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RED CELL HEMOLYSIS CATALYZED BY A FACTOR FROM RABBIT RED CELLS

I. SOME GENERAL PROPERTIES OF THE HEMOLYTIC SYSTEM

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SUMMARY

A partial elucidation of the mechanism of a unique hemolytic system has been presented. This system involves the hemolysis of red cells by a protein factor isolated from rabbit erythrocytes in the presence of ATP, Mg^{2+} and Na^+ or K^+. The involvement of two discrete steps was indicated. The first concerned a binding of the hemolytic factor to the red cell membrane and required ATP, Mg^{2+} and Na^+ or K^+. This reaction could be monitored by a stroma-inhibition assay. The ADP–ATP exchange reaction of hemolytic factor indicated that a high-energy phosphorylated intermediate may interact with the red cell membrane. The second step in the process concerned the hemolysis of the red cells. This could be made to occur in an isolated, hemolytically active hemolytic factor–erythrocyte complex which did not require ATP, other cofactors or added hemolytic factor. The equilibration of Na^+ and K^+ occurred down a concentration gradient followed by hemoglobin efflux. The hemolytic factor–erythrocyte complex could be separated from hemolytic factor binding to the red cell membrane by taking advantage of the occurrence of binding at pH 7.5 where hemolysis was inhibited. Hemolysis by the hemolytic factor–erythrocyte complex can also be completely inhibited by 1.0 mM *N*-ethylmaleimide. The overall hemolysis reaction was inhibited by increasing concentrations of Na^+, K^+ and sucrose. The hemolytic factor-binding step was activated by Na^+ and K^+ while sucrose had no effect. ATP, a substrate for the binding step, was found to inhibit overall hemolysis and its component steps of hemolytic factor binding and hemolytic factor–erythrocyte complex hemolysis at higher concentrations ADP which was not required for hemolysis, inhibited in a manner similar to that of ATP. A comparison of the above inhibitory effects was made between the overall hemolytic reaction and its component parts and a scheme for the hemolytic process was presented.

INTRODUCTION

In 1954, Snyder *et al* [1], while studying a hemolysis test for typhus rickettsiae, first observed ATP-activated hemolysis of rabbit erythrocytes, particularly after the addition of rabbit hemolysis.

This phenomenon was subsequently investigated by Borek *et al.*[2–5], who described some of the properties of this hemolytic system. Using the crude hemolysate isolated from rabbit erythrocytes, these investigators ascertained an ATP and Mg^{2+} requirement for the reaction and described a number of inhibitors of the system[2], the most significant of which was red cell stroma. This type of hemolysis was inhibited by stroma only when preincubated with the hemolytic factor in the presence of ATP and Mg^{2+}.

Later, the enzyme complex was further purified by a single $(NH_4)_2SO_4$ fractionation between 30 and 50 % saturation followed by dialysis. Utilizing this preparation, the rate and amount of ATP disappearance was determined for both the stroma inhibition and hemolysis reactions[3]. Subsequently, the role of Na^+ and K^+ in the reaction was studied and the requirement for one of these cations for the hemolytic system was observed. The activating effect of Na^+ or K^+ upon the stroma inhibition was also ascertained[4]. Finally, utilizing some rather preliminary data involving $^{22}NaCl$, ^{42}KCl and [^{14}C]sucrose equilibration in the presence and absence of the hemolytic factor, these investigators made the possibly correct prediction that alteration of membrane permeability rather than active ion transport resulted in this type of red cell hemolysis[4]. This paper is concerned with data on some of the general properties of this complex hemolytic system which are directed toward a partial description of its mechanism. Evidence will be presented to show that this type of hemolysis involves two separable steps; a binding of the enzyme to the red cell membrane which requires ATP, Mg^{2+} and Na^+ or K^+ to form an hemolytic factor–erythrocyte complex and the disruption of red cell membrane permeability which results in cation equilibration followed by hemolysis. Inhibition of this type of hemolysis by several required cofactors and other components will also be described.

