelling

Swelling was estimated by the decrease in the absorbance of a cell suspension at 700 mμ. In a typical experiment, 0.3 ml of packed cells was incubated at 37° in a Dubnoff shaker with 5.4 ml of 1.58 mM menadione dissolved in saline–ethanol or in saline–sucrose–ethanol. Control cells were incubated in solutions which did not contain menadione. At stated time intervals, 0.2 ml of the cell suspension was placed in a cuvette and diluted with 3.0 ml of either isotonic saline or saline–sucrose. Absorbance was measured with a Beckman DU spectrophotometer. Hemolysis was measured in other aliquots withdrawn from the cell suspensions at the same time intervals.

Cell morphology

For phase contrast microscopy, packed cells were suspended in 1.58 mM menadione in saline–ethanol to a final concn. of 1.68 μl cells/ml. In control experiments, the same concentration of cells was suspended in saline–ethanol alone. A 0.05-ml aliquot of the cell suspension was placed in a Prior tissue culture chamber maintained at 37° and examined with an inverted Wild phase contrast microscope.

For scanning electron microscopy, 0.3 ml of packed cells was incubated at 37° in a Dubnoff shaker with 5.4 ml of 1.58 mM menadione in saline–ethanol. Control cells were suspended in saline–ethanol alone. At 15-min intervals, 0.2-ml aliquots of the suspension were removed, diluted to 2.0 ml with saline–ethanol and centrifuged at 5000 × *g* for 2 min. The cells were resuspended in 1 ml of saline–ethanol and fixed with 0.25% glutaraldehyde for 2 h at room temperature. After fixation, the cells were washed three times with 3 ml of deionized water. Small drops of the fixed cell suspension

were placed on aluminum foil disks and dried in air. The disks were coated with gold and examined on a Cambridge scanning electron microscope.

Binding of [^{14}C]menadione to erythrocyte ghosts

Erythrocytes were isolated as previously described and hemoglobin-free ghosts were prepared by the method of DODGE *et al.*[22]. The ghost suspension was diluted to the original packed cell volume with isotonic saline. Aliquots of the ghost suspension, 2.0 ml, were incubated for 20 min at 37° with 5.0 ml of 2.0 mM PCMB dissolved in isotonic saline or 5.0 ml of isotonic saline alone. The suspension was centrifuged for 40 min at 20 000 × *g* and the supernatant was removed. Ghosts were then incubated for 60 min at 37° with several concentrations of [^{14}C]menadione (0.043 μC/μmole) dissolved in saline–ethanol to a final volume of 5.0 ml. The ghost suspensions were then washed three times with 20 ml of saline–ethanol and diluted to 2.0 ml with saline–ethanol. The saline–ethanol solutions were warmed to 37° to prevent the precipitation of menadione at high concentrations. Aliquots, 0.2 ml, were diluted with 10 ml of BRAY's solution[23] and counted in a Packard Tri-Carb liquid scintillation counter.

RESULTS

Effect of GSH loss and hemoglobin oxidation on hemolysis

Hemolysis experiments with normal rat erythrocytes are summarized in Table I. Lysis increased when cells were incubated with increasing concentrations of menadione or PCMB; however, there was no correlation between either the reduced GSH and methemoglobin levels and the degree of hemolysis. When lower concentrations of

TABLE I

EFFECT OF MENADIONE, THIODIONE AND PCMB ON GSH CONTENT, METHEMOGLOBIN FORMATION AND HEMOLYSIS IN NORMAL RAT ERYTHROCYTES

Control, menadione and thiodione suspensions were incubated in saline–ethanol for 60 min at 37°. PCMB suspensions were incubated in 0.15 M NaCl for 60 min at 37°. Packed cells contained 67 mg/100 ml GSH and 28.3 g/100 ml hemoglobin. Other experimental details are described in the text.

