Ak leukemia, and identified in our laboratory in 1953(12), and later grown in tissue culture by Eddy and her associates(13). The main differences between agglutinating property of the parotid tumor virus,† and that of leukemic filtrates, are as follows: a) the parotid tumor virus agglutinates‡ when either fresh or heated (60°C ½ hr), whereas leukemic filtrates agglutinate only after heating (55°C ½ hr); b) the parotid tumor virus elutes, and for that reason the tests must be carried out at refrigerator temperature, whereas the leukemic agglutinin described here does not elute; c) murine erythrocytes are more sensitive than those of guinea pigs to the agglutinating action of leukemic filtrates.

Summary. 1) Heated (55°C ½ hr) passage A leukemic filtrates had an agglutinating effect on mouse erythrocytes in dilutions up to 1:160, occasionally higher. Fresh filtrates did not agglutinate. 2) Agglutinin could be separated from leukemic agent by ultracentrifugation. 3) Tests were done at +4°C but could also be carried out at room temperature; the agglutinin did not elute. 4) Heated (55°C ½ hr) filtrates from spontaneous Ak leukemias agglutinated only in some instances, and in higher concentrations. Whether the agglutinating potency of some Ak leukemic filtrates is related to their ability to induce leukemia, remains to be determined. 5) Fresh or heated filtrates from pooled normal mouse organs agglutinated only in some instances, and in higher concentrations. 6) The agglutinating potency of heated leukemic passage A filtrates could be inhibited by specific rabbit immune serum to 1:128 dilution. Normal rabbit serum had an inhibiting effect only in 1:4 to 1:16 dilutions.

† This refers to *tissue-culture-grown* parotid tumor virus, which agglutinates rbc *in vitro*, and also induces high incidence of parotid, and other tumors, following inoculation into newborn mice. Curiously, however, fresh filtrates prepared *from parotid tumors,* do not agglutinate, and only occasionally induce parotid, and other tumors on inoculation tests.

‡ After this manuscript was completed, the activation of hemagglutinin in certain low-potency preparations of parotid tumor (polyoma) virus, by heating to 56°C ½ hr, was also reported (Hartley, J. W., and Rowe, W. P., *Virology,* 1959, v7, 249).

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Hyperlipemia and Hemolysis I. Interaction of Sodium Oleate with Human Erythrocytes. (24851)

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The lytic action of fatty acids on washed mammalian erythrocytes suspended in isotonic saline has long been recognized, although the precise mechanism remains speculative(1,2). Lysis of the same erythrocytes, when suspended in homologous plasma, requires significantly higher concentrations of fatty acid since normal plasma contains potent inhibitors of fatty acid hemolytic activity(3-6). Despite this marked hemolysis-inhibitory property of plasma *in vitro,* evidence has accumulated that *in vivo* hemolysis

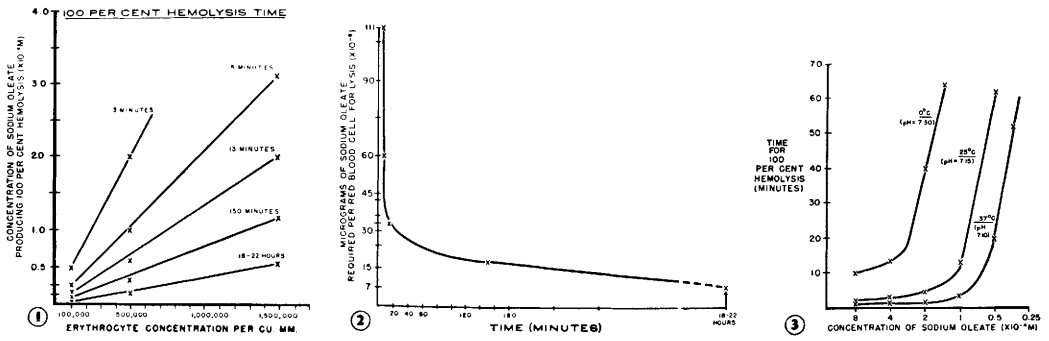

FIG. 1. Relation of human erythrocyte concentration to concentration of oleate required to induce 100% hemolysis at comparable rates at 37°C, pH 7.1.

FIG. 2. Relation of quantity of sodium oleate available per human erythrocyte (in 1.0 ml vol) to 100% hemolysis time at 37°C, pH 7.1. (Data derived from Fig. 1.)