METHODS

Isolation of the hemolytic factor

Heparinized whole rabbit blood was purchased from Pel-Freez Biochemicals, Inc., Rogers, Ark. The red cells were washed three times in 0.15 M NaCl after removal of the plasma, by centrifugation. During the washing procedure, most of the white cells and platelets were removed by suction. The washed, packed red cells were frozen at −20 °C overnight before enzyme isolation. Any fraction of the red cells not used could be stored for at least several months at −70 °C without loss of enzyme activity.

Red cells obtained from about 0.67 l of whole blood were thawed and resuspended by homogenization in 5 vol. of 1 mM dithiothreitol and stored at 3 °C for 30 min with occasional shaking. The hemolysate was centrifuged at 20000 × *g* for 40 min, the supernatant was decanted and the stroma discarded. The supernatant fraction was adjusted to 30 % saturation, centrifuged and readjusted to 40 % saturation with solid $(NH_4)_2SO_4$. The 30–40 % saturated precipitate was dissolved in 4 mM dithiothreitol and mixed with 10 vol. of cold 50 % saturated $(NH_4)_2SO_4$. The precipitate was obtained by centrifugation at 20000 × *g* for 15 min, redissolved in 4 mM dithiothreitol and again mixed with 10 vol. of 50 % saturated $(NH_4)_2SO_4$. The precipitate was collected by centrifugation (20000 × *g*, 15 min), dissolved in a minimum volume with 4 mM dithiothreitol and centrifuged at 20000 × *g* for 10 min

to remove a small amount of insoluble material. The supernatant fraction was dialyzed twice for 40 min against 50 vol. of 1 mM dithiothreitol at 3 °C. The yellow-amber solution was stored at −70 °C after adjusting the final dithiothreitol concentration to 4 mM. At this temperature the factor is stable for at least 2 months. All steps in the isolation procedure were carried out at 3 °C and only glass-distilled water was used.

The average yield obtained was about 1000 units of hemolytic activity averaging a specific activity of 45 units/mg protein. A unit is defined as a 5 % hemolysis increase per min measured at 540 nm where hemolytic activity ranges from 10 to 40 % under the usual conditions for the hemolysis assay.

Further purification of hemolytic factor by the use of such techniques as Sephadex filtration, cellulose ion-exchange resins and calcium phosphate gel resulted in excessive enzyme instability and inactivation. The preparation in its present form is too crude to allow for analysis of its composition and structure

The hemolysis assay

The final cofactor concentrations in the 1.0-ml incubation medium were 1 mM ATP, 1 mM $MgSO_4$, 100 mM NaCl, 10 mM Tris–phosphate and 0 10 of packed red cells The final pH was 6.0. The red cells* were washed twice in 0.15 m NaCl–0.01 M Tris–phosphate at pH 6.0, to ensure that they were at the incubation pH. The washed red cells were resuspended in an equal volume of 0.15 M NaCl prior to addition to the incubation medium. The reaction was started by the addition of about 0.3 unit of hemolytic factor. After 15 min at 30 °C, the reaction was stopped by addition of 9.0 ml of 0.15 M NaCl containing 0.1 mM $ZnCl_2$. The surviving cells and ghosts were removed by centrifugation and the degree of hemolysis was determined as an absorbance reading at 540 nm. The results are also expressed as percent hemolysis which is the percentage of the total hemoglobin released.

The stroma inhibition assay

This consisted of a preincubation step during which the stroma interacted with hemolytic factor and inhibition occurred, followed by an incubation step in which the decrease in hemolysis was measured for an aliquot of preincubation mixture.

