Effect of Ionic Strength on Terminal Transformation in Immune Hemolysis. (29093)

LOUIS G. HOFFMANN AND ANN T. MCKENZIE (Introduced by M. M. Mayer)
Johns Hopkins University School of Medicine, Department of Microbiology, Baltimore, Md.

Mayer and Levine(1) described a reaction step in the lysis of sheep erythrocytes by antibody and complement which follows the action of all of the complement components on erythrocytes sensitized with antibody and takes place in the absence of these components from the fluid phase. They called this reaction step the terminal transformation and referred to erythrocytes which have reacted with antibody and all the complement components, but which have not yet lysed, as E*. They also observed that an increase in the NaCl concentration in the medium from 0.143 M to 0.19 M does not affect the reaction.

In several recent studies(2-4) on the effect of ionic strength (I) on immune hemolysis and on some of the individual reaction steps which comprise this process, the ionic strength of the reaction medium has been varied over a wide range while maintaining constant osmolarity by substituting K_2SO_4, or uncharged substances such as sucrose, mannitol or inositol for the NaCl usually employed. None of these investigators, however, appears to have studied the possibility that these changes might also affect the terminal transformation. The present communication records an experiment which shows that this step in immune hemolysis is markedly inhibited in buffers of low ionic strength and that the degree of inhibition depends on the nature of the substance used as a substitute for NaCl.

A veronal-buffered NaCl solution (I = 0.147) was prepared according to the "Alternative Procedure" of Kabat and Mayer (5), except that bivalent cations were omitted. Veronal-buffered solutions of glucose, mannitol and sucrose* (each 0.284 M) were prepared by dissolving appropriate amounts of these substances and 1.019 g/l of sodium 5,5'-diethylbarbiturate in water and adjusting to pH 7.4-7.5 by addition of N HCl before adjusting to volume. Buffers of I = 0.0617 were prepared by mixing 3 volumes of veronal-buffered glucose, mannitol or sucrose with 2 volumes of veronal-buffered NaCl solution. Gelatin (USP) was added to the solutions after mixing, to a concentration of 0.1%. When desired, 1.0 ml of a stock solution 0.15 M in $CaCl_2$ and 1.00 M in $MgCl_2$ was added per liter of veronal-buffered NaCl after preparation. The conductivities and pH values of the final solutions were as follows:

Solution	Conductivity Ω^{-1} cm^{-1}	pH
Veronal-buffered NaCl, I = .147	.00824	7.3_5
Veronal-buffered NaCl-glucose, I = .0617	.00338	7.3
Veronal-buffered NaCl-sucrose, I = .0617	.00324	7.3_5
Veronal-buffered NaCl-mannitol, I = .0617	.00333	7.4

Sensitized sheep erythrocytes and complement† were prepared according to Kabat and Mayer(5). The lot of complement used had a titer of 221 $C'H_{50}$/ml.‡ To prepare the E*, $5 \cdot 10^9$ sensitized erythrocytes suspended in 74.0 ml of veronal-buffered NaCl solution containing $CaCl_2$ and $MgCl_2$ in a 250 ml Erlenmeyer flask were brought to 37.0° in a water bath equipped with a wrist-action shaker, and 1 ml of a 1/14 dilution of complement in the same diluent was injected into the flask by means of a 1 ml syringe fitted with a 1½-in, #17 needle. A control flask containing $5 \cdot 10^9$ sensitized erythrocytes in 75.0 ml received no complement; its contents were subjected to exactly the same treat-

* Glucose and sucrose used were Fisher Certified Reagents; mannitol was purchased from California Foundation for Biochemical Research, Los Angeles.

† Complement was obtained as fresh frozen guinea pig serum from Probio, Inc., Nyack, N. Y., and was stored at −40° until used.

‡ $C'H_{50}$ = 50% hemolytic unit of complement, determined under standard conditions(5).

