An Adenosinetriphosphate-Activated Hemolytic System

III. *Effect of rabbit hemolytic factor on the membrane permeability of human red cells*

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ABSTRACT Lysis of human red cells *in vitro* by an enzyme obtained from rabbit red cell hemolysates and the inhibition of this lytic activity by human stroma have been shown to require Mg^{++} and ATP, and ATP utilization has been demonstrated in both reactions. We find that sodium or potassium ions are also required for the lytic phenomenon and that they enhance the inhibition. The rate of hemolysis is not affected by the internal concentrations of these ions but depends only on the external concentration. The rate of influx and efflux of $Na^{22}Cl$ and $K^{42}Cl$ in surviving red cells is greatly enhanced both during and after treatment with rabbit hemolytic factor whereas the entry of C^{24}-sucrose, a small foreign molecule, is mediated only in the presence of hemolytic factor. Glycolysis neither protects against lysis nor enhances the activity of this system, and cardiac glycosides which are known inhibitors of active transport of ions also have no effect. It appears that lysis in this system is not brought about by increased active transport of ions into the cell but that the rabbit factor degrades or combines with some membrane component, altering permeability and resulting in increased diffusion, first of sodium and potassium ions and other small molecules, and finally of large molecules (hemoglobin) out of the cell.

Water hemolysates of rabbit red cells contain an enzyme which is capable of lysing intact human erythrocytes and this lytic action is inhibited by preincubation with human stroma prior to the addition of human red cells to the system (1, 2). Both the lytic activity of the rabbit factor and the inhibitory

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activity of human stroma are manifested only in the presence of ATP[1] and Mg^{++} in the external medium. As has been demonstrated, ATP is utilized during the lytic process and is bound to purified human stroma during inhibition of hemolytic factor by stroma (2). It has also been shown that rabbit factor labeled with S^{35} *in vivo* prior to isolation attaches to the stroma in the reaction.[2] The question then arises as to the mode of action of the factor on the red cell, the end result of which is the release of hemoglobin.

One interesting possibility is a disturbance of the normal pump mechanism of the cell causing an increase in the cellular ionic and water content to the point at which rupture of the cell membrane takes place. In this case the external ATP might serve to provide a source of high energy phosphate for the transport of ions into the cell in a manner similar to that postulated for the action of intracellular ATP (3, 4). Another possibility is that the rabbit factor destroys the integrity of the red cell membrane by altering some component of the complex membrane structure and that ATP is involved in this reaction.

In connection with the first of these possible mechanisms it was of interest to study the effect of sodium and potassium ions on the hemolytic activity of the rabbit factor and on the inhibitory activity of human stroma, and to determine the influx and efflux of these ions in human red cells during and after treatment with hemolytic factor under various conditions. The results of these and related studies are presented below.

Materials and Methods

PREPARATION OF DILUENTS AND GLASSWARE Glass-distilled water was used in the preparation of solutions and for rinsing glassware as previously described (1).

ATP Obtained as the dibarium salt from Mann Research Laboratories and converted to free ATP by addition of a calculated amount of sulfuric acid and neutralization to pH 6 with tris.

C^{14}-SUCROSE, UNIFORMLY LABELED Product of Volk Co. (Chicago), having a specific activity of 5.8 μc. per mg.

$Na^{22}Cl$, 0.9 PER CENT SALINE Product of Abbott Laboratories, having a specific activity of 28.6 μc. per mg. of NaCl.

$K^{42}Cl$ Obtained from Oak Ridge National Laboratory with a specific activity of 257 mc. per gm.

ISOTONIC TRIS-HISTIDINE·HCl BUFFERS Prepared by adding 0.3 M tris to 0.15 M histidine·HCl to obtain the desired pH.

[1] The following abbreviations are used: ATP, adenosinetriphosphate; GSH, glutathione; EDTA, ethylene-diaminetetraacetic acid; tris, tris(hydroxymethyl)aminomethane; HF, rabbit hemolytic factor.

[2] Unpublished experiments.

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HUMAN ERYTHROCYTES Human blood (group O, Rh-positive), 1 or 2 days old was centrifuged and the plasma and buffy coat were discarded. The cells were washed three times at 4° in a mixture consisting of 1 part of 0.25 M sucrose and 1 part of isotonic tris-histidine·HCl buffer, pH 7, and finally suspended to 24 per cent in the same medium unless otherwise specified. The cell suspension was usually freshly prepared each day.

