Effect of Ionic Strength upon Hemolysis of Paroxysmal Nocturnal Hemoglobinuria Erythrocytes.* (30634)

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The hemolysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNHE) displays the same cation requirements and pH optimum as classical immune hemolysis when human serum is used as the source of complement (C′)(1,2). A variety of activators of the first complement component (C′1), or C′1 esterase *per se*, can enhance PNHE lysis. This phenomenon suggests that PNHE hemolysis differed from classical immune sensitized sheep red cell (EA) hemolysis in that the early steps of C′ activation occur in the fluid phase, leading to direct attack of PNHE by late acting complement components(3). More recent observations made in this laboratory have confirmed this speculation; C′3a isolated by TEAE chromatography is able to attach directly to PNHE, and the resultant intermediate complex, $PNHEC'_{3a}$, resembles in all respects its counterpart in immune hemolysis, $EAC'_{1,4,2,3a}$. Both intermediates lyse in high dilutions of human serum in the absence of Ca^{++} or Mg^{++}, and both are lysed by a mixture of C′3b and C′3c isolated by DEAE cellulose chromatography. These observations led to the conclusion that PNHE hemolysis in acidified human serum *in vitro* is mediated by the C′ system(4).

The role of ionic strength in controlling the efficacy of classical immune hemolysis by C′ has been the subject of several recent reports (5-8). These studies have demonstrated that reduction in ionic strength results in substantial increase in C′ titer. The present report concerns the effect of reduced ionic strength on PNHE lysis. The results demonstrate that PNHE lysis, like C′ dependent immune hemolysis, is enhanced by low ionic strength.

Materials and methods. The following have all been described previously: The preparation of barbital buffered saline (BBS) at various pH's(2); the collection and storage of human serum(3); collection and storage of normal human red cells and PNHE(3); estimation of acid hemolysis *in vitro* with or without various additions to the hemolytic system(3); isolation of C′1 esterase(3); isolation of C′3a(4); preparation and lysis of $PNHEC'_{3a}$(4). Isotonic buffers of varying ionic strength were prepared using sucrose-BBS or mannitol-BBS as described by Rapp and Borsos(8).

Human serum at various ionic strengths was prepared as follows: a series of buffers at $\mu = 0.150, 0.122, 0.094, 0.065, 0.037,$ and 0.009 was prepared using proportionate amounts of BBS and either mannitol-BBS or sucrose-BBS, pH 6.5. Ten ml serum was dialyzed for 16 hours at 4°C, against 500 ml of each buffer. Following dialysis, precipitate formation increased in quantity as ionic strength decreased, starting at $\mu = 0.094$. Preliminary tests showed that the results were not altered if hemolysis was performed in the presence or absence of the precipitate, therefore it was usually removed by centrifugation before the hemolytic experiment. The sera were then all readjusted to pH 6.5 with one or two drops of 0.3 N HCl and were left on ice until used.

In studying the effects of ionic strength on the hemolysis of PNHE, 0.05 ml of a 20% PNHE suspension in 0.15 M NaCl was added to 1 ml of serum. In the experiments utilizing polyinosinic acid (poly I) or C′1 esterase the cells and serum were mixed in similar volumes, and 0.1 ml of the material in question dissolved in 0.15 M NaCl was immediately added. All hemolytic tests using $PNHEC'_{3a}$ were performed in serum containing 0.0075 M Na_3HEDTA. All ionic strengths have been corrected for contributions made by the various additions; the ionic strength

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TABLE I.

	20% suspension PNHE (ml)	20% suspension normal red cells (ml)	Serum ionic strength* .150 (ml)	.123† (ml)	.096† (ml)	.069† (ml)	.042† (ml)	O.D. 540 mμ	% lysis
A. PNH red cells	.05		1.0					.910	45.9
+ serum	.05			1.0				.915	46.2
	.05				1.0			1.004	50.8
	.05					1.0		1.625	82.1
	.05						1.0	1.785	90.2
	.05		1.0					1.980	100 (F and T)‡
B. PNH red cells	.05		1.0					.010	.5
+ heated serum	.05			1.0				.048	2.4
(56°, 30 minutes)	.05				1.0			.060	3.0
	.05					1.0		.080	4.0
	.05						1.0	.075	3.8
C. Normal red cells		.05	1.0					.012	.4
+ serum		.05		1.0				.038	1.3
		.05			1.0			.023	.8
		.05				1.0		.025	.9
		.05					1.0	.036	1.2
		.05	1.0					2.95	100 (F and T)‡

* In this and all subsequent graphs and tables, the ionic strength shown is the final one obtaining after all additions have been made.
† Dialyzed *versus* mannitol-BBS.
‡ Freeze and thaw.

of 0.0075 M Na_3HEDTA was taken as 0.045.

