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Received February 20, 1959. P.S.E.B.M., 1959, v101.

Hyperlipemia and Hemolysis. II. Interaction of Sodium Oleate with Human Erythrocytes in Homologous Plasma. (24871)

SHELDON E. GREISMAN

Depts. of Medicine and Microbiology, University of Maryland, School of Medicine, Baltimore

Previous studies(1) on the interaction of sodium oleate with washed human erythrocytes suspended in buffered isotonic saline indicate that erythrocyte-oleate binding precedes hemolysis; once lytic quantities are bound, repeated iced saline washings fail to dissociate oleate or check hemolysis, and following lysis, hemolytic substances are released. The present studies consider the interaction of sodium oleate with washed human erythrocytes suspended in homologous plasma. This investigation is a continuation of studies concerned with intermediate pathways for *in vivo* hemolysis reported during conditions associated with rapid increases in serum unesterified fatty acid(2-5).

Methods and materials. Sodium oleate (Baker) was employed as the fatty acid; all solutions were prepared in sodium phosphate buffered saline, ionic strength 0.15. The pH of buffered oleate solutions, measured with glass electrode, was 7.10 at 37°C and 7.20 at 25°C. All oleate solutions remained at room temperature 24 hours prior to use since hemolytic potency declined upon standing, rapidly at first, then more slowly. Mechanical fragility of human erythrocytes suspended in fresh heparinized plasma or in buffered saline was determined at 25°C with pipette shaker generating 2 inch horizontal strokes at 220/minute; all suspensions were tested in 1 x 12 cm test tubes. On completion of shaking, the tubes were spun at 2500 rpm, 0°C, 15 minutes and supernatant hemolysis determined by a Klett-Summerson photoelectric colorimeter (550 mu. filter).

Results. Binding of oleate by plasma. Ability of mammalian plasma and plasma proteins to bind fatty acid and neutralize hemolytic activity has been documented extensively (6-9). The present data are consistent with these findings. The assumption is made that human plasma inhibition of oleate hemolysis is dependent on plasma oleate binding. Table I indicates capacity of bovine serum albumin (Armour, Fraction V) to render exogenous oleate hemolytically inert; 1 mole of bovine serum albumin binds the hemolytic equivalent of 5 to 6 moles exogenous sodium oleate at pH 7.1, 37°C. These results do not reflect *total* oleate binding capacity of bovine serum albumin since initial oleate albumin levels were not determined. Lysis inhibition by bo-

TABLE I. Capacity of Bovine Serum Albumin to Bind Exogenous Sodium Oleate.

	Exogenous sodium oleate ($\times 10^{-7}$ moles)	Bovine serum albumin* ($\times 10^{-7}$ moles)
	24	4.6
	12	2.3
	6	1.1
	3	.5
Mean	11	2.1

* Minimum quantity required to prevent oleate lysis of washed human erythrocytes at 37°C, pH 7.1. Each value is mean of 3 determinations.

TABLE II. Inhibition of Oleate Hemolysis by Human Plasma.

2.5 ml sodium oleate pretreated with .10 ml of:	Final oleate conc. ($\times 10^{-4}$ M) 4 37°C	4 25°C	1 37°C	1 25°C
Isotonic buffered saline	1.1*	1.5	2.0	5.0
Homologous human plasma	60	60	60	60
Idem, albumin	4.0	24	60	60
" globulin	1.8	3.2	10	30

* Time (min.) for 100% hemolysis after addition of 0.5 ml aliquots of buffered saline containing 5×10^5 washed human erythrocytes to 2.6 ml of oleate preparations. Each value is mean of 3 determinations.

vine serum albumin was not increased by prior incubation with oleate, confirming reported rapid fatty acid binding by serum albumin (10). Oleate hemolysis inhibition was also consistently obtained with normal human plasma and the corresponding plasma albumin and globulin fractions prepared by ammonium sulfate fractionation as described previously (11); typical findings are given in Table II. It is stressed that to inhibit hemolysis, plasma or plasma proteins must be added to oleate *prior* to insertion of washed erythrocytes; when added after erythrocyte-oleate contact, plasma accelerates hemolysis(11).

Firmness of human plasma-oleate binding was tested by studying the effect of mechanical agitation on washed human erythrocytes

TABLE III. Effect of Oleate on Mechanical Fragility of Human Erythrocytes in Fasting Homologous Plasma in 4 Subjects.

	Fasting homologous plasma		Fasting homologous plasma + sodium oleate (8×10^{-6} moles oleate/1 ml plasma)*	
	Shaking time (min.)			
	0	180	0	180
	.02†	.02	.02	.32
	.04	.05	.05	.45
	.03	.04	.04	.81
	.02	.04	.04	.96
Mean	.03	.04	.04	.63

* This concentration of sodium oleate in buffered isotonic saline produces 100% hemolysis at 25°C in 2 min. without mechanical agitation.