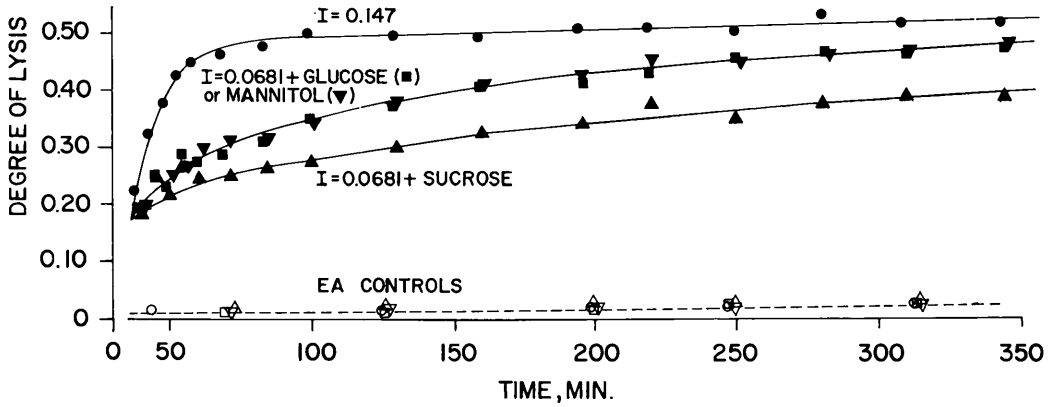

FIG. 1. Kinetics of lysis of a single E* preparation at 37.0° in 4 different buffers: I = .147 (●); I = .0681 + glucose (■); I = .0681 + sucrose (▲); I = .0681 + mannitol (▼). Aliquots (2.0 ml) of the same suspension of E* were pipetted into flasks containing the buffers listed. Samples were removed and added to 5 volumes of ice-cold buffer (I = .147) and centrifuged immediately. Supernatant fluids were collected as soon as the centrifuges stopped. Hemoglobin concentrations were measured in terms of absorbancy at $\lambda = 412$ mμ, and degrees of lysis calculated by reference to appropriate complete lysis controls. EA controls were subjected to the same treatments, except for omission of complement in the preparatory step.

ments as those of the first flask. After 10 minutes' incubation, the contents of each flask were poured into a 250 ml centrifuge bottle containing 150 ml of veronal-buffered NaCl solution without bivalent metal ions which had been kept at 0°, and immediately centrifuged at 2,000 × *g* at 0° for 10 min. The supernatant fluids were removed by suction and the sedimented cells were resuspended in 10 ml of veronal-buffered NaCl solution without bivalent cations at 0°.

Aliquots of 2.0 ml of each cell suspension were added to 125 ml Erlenmeyer flasks containing 25.0 ml of veronal-buffered NaCl solution, I = .147, 25.0 ml of veronal-buffered NaCl-glucose solution, I = .0617, 25.0 ml of veronal-buffered NaCl-sucrose solution, I = .0617, or 25.0 ml of veronal-buffered NaCl-mannitol solution, I = .0617; all of these flasks had been placed on a wrist-action shaker in a 37.0° water bath and allowed to attain that temperature beforehand. None of the diluents contained added bivalent metal ions. The flasks were closed with stoppers except during periods of frequent sampling.

A 1.0 ml sample was removed from each flask immediately after addition of the cells, followed by additional samples at appropriate time intervals. Each sample was immediately pipetted into 5.0 ml of ice-cold veronal-buffered NaCl solution (I = .147) containing no added bivalent metal ions, mixed, and centrifuged within 1 minute after removal from the reaction flask at 1,000 × *g* for 5 min at room temperature (approx. 23°). The supernatant fluids were decanted immediately after centrifugation ceased, and their absorbancies at $\lambda = 412$ mμ were determined in a Beckman model DU spectrophotometer, using a 1 cm light path. The absorbancy corresponding to complete hemolysis for each flask was taken as the average value obtained from five 1.0 ml samples taken at intervals of 1 hour, which were lysed with 5.0 ml of distilled water each and centrifuged in the manner described above; individual values showed no perceptible change with time.