HUMAN STROMA Crude and purified, hemoglobin-free, stroma were prepared as previously described (2).

HEMOLYTIC FACTOR FROM RABBIT ERYTHROCYTES A partially purified preparation obtained by ammonium sulfate fractionation of rabbit red cell hemolysate (2) was used. Prior to use, 5 ml. of the enzyme solution was dialyzed against 1000 times its volume of 0.25 M sucrose containing 0.001 M EDTA and 0.002 M tris-histidine·HCl buffer, final pH 7, for 6 hours at 4°, the dialyzing medium being renewed after 3 hours.

HEMOLYSIN TEST AND DETERMINATION OF INHIBITORY TITRE OF STROMA Both procedures were carried out as previously described (2) unless otherwise specified. Sodium and potassium concentrations of media and of cells lysed in 0.2 per cent ammonium hydroxide were determined in a process and instrument flame photometer. Hemoglobin was determined in a Beckman model-DU spectrophotometer by measuring the optical density at 540 mμ of an appropriate aliquot of red cells lysed in 0.2 per cent ammonium hydroxide using a solution of crystalline hemoglobin as a standard. C^{14} was assayed by plating suitable aliquots of cell lysates containing about 30 mg. of hemoglobin on nickel planchets which were dried and counted in an automatic nuclear D-47 gas-flow counter. All values were corrected for background and for self-absorption to 30 mg. Na^{22} and K^{42} were counted in a nuclear DS-5 scintillation well counter.

Experimental

EFFECT OF EXTERNAL SODIUM AND POTASSIUM CONCENTRATIONS ON HEMOLYTIC ACTIVITY It was observed that in an essentially sodium- and potassium-free medium higher concentrations of hemolytic factor were required to effect lysis of red blood cells than when either Na^+ or K^+ was present. This proved to be the case over a pH range of 5.4 to 7.2, the optimum pH for hemolytic activity being 6.2.

As shown in Fig. 1, the degree of hemolysis in this system, keeping the concentration of hemolytic factor and of red cells constant, increased with increasing Na^+ concentration and reached a plateau when the medium contained 15 m.eq. per liter of Na^+. When K^+ was used, although maximal activation of hemolysis was also reached at 15 m.eq. per liter, in some experiments a slight gradual decrease in hemolysis, as compared to the value at

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15 m.eq. per liter, was observed as the K^+ concentration was increased from 15 to 52 m.eq. per liter.

In another experiment in which no hemolysis occurred in a sodium- and potassium-free medium, subsequent addition of high concentrations of either Na^+ (71 m.eq. per liter), K^+ (71 m.eq. per liter), or of both ions (36 m.eq. Na^+ and 36 m.eq. K^+ per liter) resulted in identical hemolytic titres, showing

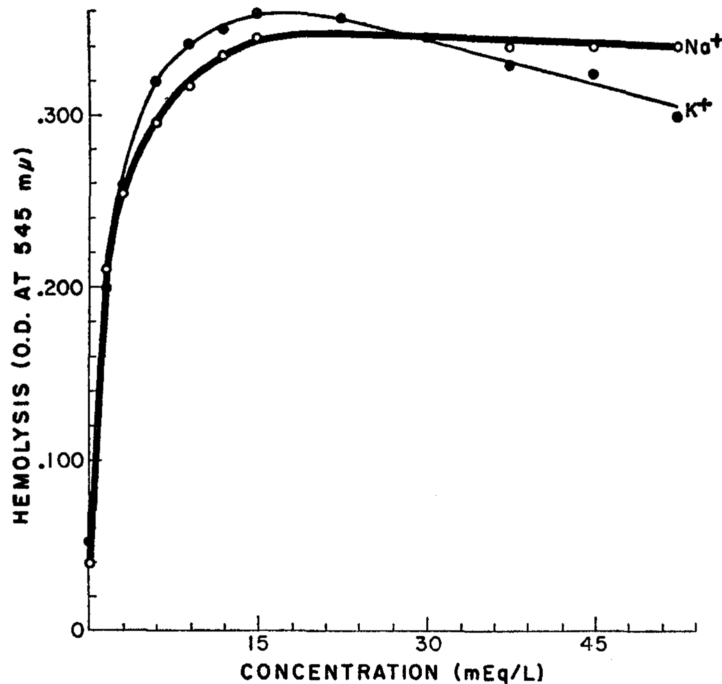

FIGURE 1. Effect of external Na^+ and K^+ concentration on hemolysis. Each hemolysin tube contained a final concentration of 1×10^{-3} M EDTA, 0.5 mg./ml. GSH, 5 per cent human red blood cells, 0.5 hemolytic units of rabbit factor, and NaCl or KCl as specified. The diluent was a 1:1 mixture of isotonic sucrose and isotonic tris-histidine·HCl buffer. pH = 6.25 Total volume = 1 ml. Incubation was carried out at 34° for 30 minutes.

that sodium and potassium were equally effective in activating hemolysis and that neither exerted an inhibitory effect on the other.