Compound	*Concn. (mM)*	*GSH loss (%)*	*Methemoglobin (%)*	*Hemolysis (%)*
Control	–	0	0	0
Menadione	1.50	92	73	95
	0.90	90	76	32
	0.75	92	72	18
	0.30	86	74	0
Thiodione	1.50	91	63	0
	0.90	88	66	0
	0.30	91	42	0
PCMB	1.50	43	0	93
	0.75	25	0	88
	0.30	23	0	10
	0.10	22	0	0

menadione were incubated with cells, little or no lysis occurred even though there was considerable methemoglobin formation and loss of GSH. Significant hemolysis was found only at higher concentrations of menadione. Thiodione did not hemolyze the cells even though thiodione caused the same GSH loss and methemoglobin formation as menadione. PCMB hemolyzed the cells although less than 45 % of the GSH was lost and no methemoglobin was formed.

Lipid peroxide formation and hemolysis

The relationship between lipid peroxide formation and lysis was studied with menadione and dialuric acid. With normal erythrocytes, no lipid peroxides were formed when cells were incubated in saline–ethanol with either compound and only those cells treated with menadione were hemolyzed (Table II).

TABLE II

EFFECT OF MENADIONE AND DIALURIC ACID ON LIPID PEROXIDE FORMATION AND HEMOLYSIS IN NORMAL RAT ERYTHROCYTES

Suspensions were incubated in saline–ethanol at 37°.

Time (min)	*Control*		*Menadione*		*Dialuric acid*	
	2-Thiobarbituric acid value ($A_{534\ m\mu}$)	*Hemolysis (%)*	*2-Thiobarbituric acid value ($A_{534\ m\mu}$)*	*Hemolysis (%)*	*2-Thiobarbituric acid value ($A_{534\ m\mu}$)*	*Hemolysis (%)*
0	0.034	0	0.025	0	0.041	0
15	0.034	0	0.012	0	0.035	0
30	0.029	0	0.014	0	0.031	0
45	0.039	0	0.018	31	0.049	0
60	0.042	0	0.019	87	0.046	0

In another series of experiments, erythrocytes were obtained from vitamin E-deficient rats and incubated with either menadione or dialuric acid in a medium which contained the non-penetrating solute sucrose. Lipid peroxides were formed during incubation with dialuric acid and the cells were hemolyzed (Fig. 1). Lipid peroxides were not formed when vitamin E-deficient cells were incubated with menadione (Fig. 1) and the cells were not hemolyzed. Thus sucrose prevented menadione-hemolysis in E-deficient cells but had no effect on dialuric acid-hemolysis in E-deficient cells.

Membrane permeability

The prevention of menadione-hemolysis by sucrose, a non-penetrating solute, suggested that menadione altered membrane permeability to cations. Direct evidence for a change in membrane permeability was obtained by measuring K^+ efflux from normal cells incubated with menadione in saline–ethanol (Fig. 2). K^+ efflux was detected at 5 min and increased when the concentration of menadione was increased. Approx. 84 % of the intracellular K^+ was released from the cells in 30 min. No hemolysis was observed during this time period.

The leakage of K^+ from cells also occurred when cells were suspended in either saline–phosphate–ethanol or saline–sucrose–ethanol and incubated with menadione;

however, hemolysis occurred at 60 min only with cells incubated with menadione in saline–ethanol (Table III). Thus phosphate and sucrose, both non-penetrating solutes, prevented menadione-hemolysis with normal cells even though K^+ efflux was observed. In another experiment, intracellular K^+ release was not found when a cell suspension was incubated in saline–ethanol for 30 min with 1.50 mM thiodione.