† % hemolysis of 0.5 ml washed packed erythrocytes suspended in 2 ml plasma. Each value is mean of 3 determinations at 25°C. Hemolysis determinations on non-shaken erythrocyte suspensions were performed after 180 min.

suspended in fasting homologous plasma and in fasting homologous plasma pretreated with 8×10^{-6} moles sodium oleate/ml. The results (Table III) suggest that human plasma oleate binding *in vitro* is not dissociated appreciably by mechanical agitation. Thus 3 hours of vigorous shaking of human erythrocytes suspended in fasting homologous plasma increased mean lysis from 0.03 (control) to 0.04%; despite pretreatment of plasma with sufficient oleate to lyse 100% of unshaken erythrocytes in isotonic saline within 2 minutes (at comparable pH and temperature), hemolysis increased from 0.04 (control) to but 0.63%. Sufficient exogenous oleate was therefore dissociated by shaking to account for only 0.58% hemolysis. The diminutive

TABLE IV. Effect of Oleate on Mechanical Fragility of Human Erythrocytes in Buffered Isotonic Saline in 4 Subjects.

	Control erythrocytes		Oleate pretreated erythrocytes*	
	Shaking time (min.)			
	0	60	0	60
	.03†	.19	.19	.74
	.03	.20	.15	1.13
	.04	.10	.10	.46
	.02	.15	.14	.63
Mean	.03	.16	.15	.74

* 0.5 ml packed washed erythrocytes suspended in 2.5 ml sodium oleate, 0.5×10^{-4} M, pH 7.2, 25°C and then immediately spun at 2500 rpm, 0°C for 3 min. followed by 5 buffered saline washings at 0°C, pH 7.2. Control erythrocytes treated identically except buffered saline substituted for oleate.

† % hemolysis of 0.5 ml packed erythrocytes suspended in 2 ml isotonic buffered saline (pH 7.2). Each value is mean of 3 determinations at 25°C. Hemolysis determinations on non-shaken erythrocyte suspensions were performed after 60 min.

quantity of oleate dissociated was further appreciated upon finding that oleate increases not only static but also mechanical fragility of human erythrocytes (Table IV). It is probable therefore that even less exogenous oleate was dissociated by mechanical agitation than would be required for 0.58% hemolysis in a static system.

Competitive oleate binding by human erythrocytes and homologous plasma. The preceding experiments concern hemolysis inhibition when oleate is *pretreated* with human plasma. Hemolytic activity of oleate

TABLE V. Competitive Oleate Binding by Human Erythrocytes and Homologous Plasma.

Lytic system	Hemolytic system (Plasma + sodium oleate) + erythrocytes	Hemolytic system (Plasma + erythrocytes) + sodium oleate
1	4.5*	.15
2	7.5	.40
3	7.5	.60

* Minimum sodium oleate ($\times 10^{-6}$ moles) required for detectable hemolysis at 37°C, pH 7.4. Each value is mean of 3 determinations.

upon *simultaneous* contact with plasma and erythrocytes was determined. Sodium oleate was added in increasing quantities to a series of tubes containing 0.6 ml normal fasting human heparinized plasma at 37°C. To a parallel series of tubes containing 0.6 ml aliquots of the same plasma, 0.4 ml packed washed homologous erythrocytes were added and all tubes mixed by inversion. Within subsequent 3 minutes, sodium oleate was added to tubes containing erythrocytes and erythrocytes were added to tubes containing oleate. All tubes were inverted 5 times immediately after each addition. Composition of the 2 series of tubes at completion of mixing was identical, the sole difference being that varying quantities of oleate were permitted to react with human plasma at 37°C in presence and absence of homologous erythrocytes. All tubes were left undisturbed at 37°C for 2 hours. The minimal quantities of sodium oleate producing visible hemolysis were then recorded. Typical results are given in Table V. The results suggest significant competition between plasma and homologous erythrocytes for binding *exogenous* oleate. By this mechanism, *in vitro* oleate hemolysis protection by human plasma can be reduced 30-fold.