In general, when studies on the hemolytic system involved the addition of potassium, the concentration of this ion was kept within a range of 15 to 30 m.eq. per liter in order to avoid any complications that might arise from a possible inhibitory effect of excess potassium.

EFFECT OF CELLULAR SODIUM AND POTASSIUM CONCENTRATION ON THE RATE OF HEMOLYSIS Aliquots of human blood obtained from a blood bank

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in ACD solution were centrifuged and the cells were washed with and stored in the following media for 5 days at 4°.

(*a*) Sodium medium—containing NaCl (0.11 M), Na_2HPO_4 (0.025 M), $MgCl_2$ (0.002 M), inosine (0.0037 M), and glucose (0.01 M) as described by Post and Jolly (5). pH = 7.4.

(*b*) Tris medium—containing tris (0.3 M), H_3PO_4 (0.025 M), $MgCl_2$, inosine, and glucose as above and HCl to bring the pH to 7.4.

(*c*) Cells stored in plasma-ACD solution served as a control.

The storage medium was renewed every 2 days. On the 6th day, the cells were separated from their respective storage media by centrifugation and washed three times with isotonic sucrose buffered with tris-histidine·HCl, pH 7, and suspended to 24 per cent in this medium before use. The cells stored in sodium medium were found to contain 38.7 m.eq. of Na^+ and 56.2 m.eq. of K^+ per liter; tris-stored cells contained 2.9 m.eq. of Na^+ and 32.3 m.eq. of K^+ per liter; and the control cells had 19.1 m.eq. of Na^+ and 81.3 m.eq. of K^+ per liter.

The rate of hemolysis of these cells by rabbit enzyme was then followed over a period of 60 minutes in a sodium- and potassium-free medium and in a medium containing 97 m.eq. of Na^+ and 26.5 m.eq. of K^+ per liter. As can be seen in Fig. 2, all three types of cells lysed at a much greater rate in the presence of external Na^+ and K^+ than in their absence. The small differences observed among the three groups of cells are probably not significant since the nature of the experiment was such that each cell group was run independently, necessitating a separate dilution of enzyme for each.

In the tris-histidine medium the Na^+- and K^+-depleted cells did not lyse at all over the period tested. However, both the control and Na^+-rich cells started to lyse at a very slow rate in this medium, probably due to the small amounts of Na^+ and K^+ diffusing out into the medium from the cells. After 30 minutes enough cells had lysed to contribute an additional amount of Na and K ions to the medium and lysis then proceeded at a greatly increased rate. The failure of the Na^+- and K^+-depleted cells to lyse in a tris-histidine medium can be ascribed to the fact that the total sodium and potassium content of these cells had been reduced by the storage conditions to the point where the concentration of the ions which could still diffuse out into the medium was too low to initiate the lytic process.

It appears, then, that differences in the internal sodium and potassium concentration of the red cell have little, if any, effect on the rate of hemolysis in this system and that the activation of hemolysis is brought about only by the sodium or potassium ions present in the external milieu.