FIG. 3. Effect of temperature on 100% hemolysis time of human erythrocytes exposed to varying concentrations of oleate. Each value represents mean of 5 determinations.

occurs during conditions associated with rapid increases in serum "free" fatty acids.* Thus *in vivo* hemolysis has been reported following alimentary hyperlipemia(8), intravenous infusions of hyperlipemic plasma or lipid emulsions(9,10), or injection of heparin into lipemic animals(11). The mechanisms underlying such *in vivo* hemolysis have not been entirely defined. The present report is the initial observation which may clarify certain intermediate hemolytic pathways.

Methods and materials. Blood of all major groups was collected from normal subjects utilizing citrate or heparin and the erythrocytes separated after 15 minutes at 2500 rpm, 0°C. Red blood cells were washed 3 times in sodium phosphate buffered saline, pH 7.15, ionic strength 0.15. The final erythrocyte suspension was adjusted by Klett-Summerson photoelectric colorimeter (filter 550 mu) so that addition of 0.5 ml of the standardized erythrocyte suspension to 2.5 ml of solution resulted in final concentration of 5 x 10^5 cells/cu mm. Sodium oleate (Baker) was employed as fatty acid; all solutions were prepared in phosphate buffered saline. The effect of temperature on pH of buffered oleate solutions as measured with glass electrode was as follows: 37°C, pH = 7.10; 25°C, pH = 7.15; 0°C, pH = 7.30. With certain specified exceptions, 2.5 ml volumes of fatty acid solutions (or saline controls) were utilized throughout, to which 0.5 ml aliquots of standardized washed erythrocyte suspension were added. All fatty acid solutions remained at room temperature 24 hours prior to use since hemolytic potency declined upon standing, rapidly at first, then more slowly. This phenomenon has been noted previously(12). Surface tension of fatty acid solutions was estimated by measuring rise of fluid into clean glass capillary tubes of 1 mm internal diameter; reproducibility was obtained within ± 4.2%. When this method was checked against reported measurements of temperature effect on surface tension of distilled water (13), the values agreed within ± 5%.

Results. The data presented in each experiment, summarized in Figures and Tables, are representative of similar experiments performed on human erythrocytes obtained from 5 normal subjects:

Effect of erythrocyte concentration on rate of oleate hemolysis. The time required for 100% lysis of human erythrocytes by varying concentrations of sodium oleate at 37°C, pH 7.1, varied with concentration of erythrocytes. Dervichian(14) reported similar results for horse erythrocytes suspended in lauric acid. For any fixed time interval within the ranges tested, concentration of sodium oleate necessary for 100% hemolysis bears a linear relation to concentration of erythrocytes to be lysed (Fig. 1). This finding indicates a stoichiometric interaction of oleate with hu-

* Since present evidence indicates that "free" fatty acids are not free but bound reversibly by serum proteins, the term unesterified fatty acid is preferable(7).

man erythrocytes and suggests that for 100% hemolysis at any given time interval at 37°C, pH 7.1, each erythrocyte binds (or inactivates) a fixed quantity of fatty acid.

Effect of "absorption" of oleate solutions with human erythrocytes. Studies to test the binding of oleate by human erythrocytes were performed by assaying ability of oleate solutions to lyse fresh human erythrocytes after "absorption" with aliquots from the same erythrocyte suspension. Typical results are indicated in Table I. Prolongation in hemolysis time of the supernatant solution obtained after oleate-erythrocyte contact indicates that human erythrocytes bind (or inactivate) oleate; greater binding occurs at 37°C than at 0°C and after increased contact time. The paradoxical increase in hemolytic activity of the supernatant oleate solution when contact is prolonged so that appreciable lysis of the "absorbing" erythrocytes occurs, suggests that following rupture of erythrocyte, hemolytic substances are released.

Evidence for release of hemolytic factors following oleate hemolysis. Standardized suspensions of human erythrocytes were exposed to varying concentrations of sodium oleate at 0°C, pH 7.3 for 30 seconds and spun down

TABLE I. Binding of Sodium Oleate by Human Erythrocytes.

2.5 ml oleate solutions (2×10^{-4} M) "absorbed" with 0.5 ml standardized human erythrocyte suspensions at:		Optical density of "absorbed" oleate solutions	100% lysis time of 0.2 ml fresh standardized human erythrocyte suspensions added to 1 ml "absorbed" oleate solutions at 37°C†
Temp., °C	Time*	(550 mu)	(min.)
0	0	0	7
"	1	"	10
37	0	20	45
"	.5	32	60
"	1	100	12
"	1.5	400 (100% lysis)	5
Unabsorbed control oleate solution		0	2.5

* Minutes prior to centrifugation at 3000 rpm, 0°C, 3 min.