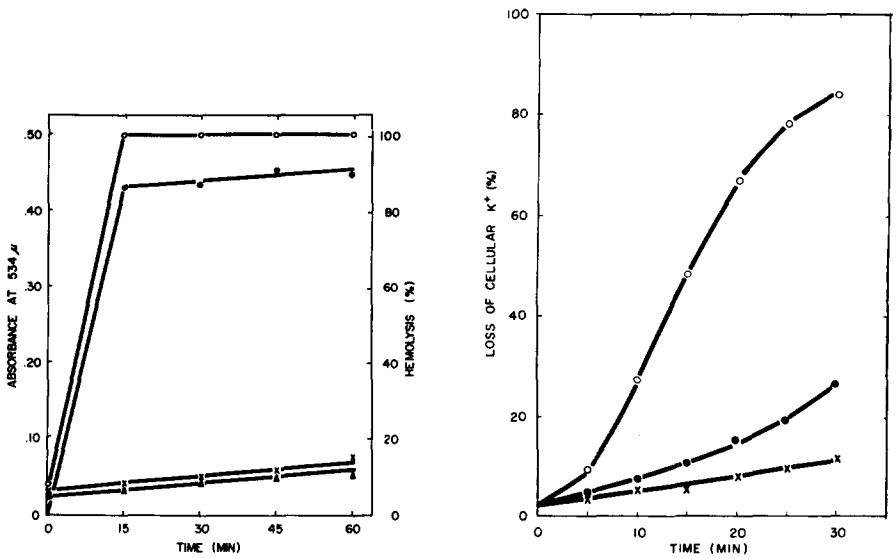


Fig. 1. Lipid peroxide formation and hemolysis found in erythrocytes from vitamin E-deficient rats. The incubation medium contained saline–sucrose–ethanol and either menadione or dialuric acid. Lipid peroxides ($A_{534\,m\mu}$) were formed when cells were incubated with dialuric acid (●—●). No lipid peroxides were formed when cells were incubated with menadione (▲—▲) or saline–sucrose–ethanol alone (×—×). Cells were hemolyzed by dialuric acid (○—○). No hemolysis was observed with menadione or saline–sucrose–ethanol alone.

Fig. 2. Menadione induced K^+ loss observed with normal rat erythrocytes. Cells were incubated with 1.50 mM menadione in saline–ethanol (○—○), 0.30 mM menadione in saline–ethanol (●—●) and saline–ethanol alone (×—×). No hemolysis occurred during this time period.

TABLE III

EFFECT OF MENADIONE AND NON-PENETRATING SOLUTES ON K^+ LOSS AND HEMOLYSIS IN NORMAL RAT ERYTHROCYTES

Incubation	*30 min*		*60 min*
	K^+ loss (%)	*Hemolysis* (%)	*Hemolysis* (%)
Saline–ethanol	12	0	0
Saline–ethanol + menadione	88	0	95
Saline–phosphate–ethanol	19	0	0
Saline–phosphate–ethanol + menadione	75	0	0
Saline–sucrose–ethanol	13	0	0
Saline–sucrose–ethanol + menadione	87	0	0

Prelytic swelling

When normal erythrocytes were incubated in saline–ethanol alone a small increase in absorbance at 700 mμ which suggested shrinking was observed in the first 10 min (Fig. 3A). When menadione was added to the incubation medium the initial increase was followed by a significant decrease in absorbance. This absorbance decrease within the first 30 min of the incubation period, a time interval in which little or no