EFFECT OF Na^+ AND K^+ ON THE INHIBITORY TITRE OF HUMAN STROMA

It has been shown (1, 2) that when rabbit hemolytic factor is preincubated

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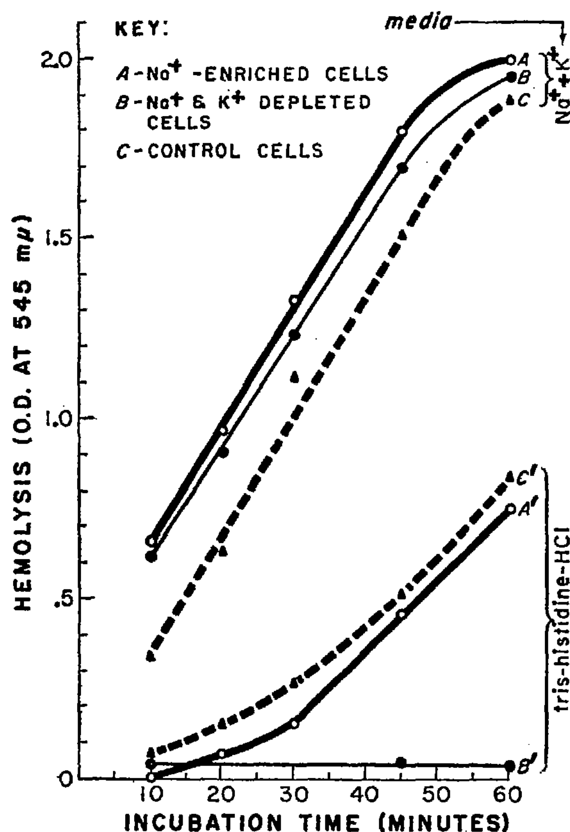

FIGURE 2. Effect of cellular Na^+ and K^+ concentration on the rate of hemolysis of red cells. Each tube contained 1.0 ml. of medium, 0.2 ml. of a 24 per cent suspension of human red cells as specified below, and 0.05 ml. of rabbit hemolytic factor. Incubation was carried out at 34°.

Curve A, Na^+-enriched cells in $Na^+ + K^+$ medium.

Curve B, Na^+- and K^+-depleted cells in $Na^+ + K^+$ medium.

Curve C, Control cells in $Na^+ + K^+$ medium.

Curve C^1, Control cells in tris-histidine·HCl medium.

Curve A^1, Na^+-enriched cells in tris-histidine·HCl medium.

Curve B^1, Na^+- and K^+-depleted cells in tris-histidine·HCl medium.

The *tris-histidine·HCl medium* was composed of $MgCl_2$ (2.4×10^{-3} M), GSH (0.6 mg./ml.), ATP (1.2×10^{-3} M), and EDTA (1.9×10^{-4} M), in isotonic tris-histidine·HCl, pH 6.3.

The *Na^+-K^+ medium* contained, in addition to the above, 97.0 m.eq. of NaCl and 26.5 m.eq. of KCl per liter of medium. pH 6.3.

with either crude or purified human stroma in the presence of ATP and Mg^{++} prior to the addition of human red cells, the hemolytic factor loses its ability to lyse the red cells. Inhibition by stroma does not occur in the absence of ATP or Mg^{++}, nor will it take place in the presence of intact red cells unless

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a very large concentration of stroma is employed. It was pertinent, therefore, to see whether the inhibition of the lytic factor by stroma was also dependent upon the presence of sodium or potassium.

Purified human stroma was incubated (for 15 minutes at 34°) with rabbit hemolytic factor, ATP, and Mg^{++}, in media containing, (*a*) 20 m.eq. per liter of K^+, (*b*) 26 m.eq. per liter of Na^+, (*c*) 13 m.eq. per liter of Na^+, and (*d*) no K^+ or Na^+. Aliquots were assayed for hemolytic activity before and after incubation in a medium containing human red cells, ATP, Mg^{++}, and