The degree of lysis for each sample was calculated by dividing the absorbancy of the supernatant fluid obtained by the value corresponding to complete hemolysis for the flask from which it was obtained. These degrees of lysis are shown as a function of time for both the E* and control preparations in Fig. 1. Since the starting time of the reaction could not be fixed, an arbitrary time axis was chosen so that t = 0 corresponds to the time of addition of the complement.

The controls show that the 4 different buffers do not differ significantly in their effect on the stability of sensitized cells which have not reacted with complement. The ex-

perimental curve for I = .147 has the same appearance as numerous other kinetic curves for the terminal transformation obtained in an earlier study(6). That study showed that these curves deviate significantly from first-order kinetics with respect to E* and that the deviation takes the form of a slow linear drift, which is quite apparent in the curve shown.

The curves for I = .0681 differ markedly from that for I = .147 and, in addition, show a dependence on the nature of the uncharged solute used to maintain osmolarity. In the case of glucose and mannitol, the inhibition of E* lysis appears to take the form of a marked reduction in the rate of lysis; the small difference between the 6 hour points for I = .147 and I = .0681 in glucose or mannitol, together with the continued convergence of these curves at that time, suggest that the ultimate degree of lysis is probably unaffected. In the presence of sucrose, however, both the rate and final extent of lysis appear to be reduced. Essentially the same results were obtained in another experiment in which different lots of buffers, cells and complement were employed.

The significance of this observation in terms of the mechanism of E* lysis is not clear, but it might be possible to explain it on the basis of the studies of Green *et al*(7), who showed that the immediate effects of the action of antibody and complement on erythrocytes are loss of K^+ and uptake of Na^+. These authors state that as a result of this equilibration between cell contents and surrounding fluid, the intracellular osmotic pressure rises, leading to disruption of the cells. When, as in our experiments, the extracellular Na^+ concentration is lowered, this process is slowed down. Whether the difference in the effects of different unionized solutes can be explained on this basis is debatable; from the available data, the difference seems to be correlated with a difference in molecular weights.

The practical significance of the inhibition of E* lysis demonstrated above is clear: In studies on the effect of ionic strength on the preceding reaction steps, it is absolutely essential that the terminal transformation be allowed to proceed at I = .147 or at least at a single ionic strength. This point is emphasized when one considers the fact that the curves in Fig. 1 originate at a common point corresponding to approx. 17% lysis, which represents the lysis which took place at I = .148 during the interval between centrifugation of the cells and their distribution to the various flasks containing the different buffers. When this baseline is subtracted and the usual 60-90 minutes' incubation period is taken into account, the data show that failure to allow E* lysis to proceed at I = .147 will result in approx. 50% inhibition at I = .0681 in the presence of glucose or mannitol, and approximately 75% inhibition at I = .0681 in the presence of sucrose.

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1. Mayer, M. M., Levine, L., *J. Immunol.,* 1954, v72, 511.

2. Wardlaw, A. C., Walker, H. G., *Immunology,* 1963, v6, 291.

3. Walker, H. G., Wardlaw, A. C., *J. Immunol.,* 1963, v90, 925.

4. Rapp, H. J., Borsos, T., *ibid.,* 1963, v91, 826.

5. Kabat, E. A., Mayer, M. M., *Experimental Immunochemistry,* Charles C Thomas, Fort Lauderdale, Fla., 2nd ed., 1961, chap. 4.

6. Hoffmann, L. G., Further Studies on the Terminal Transformation in Immune Hemolysis, Sc.M. Thesis, Johns Hopkins Univ. School of Hyg. and Public Health, 1958.

7. Green, H., Barrow, P., Goldberg, B., *J. Exp. Med.,* 1959, v110, 699.

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