† Mean of 4 determinations. (The longer the lysis time, the less hemolytically active oleate present. This relation is indicated in Fig. 1.)

TABLE II. Release of Hemolytic Substances after Oleate Lysis of Human Erythrocytes.

0.5 ml standardized human erythrocyte suspensions added to 2.5 ml sodium oleate of conc.:*	100% lysis time of washed red blood cells upon warming to 37°C†	100% lysis time of 0.05 ml fresh standardized human erythrocyte suspensions added to 2.5 ml of:†			
		Saline washing #			Lysed erythrocytes‡
		2	3	4	
($\times 10^{-4}$ M)	(min.)	(min.)			
8	2.0	>120	>120	>120	1.75
4	"	"	"	"	2.5
2	4.5	"	"	"	7.0
1	50	"	"	"	80

* Erythrocytes added to oleate solutions at 0°C, pH 7.3; after 30 sec., red cells spun down at 3000 rpm, 0°C, for 3 min., then washed 4 times with 3.0 ml buffered saline (pH 7.3) at 0°C and resuspended in a final volume of 3.0 ml buffered isotonic saline.

† Mean of 4 determinations.

‡ All tubes adjusted to same starting optical density with buffered isotonic saline to account for erythrocytes lost during washings.

within 3 minutes at 3000 rpm, 0°C. The oleate supernates were set aside for assay of residual hemolytic activity, and erythrocytes washed 4 times with 3 ml aliquots of iced buffered saline. Despite such washing, 100% hemolysis ensued upon warming to 37°C, the observed rates, (Table II) correlating with those predicted from erythrocyte binding of oleate computed from residual hemolytic activity of the oleate supernates. Tests of the last 3 iced saline washings for lytic activity at 37°C were essentially negative; the final hemolyzed supernate, however, consistently lysed additional erythrocytes. Lysis rate was significantly below that of original oleate solution and was dependent upon initial oleate concentration (Table II). These findings suggest that 1) oleate binding by human erythrocytes is not dissociated appreciably by repeated iced saline washings; 2) erythrocyte binding of oleate in fixed volume increases as the initial oleate concentration is increased; and 3) following oleate hemolysis, hemolytic substances are released. Although it seemed likely that the released lytic material was oleate or products of oleate-erythrocyte interaction, it was necessary to determine whether untreated human erythrocytes contain hemolytic factors or substances that intensify hemolytic activity of oleate traces not removed

TABLE III. Effect of Lysed Human Erythrocyte Constituents on Oleate Hemolysis.

.5 ml standardized human erythrocyte suspensions added to 2.5 ml sodium oleate (pH 7.1, 37°C) containing:		100% hemolysis time (min.) †		
		Oleate concentration ($\times 10^{-4}$ M)		
Lysate* (ml)	Isotonic saline (ml)	4	2	1
	.50	2.5	3.0	5.5
.05	.45	"	"	13
.10	.40	"	5.5	"
.20	.30	"	"	"
.50		"	6.2	30 ‡

* Lysate prepared by repeated freezing and thawing of standardized human erythrocyte suspensions followed by renewal of stroma at 4500 rpm, 0°C, 2 hr. Lysate *per se* was non-hemolytic.

† Mean of 4 determinations.

‡ When 0.50 ml lysate was added 20 sec. *after* suspension of human erythrocytes in the oleate, hemolysis time was 5.5 min.

by washing. Standardized suspensions of human erythrocytes in buffered saline were therefore lysed by repeated freezing and thawing and the stroma removed by 2 hour centrifugation at 4500 rpm, 0°C. The supernatant lysed erythrocyte constituents exhibited no detectable hemolytic activity. Table III indicates the effect of lysed erythrocyte constituents on lytic activity of sodium oleate. The findings reveal that under the defined conditions, normal erythrocyte constituents prepared by freezing and thawing retard oleate hemolysis when added to fatty acid *prior* to insertion of intact human erythrocytes (retarding effect waning as oleate concentration increases) and do not alter oleate hemolysis rate when added 20 seconds *after* oleate-erythrocyte contact. Thus the hemolytic activity observed after oleate hemolysis is probably not attributable to normal erythrocytic factors.