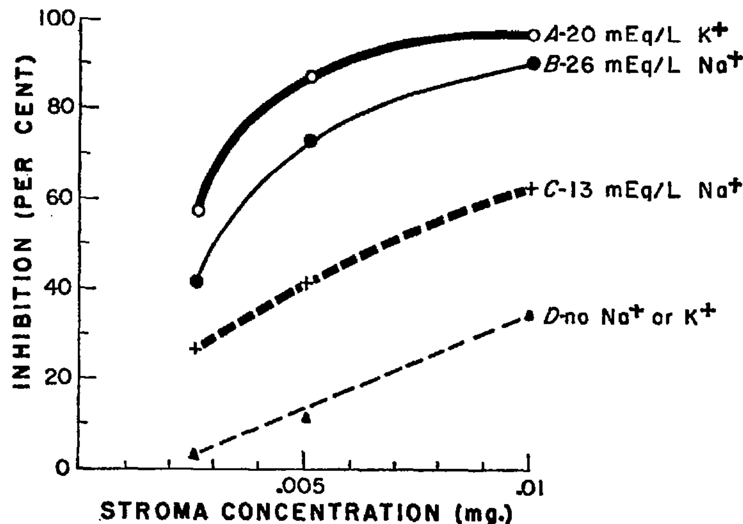

FIGURE 3. Effect of Na^+ and K^+ on the inhibitory activity of human stroma. All incubation media contained a final concentration of 6.2×10^{-3} M $MgCl_2$, 1.2×10^{-3} M ATP, 2.5 mg./ml. GSH, 2.5×10^{-2} M tris-histidine·HCl buffer, pH 7, 10 hemolytic units of rabbit factor per ml. and purified human stroma as indicated. Sucrose was added to render the solution isotonic. In addition, medium A contained 20 m.eq. per liter of KCl, medium B, 26 m.eq. per liter of NaCl, and medium C, 13 m.eq. per liter of NaCl. Incubation was carried out at 34° for 15 minutes. The total volume was 0.6 ml., of which 0.1 ml. aliquots were assayed in duplicate for hemolytic activity before and after incubation.

20 m.eq. per liter of K^+. The results, expressed as per cent inhibition of hemolysis (2), are shown in Fig. 3.

The inhibitory activity of stroma was enhanced by either sodium or potassium, although a somewhat higher concentration of either ion was required for maximal inhibition than for maximal hemolysis—15 m.eq. per liter for hemolysis as compared to 20 m.eq. per liter for inhibition. It should be pointed out that the ionic composition of the media differed only during the period of incubation of hemolytic factor with stroma, and that the subsequent assay of residual hemolytic activity of the enzyme in all cases was carried out in a medium containing an optimal concentration of K^+.

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CHANGES IN INFLUX AND EFFLUX OF Na^+ AND K^+ IN HEMOLYTIC FACTOR–TREATED RED CELLS It is generally believed that under normal conditions actively metabolizing red cells maintain their internal ionic levels constant by pumping out sodium and concentrating potassium against an external concentration gradient. Therefore, $Na^{22}Cl$ and $K^{42}Cl$ were used to determine the rate of entry and of exit of these ions in cells both during and after reaction with hemolytic factor in an attempt to determine whether or not the normal pumping mechanism of the survivors had been affected.

In the first experiment washed human red cells were preincubated for 1 hour at 34° in a glucose-inosine medium containing 17 m.eq. per liter of NaCl and 82 m.eq. per liter of KCl (in addition to the components necessary for subsequent partial lysis by rabbit hemolytic factor) in order to obtain cells in an actively glycolyzing state. The Na^+ and K^+ content of the medium was close to that of the internal cell concentration so as to minimize passive diffusion of these ions across the cell membrane. $Na^{22}Cl$ (12 m.eq. per liter; 1.8×10^9 c.p.m.) was then added to give a solution containing 7.2×10^4 c.p.m. per ml. and the cell suspension was divided into two parts, one receiving hemolytic factor and the other acting as a control. Two aliquots were withdrawn from each tube after 10 and 20 minutes' incubation and the surviving cells were immediately collected by centrifugation and washed three times at 4° with 1:1 isotonic sucrose–tris-histidine·HCl buffer at pH 7.

One aliquot of each sample of packed cells was immediately lysed with 0.2 per cent ammonia and set aside for subsequent analyses. Fresh glucose-inosine medium, lacking $Na^{22}Cl$ and hemolytic factor, was added to the duplicate aliquot to form a 20 per cent cell suspension which was reincubated for 15 minutes. The cells were then collected, washed, and lysed with ammonia as above and all the lysates were analyzed for hemoglobin, total potassium, total sodium, and Na^{22}.

As can be seen in Table I, the total sodium concentration per micromole of Hb of the hemolytic factor–treated cells remained fairly constant and equal to the controls. However, the treated cells contained 2.5 times as much Na^{22} after 10 minutes' reaction and 3.7 times as much in 20 minutes as did the control cells. On reincubation in non-radioactive medium, total sodium concentration still remained constant but the treated cells lost an average of 25 per cent of their Na^{22} whereas the controls decreased by only 12.5 per cent. After treatment with hemolytic factor, the cells contained, per micromole of Hb, 0.0171 m.eq. of K^+ as compared to 0.0194 m.eq. for the controls. On reincubation, both groups of cells increased in potassium content by 13 and 12 per cent respectively.

One would expect that if lysis in this system were caused by an active accumulation of either Na^+ or K^+ or both, that the part of the cell population which had not yet reached the point of lysis would already show a significant

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rise in the internal concentration of these ions. However, the intracellular levels of both ions in surviving cells did not change appreciably although there was a decided acceleration in the rate of exchange of Na^{22} between the cell and the medium, both during and after exposure to rabbit enzyme. This suggests that the lytic factor mediates some degradation of the membrane structure which, in turn, results in increased diffusion of Na^+ both into and out of the cell.