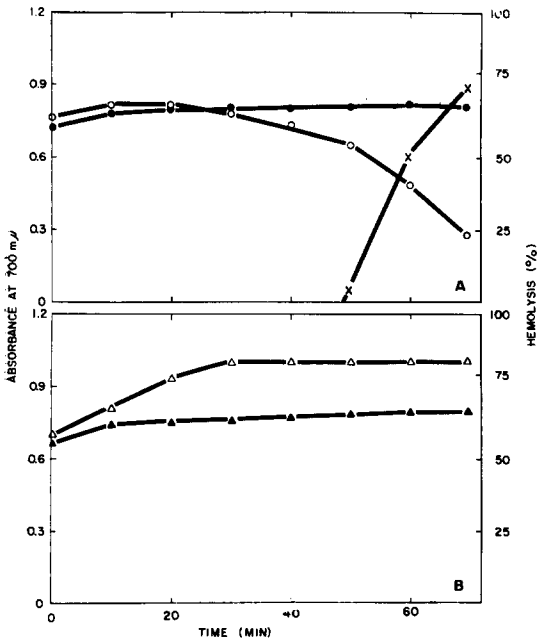
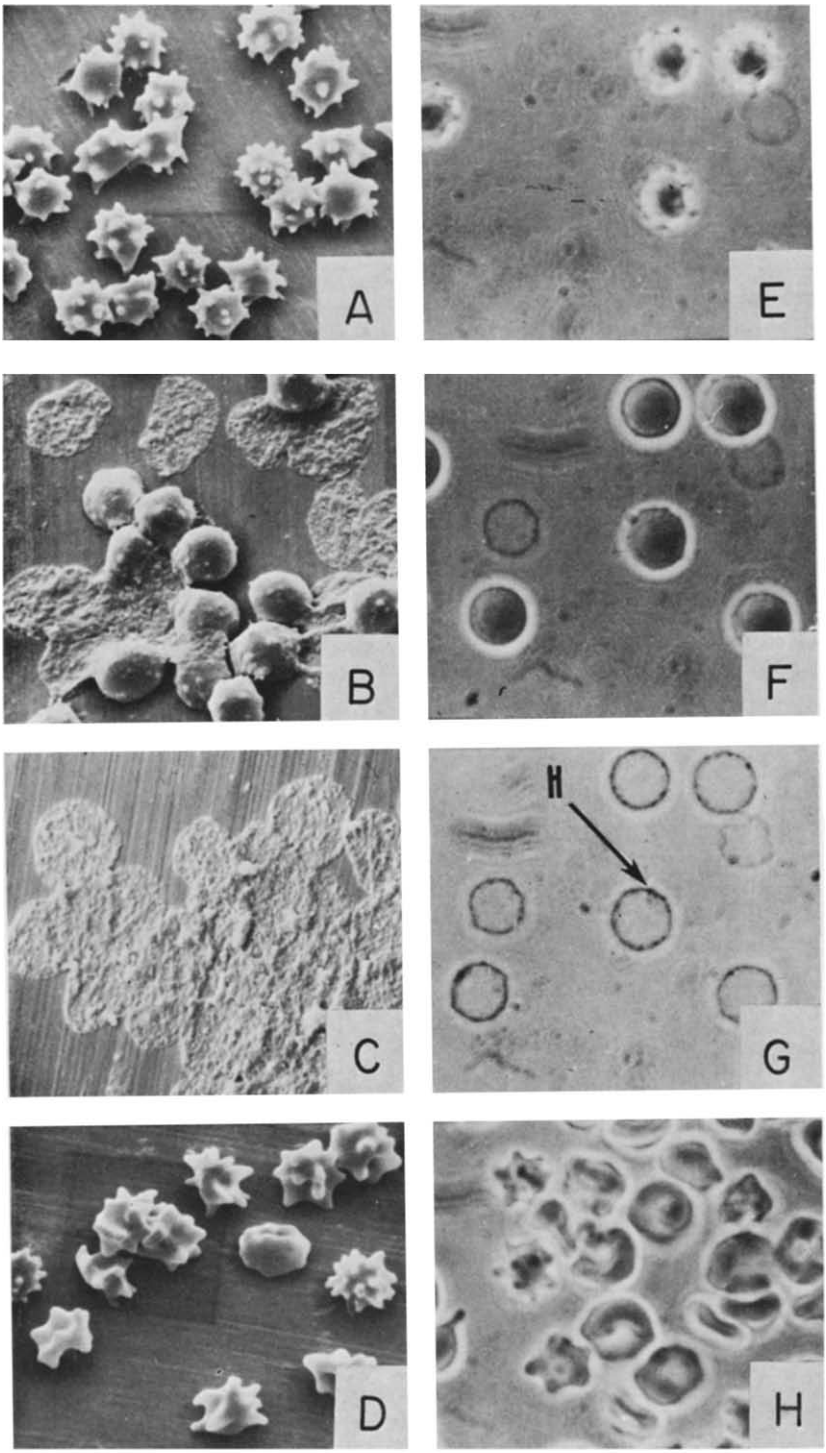