Results. Starting at $\mu = 0.096$ there is a gradual rise in PNHE lysis as ionic strength decreases. Hemolysis of PNHE reaches a maximum at $\mu = 0.042$ and diminishes as the ionic strength is then lowered to 0.016. PNHE lysis varies somewhat with the nature of nonelectrolyte used to maintain tonicity; however, in contrast to immune lysis(8), sucrose provides a more favorable milieu than mannitol. Normal red cells tested under similar conditions show no hemolysis. Heating the serum at 56° for 30 minutes destroys its capacity to hemolyze PNHE at all of the ionic strengths tested (Table I, Fig. 1).

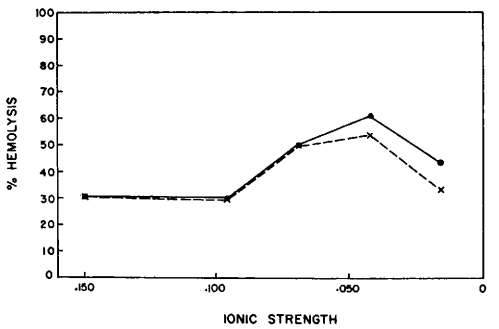

FIG. 1. Effect of ionic strength upon PNH red cell hemolysis. ●—● Sucrose-BBS. ×—× Mannitol-BBS.

Both poly I and C'1 esterase enhance PNHE lysis, and in both cases the enhancement of hemolysis observed at $\mu = 0.150$ is greater than that produced by simply lowering the ionic strength. At ionic strengths below $\mu = 0.150$ the addition of these enhancing materials does not further increase hemolysis beyond the values observed when they are used at $\mu = 0.150$; *i.e.,* the enhancing effects of a C'1 activator or C'1 esterase and decreased ionic strength are not additive (Table II).

Low ionic strength has little or no effect on the ability of isolated C'3a to combine with PNHE (Table III). The hemolysis of the intermediate complex $PNHEC'_{3a}$ in EDTA serum is inhibited by decreasing ionic strength (Table IV).

Discussion. Recent work in this laboratory has emphasized the role of the C' system in PNHE *in vitro* acid hemolysis(1-4). The present observations demonstrate another similarity between the PNHE hemolytic system and classical C' dependent immune hemolysis; both are enhanced by reducing ionic

TABLE II. Ability of Polyinosinic Acid and C'1 Esterase to Enhance PNHE Lysis at Varying Ionic Strengths.

Serum ionic strength	Test material: Saline control O.D. 540	Saline control % lysis	Poly I (7 μmol/ml) O.D. 540	Poly I (7 μmol/ml) % lysis	C'1 esterase (60 units/ml) O.D. 540	C'1 esterase (60 units/ml) % lysis
.150	.300	14.8	1.030	50.7	.632	31.1
.126	.294	14.5	1.100	54.2	.635	31.3
.102			1.060	52.3	.663	32.6
.076	.446	22	1.060	52.3	.649	31.9
.052	.555	27.3	1.063	52.4	.690	34

The hemolytic system consisted of 1 ml of serum, pH 6.5, at varying ionic strengths, .05 ml of a 20% suspension of PNHE in .15 M saline, and .1 ml of .15 M NaCl or test material, added in the order described. Degree of hemolysis was determined after 30 minutes incubation at 37°. Ionic strengths shown are the final ones calculated after all additions to the hemolytic system had been made. One hundred per cent O.D. 540 $m\mu = 2.03$ (freeze and thaw).

strength. Lysis of sensitized erythrocytes proceeds best at $\mu = 0.055$. The optimum ionic strength for PNHE lysis is 0.042.

Reduction of ionic strength increases the efficiency of immune hemolysis by several mechanisms. Included amongst these are (1) more efficient binding of C'1a to EA with resultant increase in the efficiency of $EAC'_{1,4}$ and $EAC'_{1,4,2}$ formation(8), and (2) an enhancement of the reaction $EAC'_{1,4,2} + C'3 \rightarrow$ hemolysis(5,7,8). The latter effect has been shown to be paradoxically dependent on the number of C'2 sites per cell(8). The precise intermediate steps favored by low ionic strength in the reaction between $EAC'_{1,4,2}$ and the C'3 subcomponents is unknown. The present data suggest that low ionic strength favors reactions involved in attaching C'3a to the cell, rather than later steps in immune lysis. Assuming that the C'3a capable of direct attachment to PNHE is already in an activated form, and recognizing that its attachment to PNHE is not enhanced by low ionic strength, the suggestion is made that in immune hemolysis low ionic strength favors the activation of C'3a by C'2 at the cell membrane. PNHE hemolysis, dependent upon fluid phase processes of C'3a activation, is enhanced by a similar mechanism involving fluid phase C' components. In addition, reduction in ionic strength may also serve to initiate a sequence of events involving early C' components similar to those initiated by C'1 esterase or C'1 activators, since the observation that the stimulation of PNHE lysis by reducing ionic strength and the stimulation of PNHE lysis by C'1 esterase or poly I are not additive, suggests that they operate *via* some mechanism common to both.