TABLE I

1. Incubation in $Na^{22}Cl$ medium.

Washed human red cells were suspended to 20 per cent in a medium containing NaCl (17 m.eq. per liter), KCl (82 m.eq. per liter), glucose (1×10^{-2} M), inosine (3.7×10^{-3} M), ATP (1×10^{-3} M), GSH (500 mg. per liter), $MgCl_2$ (2×10^{-3} M), EDTA (1.6×10^{-4} M), $Na^{22}Cl$ (0.48 m.eq. per liter; 7.2×10^7 C.P.M.), and tris-histidine(HCl buffer, pH 6.5, to bring the solution to isotonicity. The mixture was divided into two 25 ml. portions, one receiving 100 units of hemolytic factor and the other acting as a control. 5 ml. aliquots were removed for analysis after 10 and 20 minutes of incubation at 34°.

2. Incubation in non-isotopic medium.

Washed surviving cells from the first incubation were resuspended to 20 per cent in the same medium as described above except that $Na^{22}Cl$ and hemolytic factor were omitted.

Incubation was carried out at 34° for 15 minutes.

	Incubation in Na^{22} medium during hemolysis				Reincubation of surviving cells in non-isotopic medium		
	Time	Cell survival	Total Na^{+}*	Na^{22}	Time	Na^{22}	Na^{22} loss
	min.	*per cent*	*m.eq. per μmol Hb*	*C.P.M. per μmol Hb*	*min.*	*C.P.M. per μmol Hb*	*per cent*
1. HF-treated cells	10	65	0.00218	218	15	161	26
2. Control cells	10	100	0.00239	96.6	15	83.3	14
3. HF-treated cells	20	49	0.00227	518	15	395	24
4. Control cells	20	100	0.00227	140	15	124	11

* Average Hb content of human red cells is 5 μmol per ml. of packed cells.

In the second experiment a 5 per cent suspension of washed human red cells was incubated with hemolytic factor in a $K^{42}Cl$ medium containing 27.5 m.eq. of K^+ and less than 2 m.eq. of Na^+ per liter so that the external Na^+ and K^+ concentration was much lower than that of the internal cellular milieu. Aliquots were withdrawn at 10, 20, and 30 minutes and the surviving cells were collected by centrifugation and washed with isotonic sucrose at 4°C. The cells were then lysed and the lysate was assayed for hemoglobin, K^{42}, and total K^+. Two cell controls, one lacklng ATP and the other lacking hemolytic factor, were treated in the same manner (Table II).

The concentration of K^{42} per micromole of Hb increased with time at a similar rate in both the control cells and in the hemolytic factor–treated sur-

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vivors. However, the total potassium concentration per micromole of Hb remained constant in the controls throughout the incubation period whereas that of the treated survivors fell to 52, 34, and 17 per cent of the averaged control values after 10, 20, and 30 minutes, respectively. Therefore, the ratio of K^{42} to total potassium of the treated survivors was higher than that of the averaged control value by a factor of 1.7 after 10 minutes, 3.1 after 20 minutes, and 6.3 after 30 minutes.

TABLE II

INFLUX OF K^{42} INTO SURVIVING CELLS DURING REACTION WITH HEMOLYTIC FACTOR

Washed human erythrocytes were suspended to 5 per cent in a medium containing KCl (27.5 m.eq. per liter), Na^+ (<2 m.eq. per liter), $MgCl_2$ (2.6×10^{-3} M), GSH (0.5 mg./ml.), and potassium phosphate buffer, pH 6.5 (1×10^{-3} M). Sucrose was added to make the solution isotonic. The solution was divided into three aliquots to which the following were added at zero time:

1. Complete system. ATP (1×10^{-3} M) + hemolytic factor + $K^{42}Cl$ (1×10^6 C.P.M.).
2. HF omitted. ATP (1×10^{-3} M) + $K^{42}Cl$ (1×10^6 C.P.M.).
3. ATP omitted. Hemolytic factor + $K^{42}Cl$ (1×10^6 C.P.M.).

Incubation was carried out at 34°. The total volume was 20 ml. of which 5 ml. aliquots were withdrawn at the specified times.