Fig. 3. Prelytic swelling and hemolysis found in normal rat erythrocytes. Absorbance of cell suspensions at 700 mμ was measured. A. Incubation with menadione in saline–ethanol (○—○); incubation in saline–ethanol alone (●—●); hemolysis measured during incubation with menadione in saline–ethanol (×—×). B. Incubation with menadione in saline–sucrose–ethanol (△—△); incubation in saline–sucrose–ethanol alone (▲—▲).

hemolysis occurred (Fig. 3A), indicated prelytic swelling. Since hemolysis began after 30 min, the absorbance decrease which was observed when cells were incubated for longer periods of time was undoubtedly associated with lysis (Fig. 3A). A small increase in absorbance was observed at 10 min when cells were incubated in saline–sucrose–ethanol alone (Fig. 3B). When menadione was added to the saline–sucrose–ethanol, absorbance continued to increase for 30 min (Fig. 3B), the same time interval in which K^+ efflux occurred (Fig. 2). Sucrose prevented the absorbance decrease found with menadione in saline–ethanol.

Cells incubated in saline-ethanol alone developed projections and appeared as 'spiny' cells which were visualized by scanning electron microscopy (Fig. 4D). Irregular projections in some cells were also noted by phase contrast microscopy (Fig. 4H). 'Spiny' cells were not glutaraldehyde fixation artifacts since these projections are not formed when cells suspended in saline are fixed with glutaraldehyde and examined by scanning electron microscopy and interference microscopy[24]. During incubation with menadione in saline–ethanol the 'spiny' cells present initially (Fig. 4A) became more


A
E
B
F
C
H
G
D
H

spherical resembling prelytic spheres (Fig. 4B) and then hemolyzed (Fig. 4C). This swelling sequence was followed in 3 cells by phase contrast microscopy (Figs. 4E–G) Heinz bodies were identified after prelytic spheres were converted to ghosts (Fig. 4G). The morphological changes in the initial formation of 'spiny' cells and the subsequent formation of prelytic spheres were consistent with the initial increase and subsequent decrease in absorbance at 700 mμ.

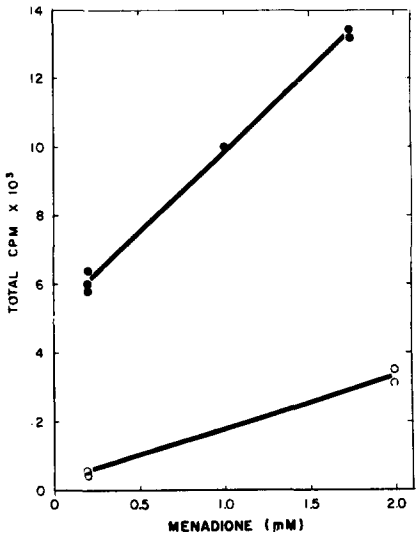


Fig. 5. Menadione binding observed with ghosts prepared from normal rat erythrocytes. Radioactivity of ghost suspensions incubated with menadione (●—●). Radioactivity of ghost suspensions pre-incubated with PCMB and then incubated with menadione (○—○).

Menadione binding to erythrocyte ghosts

The reaction between menadione and the cell membrane was studied by incubating menadione with an erythrocyte ghost suspension (Fig. 5). Menadione was bound to the ghosts and binding increased in a biphasic manner when the concentration of menadione in the incubation medium was increased. Much less menadione was bound to the ghosts when the ghosts were preincubated with 1.43 mM PCMB dissolved in isotonic saline. Preincubation in isotonic saline alone did not prevent the binding observed with menadione in these studies.