Quantitation of oleate required for lysis of human erythrocytes. As seen in Fig. 1, to shorten the 100% oleate hemolysis time, disproportionately greater increments of oleate are required for any given increment in erythrocytes. This relation can be expressed in terms of mean quantity of oleate required/erythrocyte for 100% lysis at any given time (Fig. 2). The quantity of sodium oleate required/erythrocyte for 100% lysis at 37°C, pH 7.1, becomes asymptotic with respect to time axis; a mean minimum of approximately 7×10^{-9} μg of sodium oleate/erythrocyte/1 ml is required for hemolysis to occur at a rate detectably greater than that of control untreated erythrocytes. Conversely, under the same conditions, when greater than 111×10^{-9} μg of sodium oleate are available/erythrocyte/1 ml, lysis time is no longer shortened detectably.

Influence of temperature on oleate hemolysis. Temperature variations markedly alter oleate hemolysis rates (Fig. 3). At least 2 mechanisms appear to be involved. Cooling from 37°C to 0°C not only decreases the rate (and/or amount) of erythrocyte oleate binding as indicated by Table I, but also slows the rate at which oleate induces lysis after erythrocyte binding. Thus when standardized suspensions of human erythrocytes were exposed to sodium oleate at 0°C for periods too brief to permit lysis and then washed 5 times with iced buffered saline, those aliquots warmed to 37°C consistently lysed more rapidly than controls held at 0°C. The role of increase in pH of oleate solutions from 7.1 to 7.3 upon cooling from 37°C to 0°C in retarding erythrocyte binding of oleate and slowing hemolysis after binding was not determined.

Effect of non-specific enzyme inhibitors on oleate hemolysis. To determine whether oleate hemolysis involved an enzymatic reaction, several non-specific enzyme inhibitors were tested. Addition of 0.01 M (final concentration) fluoroacetate, fluoride, or azide failed to alter the rate of oleate hemolysis of human erythrocytes. Sodium cyanide, however, added to 1×10^{-4} M sodium oleate (25°C, pH 7.2) delayed 100% lysis of 5 standardized human erythrocyte suspensions from a mean of 3.5 minutes (control) to: 12 minutes at 5×10^{-2} M, 20 minutes at 5×10^{-3} M, and 6 minutes at $5 \times 10^{4-}$ M of cyanide ion.

Role of surface tension reduction on oleate hemolysis. Addition of sodium oleate to buffered isotonic saline lowered surface tension, reduction at any given temperature increasing progressively with increasing oleate concentration. It seemed possible that oleate hemolysis rates might be a function of such surface tension alterations. Fig. 1, however, indicates that within the ranges of oleate and erythrocyte concentrations tested, the 100%

hemolysis time can be maintained constant despite varying fatty acid concentration (and hence varying surface tensions) by altering quantity of erythrocytes added. This suggests that the lowered surface tension to which erythrocytes are *initially* exposed upon introduction into oleate solutions is not an important determinant of 100% lysis time. That surface tension lowering plays no important role even during the latter phases of oleate hemolysis was suggested by experiments in which standardized human erythrocyte suspensions were added to increasing dilutions of 50 x 10^{-2} mg sodium oleate starting with 2 ml solutions; despite an 8-fold dilution and a progressive rise in surface tension, the 100% lysis time at 37°C, pH 7.1, remained constant.

Discussion. The initial step in lysis of human erythrocytes by oleate at pH 7.1 and with concentrations below 8 x 10^{-4} M appears to involve binding of fatty acid by the red blood cell. This was first suggested by the observation that the quantity of oleate required to induce 100% hemolysis within any given time period maintained a linear relation to number of erythrocytes to be lysed. Subsequent studies indicated that contact of normal washed human erythrocytes of all major blood groups with sodium oleate consistently resulted, prior to any detectable hemolysis, in removal of the oleate from solution. Such removal was not proved by chemical analysis, but was indicated by reduced hemolytic activity of the supernatant oleate solution. The alternative possibility that oleate was rendered hemolytically inert is untenable in view of subsequent observations. Moreover, recent evidence demonstrates that human erythrocytes are capable of binding radioactive sodium oleate-I^{131}(15) and sodium palmitate-1-C^{14}(16). Within concentrations employed, the lowered surface tension of oleate solutions did not influence hemolysis rates.