	Incubation time	Cell survival	Total potassium	K^{42}	K^{42} per total potassium
	min.	*per cent*	*m.eq. per μmol Hb*	*C.P.M. per μmol Hb*	*C.P.M. per m.eq. K⁺*
Complete system	10	89	0.00563	135	23,900
	20	46	0.00373	237	63,600
	30	33	0.00186	331	178,000
HF omitted	10	100	0.01000	145	14,500
	20	100	0.00982	238	24,300
	30	100	0.00984	301	30,600
ATP omitted	10	100	0.0117	155	13,200
	20	100	0.0128	221	17,300
	30	100	0.0114	300	26,300

The results obtained in this and in the previous experiment indicate that during reaction with hemolytic factor the cell membrane becomes increasingly and progressively more permeable to various cell constituents, since surviving cells which have not been damaged to the point that leakage of hemoglobin occurs already exhibit a greatly enhanced permeability to Na^{22} and to K^{42}. When the reaction takes place under conditions where the Na^+ and K^+ concentration of the external medium is close to that of the internal cellular level the surviving cells do not exhibit any appreciable change in their ionic composition. However, when the cellular concentration is much higher than that of the medium, potassium flows out of the treated cells at a faster rate than that of the controls. In still other similar experiments it was found

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that the presence of glucose and inosine in the medium did not influence the rate of hemolysis of the cells by the hemolytic system or the efflux of K^+ from the cell during reaction in a comparatively low potassium medium and, therefore, glycolysis neither enhances nor protects the cell against the effect of rabbit factor.

PERMEABILITY OF HUMAN RED CELLS TO SUCROSE DURING AND AFTER REACTION WITH HEMOLYTIC FACTOR To further investigate the apparent increased passive permeability of surviving cells during and after treatment with hemolytic factor the influx of sucrose, to which the human red cell is normally impervious, was studied.

An 8 per cent suspension of saline-washed human red cells was incubated for 30 minutes at 34° in an isotonic medium containing ATP (1.7×10^{-3} M), $MgCl_2$ (4.3×10^{-3} M), KCl (3.1×10^{-2} M), NaCl (6.9×10^{-2} M), GSH (0.34 mg./ml.), C^{14}-sucrose (1.47×10^{-6} M; 200,000 C.P.M.), tris-histidine·HCl buffer, pH 7, and rabbit hemolytic factor as follows:—

Tube A, 12.5 hemolytic units.

Tube B, 25.0 hemolytic units.

Tube C, control, no hemolytic factor.

All experiments were run in duplicate.

After incubation the surviving cells were collected by centrifugation and, after removal of stroma and supernatant fluid, were washed three times at 4° with a 1:1 mixture of isotonic sucrose and isotonic tris-histidine·HCl buffer, pH 7. After each wash the supernatant fluid was carefully removed and set aside for C^{14} assay. After the final wash, the packed cells were then lysed with 0.2 per cent NH_4OH and the lysate was analyzed for hemoglobin and C^{14}.

The proportion of original cells which survived was 76 per cent in tube A and 60 per cent in tube B and both surviving groups contained the same amount of hemoglobin per unit volume of packed cells (determined as hematocrit values using Wintrobe tubes) as did the untreated control (tube C), within the limit of error of the determination. The C^{14} content per milliliter of the final wash solution was in no case greater than 3 per cent of the value per milliliter of the corresponding packed cells and, therefore, the error produced by extracellular trapping of the radioactive medium was negligible.

The C^{14}-sucrose concentration, expressed as counts per minute of C^{14} per micromole of Hb, of the control cells was 358 (tube C), as compared to 902 (tube A) and 2,551 (tube B) for the treated survivors. In another experiment in which 67 per cent of the cells escaped lysis, the survivors were 8.5 times as radioactive as the controls. Thus it appears that during the action of rabbit factor on the red cell not only is the cell membrane rendered more pervious to normal cell constituents such as Na^+ and K^+ but also the influx of a foreign component of small molecular weight such as sucrose can take place.

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It should be emphasized that the stromata of those cells which had been lysed by hemolytic factor were removed from the surviving cells during the washing procedure prior to final water lysis and C^{14} assay. We have found that when crude human stroma is incubated for 30 minutes with C^{14}-sucrose and then subjected to the same washing procedure as described above for red cells, it retains about 1 per cent of the C^{14}-sucrose activity of the medium per milliliter of packed stroma. Since the final water lysates were not freed of stroma prior to counting, the C^{14} content of the controls most probably represents surface-adsorbed, rather than intracellular, sucrose.