The preincubation medium and volume was similar to that used in the hemolysis assay except that stroma (approx. 0.20 mg protein) was substituted for red cells and 3.0 units of hemolytic factor activity was added to start the reaction. Preincubation was at 20–21 °C for 15 min. Aliquots of 0.10 ml were withdrawn and added to prepared tubes containing all components except hemolytic factor for the hemolysis assay. After incubation at 30 °C for 15 min, the reaction was stopped and absorbance measured as previously described. The uninhibited controls contained the same preincubation mixture *minus* stroma The stroma utilized was freshly prepared in the following manner. After removal of plasma and buffy coat by centrifugation and suction, the red blood cells were washed three times in 0.15 M NaCl The packed red cells were hemolyzed in 10 vol. of water and the stroma obtained was washed three additional times with water and resuspended in 0.15 M NaCl.

* Human blood used for these experiments was obtained from the Irwin Memorial Blood Bank, San Francisco, Calif

A unit of stroma inhibitory activity is defined as a decrease of 5 % hemolysis per min in the hemolytic activity of hemolytic factor after 15 min of preincubation at 20–21 °C in the presence of stroma under the described conditions for the inhibition assay. The inhibition must be 50 % or less while the residual hemolytic activity of the uninhibited controls lies between 10 and 40 %

Results are usually expressed as percent stroma inhibition which can be calculated as follows.

$$\% \text{ stroma inhibition} = \left(1 - \frac{\% \text{ hemolysis of inhibited hemolytic factor}}{\% \text{ hemolysis of uninhibited hemolytic factor}}\right) \times 100$$

Isolation of the hemolytic factor–erythrocyte complex

In a preincubation step, 0.6 unit of hemolytic factor was reacted with 0.1 ml of packed, washed, human red cells in a total volume of 1.0 ml. The medium at pH 7.6, contained 1 mM ATP and $MgSO_4$, 100 mM NaCl and 10 mM Tris–phosphate buffer. The tubes were incubated at 20–21 °C for 15 min. During this time, hemolytic factor bound to the red cell membrane while hemolysis of the hemolytic factor–erythrocyte complex was inhibited. The complex was sedimented by centrifugation after suspension in 5 0 ml of cold 0 20 M sucrose. The supernatant fractions were removed by suction and the hemolytic factor–erythrocyte complex could be washed or used directly for the assay of spontaneous hemolysis

Assay for spontaneous hemolysis of the hemolytic factor–erythrocyte complex

To start the hemolysis reaction, 0.9 ml of a prepared media of varying composition was used to resuspend the hemolytic factor–erythrocyte sediment. Incubation proceeded at 30 °C for 15 min. The reaction was stopped by the addition of 5.0 ml of 0.15 M NaCl and centrifugation to sediment the complex. Hemoglobin release was measured by reading the absorbance at 540 nm as described in the hemolysis assay procedure

Controls containing 1 mM *N*-ethylmaleimide in the incubation medium represented the amount of extraneous hemoglobin released as a result of membrane permeability changes which may have occurred during the formation and washing of the hemolytic factor–erythrocyte complex used in the spontaneous hemolysis experiments These control values ranged from 0.10 to 0.20 $A_{540\text{ nm}}$ units and were subtracted from the absorbance readings of the assay tubes to obtain net absorbance values for spontaneous hemolysis by hemolytic factor–erythrocyte except in Curve D of Fig 6

Variations of the usual procedure for the hemolysis and stroma inhibition and other assays are described under the appropriate table or figure. Protein assays were carried out by the Lowry method[6] using crystalline bovine albumin as standard.

RESULTS

The hemolysis reaction

The rate curve for hemolytic factor-catalyzed hemolysis indicated that a lag period occurred prior to hemolysis (Fig 1, Curve A). This lag was found to be de-

pendent upon the concentration of the factor. For example, 0.75 unit of hemolytic factor caused hemolysis with a lag of 2 min compared to a 5-min lag for 0.15 unit of hemolytic factor under the same conditions of incubation. Lineweaver–Burk plots of enzyme activity showed proportionality between V and hemolytic factor concentration over a range of 5 different enzyme activities from 0.15 to 0.75 unit.

