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Reversibility of Reaction Between Human C'1 and Sensitized Sheep Erythrocytes.* (28177)

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Laporte and coworkers(1) prepared EA-gpC'$_1$, the complex between sensitized sheep erythrocytes (EA) and guinea pig (gp) C'1, by incubating EA with the midpiece fraction of hydrazine treated gp serum (R4). In extending this work to the human (hu) system a marked influence of temperature on dissociation of EA-huC'$_1$ was observed, which is described in this communication.

Materials and methods. Triethanolamine buffered saline (TBS)(2) containing 0.05% gelatin (Knox Intravenous) was used for all dilutions and washes. EA, prepared in the presence of EDTA(3), were washed and resuspended to a concentration of 5×10^8 cells/ml. All intermediate complexes prepared from EA were adjusted to this concentration unless otherwise noted. The complement reagents R1 and R4 were prepared according to the general methods described in Kabat and Mayer(4). Human R3 and the complex EA-huC'$_{1,4,2}$ were also prepared by established techniques(5). EA-huC'$_{1,4,2}$ was converted to EA-huC'$_{1,4}$ by thermal inactivation at 37°C for 45 minutes. C'1 was eluted from this complex by two 10 min extractions at 37°C with TBS containing 0.008 M trisodium ethylenediamine tetraacetic acid (Na_3-HEDTA)(1). The product, EAhuC'$_4$, was washed free of Na_3HEDTA before use.

The complex EA-huC'$_1$ was prepared by incubating one volume of R4 (usually a 1:10 dilution) with 2 volumes of EA at 37°C for 1 minute. Five volumes of TBS warmed to 37°C were added and the suspension centrifuged at room temperature for 1.5 min to minimize cooling *(vide infra)*. The sedimented cells were resuspended in TBS at 37°C, recentrifuged at room temperature for 1.5 min, resuspended in fresh TBS at 37°C and maintained at 37°C until used. Incubation of EA with R4 at 37°C for longer times than 1-3 min resulted in loss of EA-huC'$_1$ activity similar to that observed by Klein(6) in the gp system. Incubation at lower temperatures gave poor yields of EAhuC'$_1$. Tests for presence of C'1 activity bound to EA (EA-huC'$_1$, EA-huC'$_{1,4}$) were performed by mixing 1 ml of complex with 3 ml of a non-lytic dilution of R1 (usually 1:50) and incubating at 32°C for one hour (7).

Free C'1 was measured by adding test solution (1 ml) to packed EA-huC'$_4$ (1×10^9) and incubating for 5 min at 37°C to allow formation of EA-huC'$_{1,4}$. The bound C'1 was then titrated as described above. Optical density of hemolysates was determined at 541 mμ.

Results. Initial observations on dissociation of C'1 were made using the complex EA-huC'$_{1,4}$. Aliquots (1 ml) were centrifuged at 0°C or 22°C for 1.5 min, washed with 5 ml portions of TBS at either 0°C or 37°C and tested for C'1 activity. Repeated washing at 0°C decreased the C'1 activity of the complex (Table I), whereas little or no loss of activity was observed after washing at 37°C.

Stability of EA-huC'$_1$ as a function of temperature was examined by dispensing 1 ml aliquots into tubes containing 5 ml of TBS maintained at 37°C, 22°C or 0°C. After incubating for 5 min at these temperatures

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TABLE I. Change in C'1 Activity of EA-huC'$_{1,4}$ Washed at 37°C or 0°C.

No. of washes	C'1 activity 0°C	37°C
	O.D.	
1	.494	.543
2	.405	.504
3	.316	.498

tubes of the first two groups were centrifuged at 22°C (1.5 min) and the third group at 0°C. The packed cells were brought to constant temperature, 32°C, and R1 was added to determine the quantity of EA-huC'$_1$ present. The results (Table II), demonstrated that the stability of EA-huC'$_1$ was greater at 37°C than at 22°C or 0°C.

Stability of washed EA-huC'$_1$, maintained at 37°C, was measured (Table III) by removing aliquots at different times and testing for activity. A gradual decrease in activity was observed over an 80 min period.

While EA-huC'$_1$ activity was reduced by incubation at 0°C, centrifugation and resuspension in fresh TBS at 0°C completely abolished the EA-huC'$_1$ activity. This suggested that C'1 activity dissociated from EA-huC'$_1$ at low temperatures and that the reaction was reversible to some extent when the supernatant fluid remained in contact with the cells.

TABLE II. Stability of EA-huC'$_1$ Incubated at Various Temperatures.

Temperature of incubation	C'1 activity, O.D.
37°	.383
	.386
	.379
22°	.288
	.302
	.308
0°	.156
	.156
	.154

TABLE III. Stability of EA-huC'$_1$ Incubated at 37°C.