DISCUSSION

Menadione hemolyzed normal rat erythrocytes, decreased intracellular GSH and oxidized hemoglobin to methemoglobin. However, the loss of reduced GSH and the

Fig. 4. Scanning electron microscopy (A–D) and phase contrast microscopy (E–H) of menadione-treated and control rat erythrocytes. A. 'Spiny' cells after 15 min incubation with menadione (× 1900). B. Prelytic spheres after 30 min incubation with menadione (× 1900). C. Ghosts from hemolyzed cells after 45 min incubation with menadione (× 1900). D. 'Spiny' cells after 45 min incubation in saline–ethanol alone (× 1900). E. 'Spiny' cells after 25 min incubation with menadione (× 950). F. Prelytic spheres from the same cells after 60 min incubation with menadione (× 950). G. Ghosts containing Heinz bodies (H) from the same cells after 68 min incubation with menadione (× 950). H. Control cells after 60 min incubation in saline–ethanol alone. Magnification does not include photographic enlargement.

formation of methemoglobin preceded the hemolytic event. Thus low concentration of menadione caused significant changes in the concentrations of these cellular components without hemolysis. Furthermore, thiodione even at high concentrations did not cause hemolysis although as much GSH and hemoglobin were oxidized when cells were incubated with thiodione as were oxidized when cells were incubated with menadione. These experiments indicated that menadione-hemolysis was not directly associated with the loss of GSH or the oxidation of hemoglobin. Recent studies on the hemolysis induced by phenyldiazenecarboxylate suggest that the oxidation of intracellular GSH preceded the membrane damage which initiated hemolysis[25]. Thus intracellular GSH acted only in the detoxification of phenyldiazenecarboxylate.

Although menadione is an oxidant drug, no lipid peroxides were detected by 2-thiobarbituric acid in normal and vitamin E-deficient cells which were incubated with menadione. Peroxidative hemolysis was induced in E-deficient cells with dialuric acid so that this type of hemolysis could be compared with menadione-hemolysis. Peroxides were detected by 2-thiobarbituric acid when E-deficient cells were incubated with dialuric acid. Menadione-hemolysis was prevented when E-deficient cells were incubated in media which contained a non-penetrating solute such as sucrose. In contrast, peroxidative hemolysis by dialuric acid occurred even when cells were incubated in media which contained sucrose. These experiments, the absence of a positive 2-thiobarbituric acid reaction and the prevention of lysis by a non-penetrating solute, showed that menadione-hemolysis proceeded by a mechanism which did not involve extensive membrane disruption through lipid peroxidation.

The experiments with non-penetrating solutes, absorbance changes at 700 mμ and cell morphology all suggested that menadione-hemolysis was a colloid-osmotic phenomenon[26,27]. Colloid-osmotic hemolysis is preceded by a change in cation permeability, the equilibration of intra- and extracellular cation concentrations, and swelling in response to the intracellular hemoglobin concentration. Non-penetrating solutes such as sucrose and phosphate balance the osmotic pressure of intracellular hemoglobin preventing swelling and hemolysis.

A mechanism for colloid-osmotic hemolysis induced by menadione was suggested by the differences between the biological effects of menadione and thiodione and the differences between the chemical properties of these two compounds. While both menadione and thiodione are oxidizing agents, only menadione altered K^+ permeability and hemolyzed the erythrocyte. Menadione can react with sulfhydryl groups forming a 3-thioether. Thiodione is a 3-thioether derivative of menadione and this compound will not form a derivative with intracellular or membrane sulfhydryl groups. It appeared that menadione could block membrane sulfhydryl groups by binding prior to lysis. This hypothesis was supported by several experiments. Menadione was bound to ghosts and bound menadione was not removed when ghosts were re-suspended and washed several times. Furthermore, menadione binding was largely eliminated when ghosts were pre-treated with the sulfhydryl inhibitor PCMB. Several studies have shown that increased permeability to cations occurred when the membrane sulfhydryl groups of the erythrocyte were modified by PCMB[11], *p*-chloromercuribenzene sulfonate[12], and *N*-ethylmaleimide[11]. Menadione and PCMB apparently competed for the same binding sites on the erythrocyte ghost. These experiments suggest that the change in permeability and subsequent colloid-osmotic hemolysis were associated with the inhibition of membrane sulfhydryl groups by menadione.