The extent of the alteration in red cell permeability of rabbit factor–treated survivors was studied in still another way. Human red cells were incubated with hemolytic factor under conditions such that only 40 per cent of

TABLE III

UPTAKE OF $NA^{22}Cl$ AND C^{14}-SUCROSE BY HEMOLYTIC FACTOR–TREATED RED CELLS

5 ml. of a 20 per cent suspension of either control cells or hemolytic factor survivors in an isotonic medium containing 37.5 m.eq. per liter each of KCl and NaCl and tris-histidine(HCl buffer, pH 7, received, as indicated, either $Na^{22}Cl$ (4.8 µmols; 696,000 C.P.M.) or C^{14}-sucrose (1.7 µmols; 400,000 C.P.M.). After 15 and 30 minutes' incubation at 34°, 1 ml. aliquots were withdrawn for analysis.

Human red cells	Incubation time	Na^{22}	C^{14}-sucrose
	min.	*C.P.M. per µmol Hb*	*C.P.M. per µmol Hb*
Hemolytic factor survivors	15	1,468	2,350
	30	2,192	2,420
Controls	15	143	2,288
	30	224	2,206

the cells lysed. The remaining cells, after being washed free of the hemolytic system components and of stroma, were then suspended to 20 per cent in an isotonic solution containing 37.5 m.eq. per liter each of NaCl and KCl, and tris-histidine·HCl buffer, pH 7. The suspension was divided into two equal aliquots, one of which received $Na^{22}Cl$ and the other C^{14}-sucrose at zero time and both were incubated at 34°. Samples were withdrawn after 15 and 30 minutes, chilled, and centrifuged. The cells were washed three times with isotonic sucrose buffered at pH 7, lysed with 0.2 per cent NH_4OH, and assayed for hemoglobin and either Na^{22} or C^{14}. Control cells were subjected to the same procedure with the sole exception that rabbit factor was omitted during incubation with the other components of the hemolytic system. The results of a typical experiment are shown in Table III.

In agreement with previous experiments, after exposure to hemolytic factor washed, surviving red cells continued to exhibit increased permeability to Na^{22} over untreated control cells, indicating a permanent alteration in that

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part of the membrane structure directly concerned with Na^+ permeability. The behavior of these cells with respect to sucrose permeability was quite different, however, since they did not take up more C^{14}-sucrose from the medium than did the controls, nor did the C^{14} content increase significantly with incubation time. In a similar experiment the C^{14} content of both treated and untreated cells assayed immediately after addition of the labeled sucrose was already about one-third of the amount found after 15 or 30 minutes' incubation.

NON-INHIBITORY EFFECT OF CARDIAC GLYCOSIDES AND AGLYCONES ON HEMOLYSIS It has been shown that strophanthin *kombé* and other cardiac glycosides and aglycones at concentrations of 10 to 0.1 gamma per ml. inhibit active transport of Na^+ and K^+ across the red cell membrane (6). The fact that hemolysis by rabbit factor was not inhibited by concentrations of up to 0.5 mg. per ml. of a variety of such compounds (*i.e.* strophanthin *kombé*, strophanthin *gratus*, digitoxin, digoxin, lanotiside C) also argues against the involvement of an active pump mechanism in this system.

DISCUSSION

It is apparent from the experiments on sodium and potassium dependence that the lytic reaction under consideration does involve these ions, in accordance with our speculations in an earlier paper (1). It is, however, equally clear from the results of the experiments with sodium and potassium depletion that the reactive site is architecturally so located that only sodium and potassium external to the cell can react. The dependence of the inhibitory reaction by purified stroma on sodium and potassium is also in accord with the postulate that the lytic reaction involves the cell stroma.

The results of the experiments on passage of radioactive sodium and potassium ions across the cell membrane during and after the course of lytic reaction indicate that this reaction is one affecting the integrity or permeability of the membrane rather than one involving the membrane pumping mechanisms for these ions. The increased permeability to sucrose during the reaction would seem to substantiate this interpretation. The reversal of this increased permeability to sucrose after the active lytic reaction is over is in contrast to the non-reversal of the increased permeability to sodium and potassium. It is, however, in line with other work (7) in which it has been shown using Fe^{59}-labeled hemoglobin that during osmotic hemolysis of human red cells all of the hemoglobin is exchangeable but that after resuspension in isotonic medium the ghost is impermeable to hemoglobin. Although one might expect that any permanent structural degradation of the cell membrane which allows more rapid diffusion of Na^+ would also allow passage of a small molecule

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like sucrose, nevertheless sucrose enters the cell only during the period of active reaction with hemolytic factor.

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