Time of incubation, min	C'1 activity, O.D.
0	.490
20	.463
40	.460
60	.422
80	.408

This reversibility hypothesis was tested by incubating 0.5 ml aliquots of EA-huC'$_1$ at 0°C for 30 min and then at t_0 transferring the tubes to a 37°C bath. At 5 min intervals thereafter the tubes were cycled between the 37°C and 0°C baths. Immediately prior to each transfer step R1 was added to one tube to determine the amount of EA-huC'$_1$ activity. The results (Fig. 1) demonstrate that

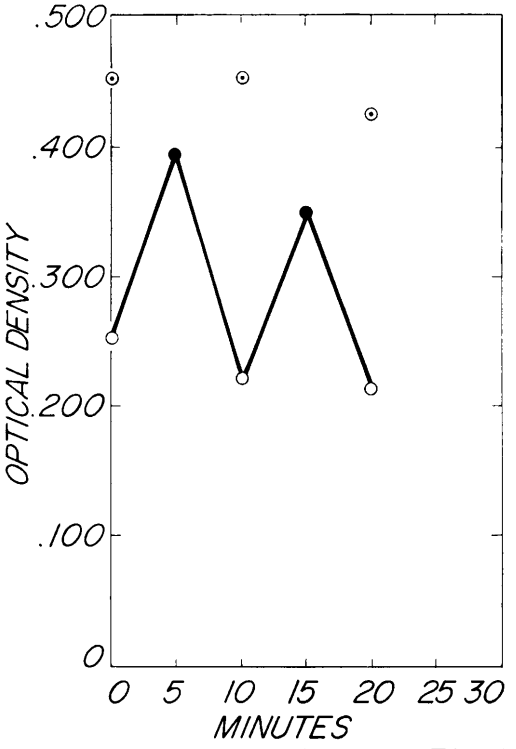

FIG. 1. Reversibility of reaction between EA and C'1 as temperature of reaction system is alternated between 0°C and 37°C at 5 min intervals; ○, activity of samples at 0°C, ●, activity of samples at 37°C, ⊙, activity of samples maintained at 37°C throughout experiment.

on incubation at 0°C EA-huC'$_1$ activity is lost whereas with each reincubation at 37°C EA-huC'$_1$ activity is restored. However the EA-huC'$_1$ activity after 5 min incubation at 37°C was lower than the EA-huC'$_1$ activity of samples maintained at 37°C throughout the experiment.

To demonstrate that C'1 was eluted in the cold, a suspension of EA-huC'$_1$ was shaken at 37°C for 10 min and centrifuged. The supernatant (37°C eluate) was reserved, and

TABLE IV. C'1 Activity of 37°C and 0°C Extractions of EA-huC'$_1$.

Dilution	C'1 activity	
	Cold eluate	Warm eluate
	O.D.	
1:5	1.149	.077
1:10	.766	.031
1:20	.476	—

the cells were resuspended to original volume in cold TBS, shaken for 10 min at 0°C, and centrifuged at 0°C. The 0°C eluate as well as the 37°C eluate were tested for C'1 activity using EA-huC'$_4$. The results (Table IV) clearly show that large amounts of C'1 are dissociated from EA-huC'$_1$ at 0°C whereas relatively small amounts of C'1 are dissociated at 37°C.

Discussion. The experiments reported establish that: 1) the complex EA-huC'$_1$, formed at 37°C, dissociates when the temperature is reduced to 22° or 0°C (Table II); 2) the dissociation is reversed by increasing the temperature to 37°C (Fig. 1); 3) C'1 activity can be isolated by elution of EA-huC'$_1$ at 0°C (Table IV); 4) the complex EA-huC'$_1$ is relatively stable at 37°C (Table III); 5) the complex EA-huC'$_{1,4}$ is more stable at 0°C than the complex EA-huC'$_1$ (Table I).

These findings are difficult to reconcile with a simple picture of Ca^{++} combining with negatively charged groups on both EA and C'1 to form the complex EA-huC'$_1$. Since the experiments reported here were carried out at pH 7.3-7.4, the negatively charged groups available for such interaction with Ca^{++} would be carboxyls. Heats of ionization of carboxyl groups(8) are small and one would therefore not expect a change in temperature from 37°C to 22°C or 0°C to markedly affect the strength of a bond between calcium and carboxyl groups. Such temperature changes, however, have been shown to have marked effects on configuration and degree of association of proteins such as gelatin(9) and tobacco mosaic virus(10). Similar temperature dependent changes in C'1 configuration or degree of association could play a role in the phenomena described here.

As noted in *Materials and methods,* samples tested for EA-huC'$_1$ activity were incubated with R1 at 32°C for one hour. If reaction (a) were a true equilibrium reaction, all

$$EA + huC'_1 \underset{0°}{\overset{37°}{\rightleftharpoons}} EA - huC'_1 \quad \text{.....(a)}$$

unwashed samples, whether taken at 0°C, 22°C or 37°C should have re-equilibrated at 32°C and shown similar activity when tested with R1. Possible explanations for the fact that the activities differed markedly (Table II) are that the R1 may have interfered with re-equilibration, or that C'4 and C'2 in R1 were inactivated by free C'1 esterase(11) present in the 22°C and 0°C samples. Studies are continuing in this area.

Summary. The complex between sensitized sheep erythrocytes and human C'1, EA-huC'$_1$ dissociated spontaneously at 22°C or 0°C. The dissociation was reversed by raising the temperature to 37°C. C'1 activity was recovered from 0°C eluates of EA-huC'$_1$.

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