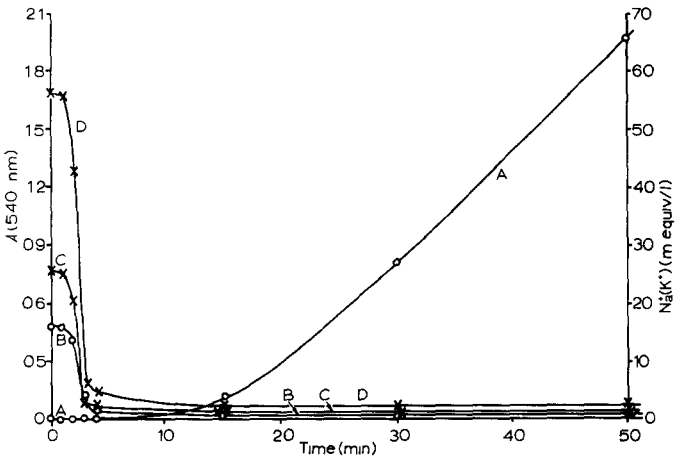

Fig 1 The sequence of events in the hemolysis process Aliquots were withdrawn for analysis at various times from the standard hemolysis incubation medium described in Methods The samples were immediately centrifuged for 2 min to remove surviving red cells Aliquots of the supernatant fractions were taken for assay of hemolysis A second aliquot of these supernatants was incubated with red cells and all cofactors required for the hemolysis assay The latter procedure indicated the extent of hemolytic factor binding to red cells as a function of residual supernatant hemolytic factor (see Table I, Expt III) The surviving red cells were washed twice with 0 25 M sucrose to remove extracellular Na^+ and K^+ The red cell pellets were extracted with 6 0 ml of water and 3 0 ml of toluene Na^+ and K^+ analysis was carried out on the water phase by atomic absorption spectrophotometry (Perkin–Elmer Model 403) Washing the surviving red cells with 0 25 M sucrose may have caused efflux of Na^+ and K^+ due to disruption of the permeability of the membrane by the enzyme In this case, efflux of Na^+ and K^+ would be a measure of the extent of this disruption by hemolytic factor Curve A, hemolysis, Curve B, Na^+_i, Curve C, unbound or soluble hemolytic factor, and Curve D, K^+_i

The necessity for ATP, Mg^{2+} and Na^+ or K^+ for hemolytic factor-catalyzed hemolysis has been previously pointed out[2,3]. This has been confirmed in numerous control experiments omitting these cofactors. It was found that washing red cells twice in 0.25 M sucrose, 0 01 M Tris–phosphate at pH 6.0 obviated the necessity of the elaborate treatment of red cells for intracellular Na^+ and K^+ depletion in order to demonstrate a requirement of Na^+ or K^+ for hemolysis[3].

The hemolytic reaction could also be observed microscopically. Only the complete system containing active enzyme, ATP, Mg^{2+} and Na^+, all of which are necessary for the reaction, produced an observed clumping effect concurrent with hemolysis. Omission of ATP or using inactive hemolytic factor in an otherwise complete system produced no agglutination.

Inhibition of hemolysis by stroma

As previously observed[2], stroma from red cells of several species including human can inhibit the hemolysis reaction when preincubated with the enzyme.

Evidence was also presented that the same cofactors were required for both hemolysis and stroma inhibition[3].

The data in Table I confirmed the requirement for ATP and Mg^{2+} in the stroma inhibition reaction although EDTA had to be utilized to demonstrate a complete dependence on Mg^{2+}.