Once human erythrocytes are exposed to oleate under conditions permitting binding of hemolytic quantities, the union is firmly established; lysis cannot be checked by repeated iced saline washings. Hemolysis now proceeds at pH 7.2 $\pm$ 0.1 as a function of the quantity of oleate bound and of temperature. The temperature effect is complex. A decrease from 37°C to 0°C slows oleate hemolysis by at least 2 mechanisms: 1) erythrocyte binding of oleate is slowed (and/or reduced), and 2) lytic action of oleate following binding is retarded. Since the quantity of oleate binding is a determinant of hemolysis rate, it might be expected that once erythrocyte oleate binding capacity is saturated, the 100% hemolysis time (at any given pH and temperature) no longer would decrease as oleate concentration increases. This indeed occurs and is attained with a mean of approximately 111 x 10^{-9} μg (36 x 10^{-17} moles) of sodium oleate/erythrocyte at pH 7.1, 37°C. Although this evidence is not conclusive for erythrocyte saturation at these levels, the values may be compared with the mean of 5.3 x 10^{-17} moles of binding sites/human erythrocyte reported for sodium palmitate-1-C^{14} determined at pH 7.45, 23°C(16). The mean minimum quantity of sodium oleate required/human erythrocyte for lysis at rates detectably faster than control untreated red cells at pH 7.1, 37°C is approximately 7 x 10^{-9} μg.

Ability of cyanide ion to retard oleate hemolysis was of interest. Whether this was related to enzymatic interference is unknown; however other non-specific enzyme inhibitors —fluoroacetate, fluoride, and azide—failed to alter oleate hemolysis rates in final concentrations of 0.01 M.

Following lysis of erythrocytes binding oleate, hemolytic factors were released. Since such factors could not be detected from normal erythrocytes lysed by repeated freezing and thawing, it seems probable that oleate (or some product of oleate-erythrocyte interaction) is liberated following oleate hemolysis. Assuming the released hemolytic material to be oleate, the mechanism of release is unknown. Fatty acid probably enters the red blood cell after initial attachment to the surface membrane(1,16); since red cell ghosts bind fatty acid as avidly as intact cells(16), it is tempting to speculate that the lytic factor released following oleate hemolysis represents that fraction of oleate transferred *into* the erythrocyte. Although the hemolytic activity of such released material was always significantly lower than that of the original

oleate solutions, it is apparent that oleate possesses the potential of inducing a decelerating hemolytic chain reaction under proper dynamic circumstances—*i.e.*, when erythrocytes binding oleate lyse after moving into areas populated by normal erythrocytes.

Summary. 1. The initial step in human erythrocyte lysis by oleate at pH 7.2 ± 0.1 and concentrations below 8 x 10^{-4} M involves erythrocyte-oleate binding; oleate binding by a standard number of erythrocytes in a fixed volume is a function of oleate concentration, temperature, and contact time. 2. Once lytic quantities of oleate are bound, hemolysis proceeds at pH 7.2 ± 0.1 as a function of quantity bound and of temperature. Lysis cannot be checked by repeated iced saline washings. A mean minimum of approximately 7 x 10^{-9} μg of sodium oleate is required/erythrocyte at 37°C, pH 7.1 for lysis at rates detectably faster than controls; the sodium oleate binding mechanism appears saturated with a mean of approximately 111 x 10^{-9} μg/erythrocyte. 3. Lowered surface tension of oleate solutions does not influence hemolysis rates under conditions tested. 4. Cyanide retards oleate hemolysis; other nonspecific enzyme inhibitors fail to do so. 5. Following oleate hemolysis, hemolytic substances are released; these probably represent oleate or products of oleate-erythrocyte interaction. It is suggested that oleate possesses the potential of inducing a decelerating hemolytic chain reaction.

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Comparative Effects of Glycogen and Antigen-Antibody Reactions on Serotonin and Histamine in the Rabbit. (24852)

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In rabbit blood, serotonin (5-hydroxytryptamine) and histamine are present in, or bound to the platelets. When antigen is added to blood from a sensitized rabbit, clumping of platelets and leucocytes occurs, and serotonin as well as histamine is released from platelets (1). During anaphylaxis in the rabbit, similar changes occur. In addition, a decrease occurs in whole blood concentration of these amines(1). This decrease is secondary to disappearance of platelets from circulating blood. The rapid fall in number of platelets and leucocytes in blood is a consistent finding during hypersensitivity reactions in animal species including dogs, monkeys, guinea pigs, and rabbits(2,3,4). In humans, sensitive to

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