Discussion. Addition of bovine serum albumin or of human plasma, plasma albumin or globulin to oleate solutions prior to insertion of washed human erythrocytes inhibits subsequent hemolysis. This fatty acid hemolysis inhibitory effect has long been recognized(6-9). If the assumption is correct that plasma inhibition of oleate lysis is dependent on oleate binding, then once oleate is bound *in vitro* by human plasma, the binding is firm. This conclusion is based upon the observation that 3 hours of vigorous mechanical agitation of human plasma containing sufficient exogenous oleate to lyse 100% of unshaken erythrocytes within 2 minutes in isotonic saline at pH 7.4 resulted in but 0.63% hemolysis; 0.58% hemolysis was attributable to exogenous oleate. Precise quantitation of oleate dissociated during mechanical agitation was not possible from hemolysis measurements since oleate increased erythrocyte mechanical fragility; the actual quantity of oleate dissociated during mechanical agitation would therefore not be comparable to that required for similar hemolysis in static systems. It could be argued that large quantities of oleate *were* dissociated from human plasma and interacted with erythrocytes during mechanical agitation but plasma inhibited lytic consequences ordinarily expected in saline media. Recent observations indicating that human plasma does not inhibit but intensifies hemolysis following oleate-erythrocyte interaction(11) precludes this possibility.

In preceding experiments, oleate was added to human plasma followed by insertion of washed erythrocytes. Obviously, erythrocytes were exposed solely to oleate the plasma was incapable of binding. Such systems generally have been the type studied previously, from which marked fatty acid hemolysis inhibitory action of mammalian plasma has been inferred. However, when erythrocytes are suspended in homologous plasma *prior* to oleate addition, the hemolysis protective potential of plasma wanes. If this finding is correlated with the preceding data, it appears that whenever exogenous oleate enters whole human blood *in vitro,* the red cells and plasma compete for binding oleate; that fraction of exogenous oleate bound by plasma is rendered hemolytically inert whereas that fraction bound by the erythrocyte constitutes the hemolytically active moiety. The oleate hemolysis protective potential of normal human fasting plasma can be reduced 30-fold by such a mechanism.

Summary and conclusions. Normal human plasma binds exogenous sodium oleate firmly *in vitro* as indicated by inhibition of oleate hemolysis despite vigorous mechanical agitation. The oleate hemolysis inhibition can be reduced 30-fold by adding oleate directly to

whole blood, rather than to plasma subsequently reconstituted as whole blood. Whenever oleate enters whole human blood *in vitro,* red cells and plasma compete for binding oleate; that fraction of oleate bound by plasma is rendered hemolytically inert whereas that fraction bound by erythrocyte constitutes the hemolytically active moiety.

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Received March 10, 1959. P.S.E.B.M., 1959, v101.

Pharmacologic Studies of a New Antihypertensive Compound N-(o-Methoxyphenyl)-N'-(3-methoxypropyl)-piperazine Phosphate (HT1479) (24872)

B. B. MORPHIS, L. W. ROTH AND R. K. RICHARDS
Dept. of Pharmacology, Abbott Laboratories, North Chicago, Ill.

In screening of compounds for antihypertensive activity certain members of a series of piperazines showed significant power to produce a fall of blood pressure, both in normotensive and hypertensive animals. Further testing of this group (all synthesized by Sommers *et al.* of Organic Chemistry Dept., Abbott Labs) yielded one (HT1479) which possessed satisfactory antihypertensive characteristics, and promised to excell in one important phase, that of relatively prolonged duration of action in anesthetized animals.

Methods. All injections were given intravenously unless otherwise specified. Blood pressure was recorded from carotid artery of cats, dogs and monkeys anesthetized with phenobarbital-pentobarbital mixture, and recorded with ink-writing mercury manometer. Blood pressure was also recorded from femoral artery of trained unanesthetized normotensive dogs. Cats were used for studies on carotid reflexes, effect of anoxia and nictitating membrane activity. Carotid sinus occlusion was achieved by 2 pneumatic cuffs which applied pressure to the carotids *in situ,* with minimum of trauma or traction. Anoxia was produced by connecting tracheal cannula to spirometer containing 100% nitrogen. Nictitating membrane experiments employed direct mechanical recording of membrane movements. The preganglionic portion of the superior cervical nerve was stimulated by square wave or 60 cycle impulses. Standardized doses of epinephrine were given before and at intervals after administration of piperazine compound. Duration and degree of inhibition of pressor response was used as semi-quantitative measure of relative potencies. Parasympatholytic action was determined in anesthetized cats by comparing depressor response to standardized doses of acetylcholine (1 μg/kg) before and after administration of drug. Antihistaminic potency of compounds was evaluated in anesthetized cats by their influence on depressor response to standardized dose of histamine (0.25 μg). Renal hypertensive rats, prepared by Grollman method (1) were used to study hypotensive effect of the compounds. Blood pressure was measured in these unanesthetized rats by photoelectric method of Kersten *et al.*(2). Direct effect on heart was studied in the Langendorff heart preparation, perfused at pressure of 40-50 cm with Ringer-Locke solution. The drugs