TABLE I

THE STROMA INHIBITION REACTION

The usual procedure involved a preincubation step in the medium described below, followed by analysis of an aliquot of the preincubation mixture for hemolytic activity. The soluble hemolytic factor (HF) term refers to a preliminary centrifugation of the preincubation medium and utilization of the supernatant or unbound hemolytic factor for analysis

Expt	*Preincubation*	% *hemolysis*	% *inhibition*
I	HF + cofactors + ATP	15.2	—
	HF + cofactors + ATP + stroma	5.7	62.5
	HF + cofactors	13.2	—
	HF + cofactors + stroma	13.2	0
II	HF + cofactors + Mg^{2+}	27.2	—
	HF + cofactors + Mg^{2+} + stroma	8.6	68.3
	HF + cofactors	24.3	—
	HF + cofactors + stroma	17.0	31.0
	HF + cofactors + 5 mM EDTA	21.3	—
	HF + cofactors + 5 mM EDTA + stroma	21.0	1.0
III	HF + all cofactors	27.1	—
	HF + all cofactors + stroma	13.9	48.7
	HF + cofactors + stroma − ATP	27.0	0
	HF + all cofactors (soluble HF assayed)	27.0	0
	HF + all cofactors + stroma (soluble HF assayed)	9.4	65.3
	HF + cofactors + stroma − ATP (soluble HF assayed)	27.1	0

The inhibition by stroma was presumably due to an irreversible binding of the hemolytic factor to the stroma. Experiment III in Table I showed that the loss of soluble hemolytic factor activity was related to the extent of inhibition by stroma. The stroma inhibition reaction was thus believed to be similar to the initial hemolytic factor binding to red cell membrane which preceded the hemolytic release of hemoglobin. Subsequent experiments to be described, involving the relative rates of hemolytic facor binding, cation equilibration and hemolysis (Fig. 1) as well as the isolation of an hemolytic factor–erythrocyte complex which was hemolytically active (Fig. 5), reinforce this concept.

Sequence of events in the overall hemolysis process

Aliquots of hemolysis incubation mixtures were taken at various times and analyzed for hemolytic factor binding, intracellular Na^+ and K^+ concentrations and hemolysis. The curves in Figs. 1 and 2 indicated the sequence and extent of these processes with and without ATP, respectively. There was no change in all three processes without ATP. In the presence of ATP, within the limits of accuracy of withdrawn, timed samples, the hemolytic factor binding and Na^+ and K^+ efflux

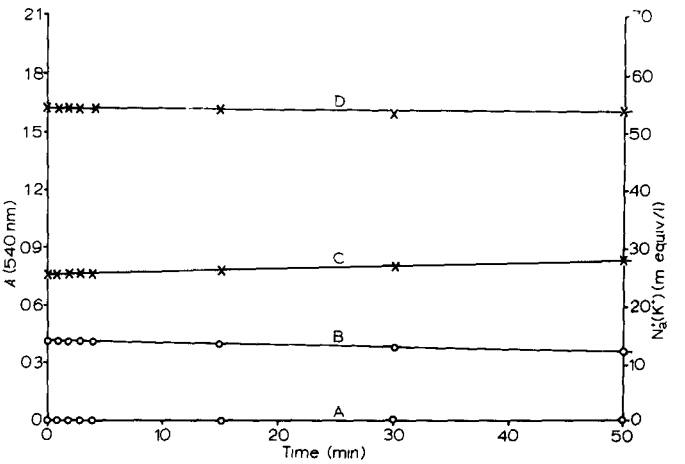

Fig 2 The effect of ATP lack on the hemolytic sequence The conditions for incubation and analysis were similar to that for the curves of Fig. 1 except that ATP was not added to the medium

TABLE II

COMPARISON OF ATP HYDROLYSIS BY HEMOLYTIC FACTOR, STROMA AND THE HEMOLYTIC FACTOR–STROMA COMPLEX

The reaction was carried out for 30 min at 37 °C It was started by the addition of the active fractions (hemolytic factor (HF), 15 units, stroma, 1 0 mg protein) and stopped by the addition of 0 2 ml of 50% trichloroacetic acid The final concentrations of cofactors used were 1 mM ATP, 1 mM $MgSO_4$, 10 mM histidine buffer and 100 mM NaCl at pH 6 5 in a final volume of 2 0 ml Analysis of phosphate was done by a modification of the Fiske and SubbaRow method[7]. These results represent an average of 8 assays

Fraction	*P_i released (μmole/h)*
(HF–stroma)	0 64
HF	0 15
Stroma	0 21
(HF–stroma) − (HF + stroma)	0 28

appeared to occur simultaneously and preceded the efflux of hemoglobin which was utilized as a measure of hemolysis.