TABLE III. Effect of Ionic Strength on Formation of $PNHEC'_{3a}$.

$PNHEC'_{3a}$ formed at ionic strength	O.D. 540 mμ	% lysis
.150	1.180	46.8
.037	1.140	45.2
100% lysis (freeze and thaw)	2.524	

Equal buttons of PNHE (washed 3 times in .15 M NaCl, and once in BBS ($\mu = .150$) or mannitol-BBS ($\mu = .037$) were exposed to .2 ml C'3a (500 μg protein/ml) in the indicated buffer for 5 min. at 37°. They were then washed 2 times at 0°C in the same buffer and suspended in 1 ml serum, pH 6.5, containing .015 M Na_3HEDTA; hemolysis was performed at 37° for 30 min. PNHE will not hemolyze under these conditions.

TABLE IV. Effect of Ionic Strength on the Hemolysis of $PNHEC'_{3a}$ by EDTA-Serum.

EDTA-serum ionic strength	PNHE O.D. 540 mμ	PNHE % lysis	$PNHEC'_{3a}$ O.D. 540 mμ	$PNHEC'_{3a}$ % lysis
.195	.011	.6	.477	30
.082	0	0	.152	9.5
.054	.017	.9	.085	5.3
100% lysis (freeze and thaw)	1.816		1.598	

The hemolytic system consisted of 1.05 ml serum, pH 6.5, at varying ionic strength containing .0075 M Na_3HEDTA and .05 ml of a 20% suspension of the appropriate cell type. Hemolysis was carried out at 37° for 30 min. Ionic strengths shown include that contributed by the EDTA (μ .0075 M EDTA = .045).

Summary. The *in vitro* acid hemolysis of red cells from patients with paroxysmal noc-

turnal hemoglobinuria (PNHE) is intensified by reducing the ionic strength to 0.042. The ability of PNHE to form an intermediate complex by direct reaction with isolated C'3a ($PNHEC'_{3a}$) is not affected by decreasing the ionic strength; the hemolysis of $PNHEC'_{3a}$ is inhibited by reduction in ionic strength. The effects of low ionic strength and first complement component (C'1) esterase or a C'1 activator upon the enhancement of PNHE lysis are not additive, which suggests that these factors share a common mechanism. The effects of ionic strength on PNHE hemolysis are similar to those observed in other complement dependent hemolytic systems.

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Extent of Tocopherol Depletion *Versus* Onset of Creatinuria in Rats Fed Saturated or Unsaturated Fat.* (30635)

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The rate of production of a specific tocopherol-deficiency sign in the rat, nutritional muscular dystrophy, has been related to the fatty acid composition of the diet and of the tissues(1-5). Onset of nutritional muscular dystrophy in the rat is marked by a rather sudden, drastic increase in the excretion of creatine in the urine and is therefore readily detected.

The present experiment was undertaken to explore the interrelations between tissue α-tocopherol levels, fatty acid composition, and *in vivo* lipid peroxidation.

Experimental. Male weanling rats of the Sprague-Dawley strain were fed a tocopherol-deficient diet containing 23.7% casein, 58.2% cerelose, 4.9% salts 446(6), vitamins(7) made up in cerelose 0.7%, and 12.5% fat specially prepared to remove tocopherol(1). The diet contained 0.04 ppm of selenium as determined by neutron activation analysis.† Groups 1 & 2 were fed a tocopherol-free (<0.25 μg/g) fat containing 6.2% saturated, 8.2% monoenoic, 5.9% dienoic, and 23.7% trienoic acids, and having an iodine value(8) of 82.0. Groups 3 & 4 were fed tocopherol-free (<0.1 μg/g) coconut oil. Groups 2 & 4 received oral supplements of *d*-α-tocopheryl acetate 3 times a week.

Rats were sacrificed by dislocation of the cervical vertebrae. Small samples of the gastrocnemius and quadriceps muscle were excised for fatty acid analyses by gas-liquid chromatography as described previously(1). The remainder of the rat was then saponified in its own weight of 30% ethanolic potassium hydroxide containing 2-3% pyrogallol and an amount of 5-methyl-C^{14}-*d*-α-tocopherol (specific activity 1.1 μC/μmole) comparable to the quantity of α-tocopherol anticipated to

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