ACKNOWLEDGMENTS

This study was supported in part by research grants GM-09506 and HE-11896 from the National Institutes of Health (U.S.A.).

REFERENCES

1 O. BROBERGER, L. ERNSTER AND R. ZETTERSTROM, *Nature*, 188 (1960) 316.
2 J. D. HARLEY AND H. ROBIN, *Nature*, 193 (1962) 478.
3 E. BEUTLER, *Nature*, 196 (1962) 1095.
4 G. COHEN AND P. HOCHSTEIN, *Biochemistry*, 3 (1964) 895.
5 C. C. TSEN AND H. B. COLLIER, *Can. J. Biochem. Physiol.*, 38 (1960) 957.
6 H. S. JACOB AND S. E. LUX, *Blood*, 32 (1968) 549.
7 L. F. FIESER, *Ann. Internal Med.*, 15 (1941) 648.
8 R. E. BENESCH AND R. BENESCH, *Arch. Biochem. Biophys.*, 48 (1954) 38.
9 R. F. SHEETS, H. E. HAMILTON, E. L. DEGOWIN AND R. L. KING, *J. Appl. Physiol.*, 9 (1956) 145.
10 C. C. TSEN AND H. B. COLLIER, *Can. J. Biochem. Physiol.*, 38 (1960) 981.
11 H. S. JACOB AND J. H. JANDL, *J. Clin. Invest.*, 41 (1962) 779.
12 R. M. SUTHERLAND, A. ROTHSTEIN AND R. I. WEED, *J. Cell Physiol.*, 69 (1967) 185.
13 H. H. DRAPER, J. G. BERGAN, M. CHIN, A. S. CSALLANY AND A. V. BOARO, *J. Nutr.* 84 (1964) 395.
14 R. G. MARTINEK, *Clin. Chem.*, 10 (1964) 1078.
15 W. J. NICKERSON, G. FALCONE AND G. STRAUSS, *Biochemistry*, 2 (1963) 537.
16 L. FRIEDMAN, W. WEISS, F. WHERRY AND O. L. KLINE, *J. Nutr.*, 65 (1958) 143.
17 R. F. SHEETS, H. E. HAMILTON AND E. L. DEGOWIN, *Proc. Soc. Exptl. Biol. Med.*, 91 (1956) 423.
18 A. HAINLINE, JR., in D. SELIGSON, *Standard Methods of Clinical Chemistry*, Vol. II, Academic Press, New York, 1958, p. 52.
19 K. A. EVELYN AND H. T. MALLOY, *J. Biol. Chem.*, 126 (1938) 655.
20 E. BEUTLER, O. DURON AND B. M. KELLY, *J. Lab. Clin. Med.*, 61 (1963) 882.
21 K. M. WILBUR, F. BERNHEIM AND O. W. SHAPIRO, *Arch. Biochem. Biophys.*, 24 (1949) 305.
22 J. T. DODGE, C. MITCHELL AND D. J. HANAHAN, *Arch. Biochem. Biophys.*, 100 (1963) 119.
23 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
24 H. J. KAYDEN AND M. BESSIS, *Blood*, 35 (1970) 427.
25 N. S. KOSOWER, K. SORG AND E. M. KOSOWER, *Biochim. Biophys. Acta*, 192 (1969) 23.
26 W. WILBRANDT, *Arch. Ges. Physiol.*, 245 (1941) 22.
27 M. H. JACOBS AND D. R. STEWART, *J. Cell. Comp. Physiol.*, 30 (1947) 79.