The hemolytic enzyme as an ATPase

The hemolytic factor can hydrolyze ATP independently but, as shown in Table II, the hemolytic factor–stroma complex, when formed by hemolytic facor and stroma interacting under stroma inhibition preincubation (binding) conditions, has greater ATPase activity than the sum of hemolytic factor and red cell stroma acting alone. This ATPase activity is linear for at least 60 min as shown in Fig. 3.

Free hemolytic factor and hemolytic factor–stroma complex both have the ability to catalyze the ADP–ATP exchange reaction (Fig 4) indicating the formation of a high-energy phosphorylated intermediate

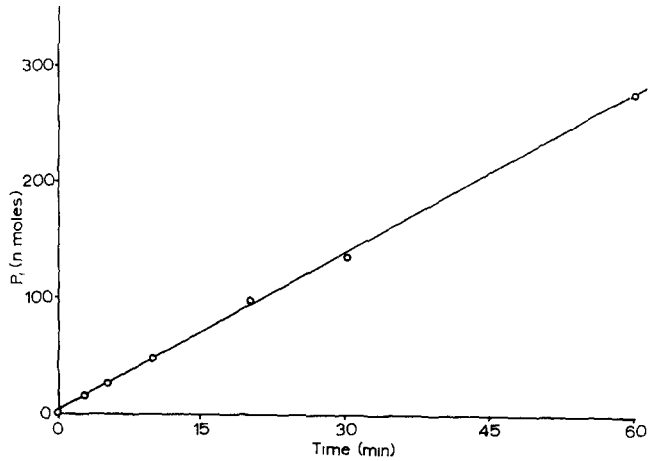

Fig. 3. Kinetic curve of ATP hydrolysis by hemolytic factor–stroma. The incubation and analysis were similar to that described in Table II. 6 units of hemolytic factor and 0.4 mg of stroma protein were used for this reaction.

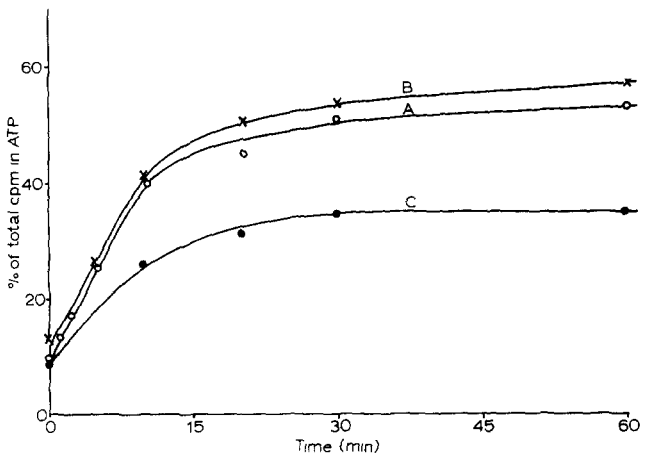

Fig. 4. Comparison of the rate of ADP–ATP exchange for hemolytic factor and the hemolytic factor–stroma complex. All tubes contained 1 mM ATP, 0.9 mM [8-^{14}C]ADP (7.5 mC$_i$/mmole), 1 mM $MgSO_4$, 15 mM NaCl, 10 mM histidine, pH 6.5 and 30 μg (1.2 units) of hemolytic factor in a total volume of 60 μl (Curve A) or 56 μg of hemolytic factor complexed with 3.5 μg of stroma protein in a total volume of 80 μl (Curve B). The hemolytic factor–stroma complex can be formed by incubating hemolytic factor with stroma under the conditions described in Methods for the stroma inhibition assay. Hemolytic factor–stroma was obtained by centrifugation and used for the ADP–ATP exchange reaction. Adenylate kinase exchange activity (Curve C) was determined by a similar incubation in the absence of ATP so that this curve represents a maximum contribution of adenylate kinase activity in Curves A and B since the presence of ATP in the medium would depress exchange by adenylate kinase under these experimental conditions. After the reaction was stopped by a 1-min heating period at 100 °C, samples of the supernatant fraction were separated into ATP, ADP and AMP components by electrophoresis on cellulose acetate. The spots were identified and marked by the use of ultraviolet light, cut out and placed in scintillation fluid for counting. The Beckman Model R-101 microzone electrophoresis apparatus was used for nucleotide separation. Electrophoresis was carried out at 250 V for 30 min on cellulose acetate using a 0.25 M ammonium acetate buffer at pH 3.6 and 0.05 g EDTA per l. The scintillation fluid was a mixture of PPO, dimethylPOPOP and toluene. Samples were counted in a Packard scintillation counter Model 3320.

Isolation of the hemolytically active hemolytic factor–erythrocyte complex

It was hypothetically possible to achieve the isolation of the hemolytic factor–erythrocyte complex by taking advantage of the basic differences between the binding and hemolysis steps. The activation and inhibition of hemolytic factor binding and hemolysis, respectively, by Na^+ and K^+ (see Figs 7 and 11) or the greater inhibition of hemolysis relative to binding at pH 7.5 or higher are examples. The latter method was preferentially utilized for hemolytic factor–erythrocyte formation and isolation.

Thus, the hemolytic factor–erythrocyte complex can be produced and washed free of unbound hemolytic factor and cofactors with little loss of intracellular cations and hemoglobin. When the pH is re-adjusted to 6.0, there is a rapid equilibration of cations and loss of hemoglobin which increases with increased preincubation time without the necessity of ATP or hemolytic factor addition This process can be completely inhibited by 1 mM *N*-ethylmaleimide as demonstrated by Curves C and D in Fig. 5 This sulfhydryl agent was also shown to be a potent inhibitor of overall

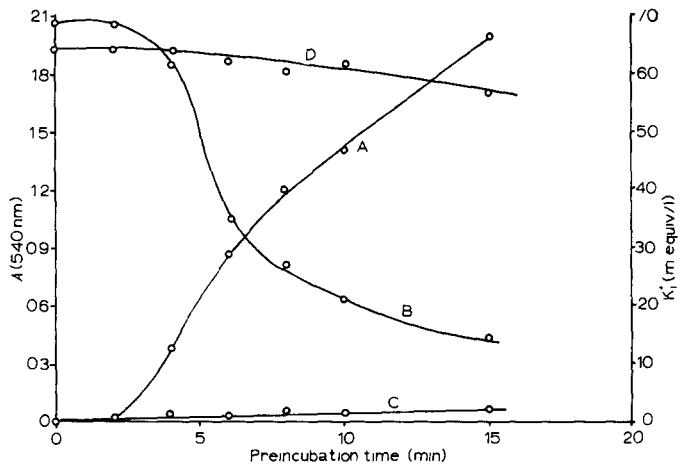

Fig 5 The K^+ equilibration and spontaneous hemolysis of the hemolytic factor–erythrocyte complex as a function of preincubation time Hemolytic factor and red cells were incubated at 20–21 °C with required cofactors for hemolysis at pH 7 5 Under these conditions, hemolysis was prevented but hemolytic factor binding could occur At various times, 1.0-ml samples were withdrawn, added to 9 0 ml of cold 0 2 M sucrose and centrifuged The absorbance readings of the supernatant fractions indicated that no hemolysis had occurred during the preincubation period The hemolytic factor–erythrocyte pellets were resuspended in the usual cofactor medium at pH 6 0, *minus* ATP, for hemolysis after carefully removing the remaining supernatant by suction Incubation was carried out at 30 °C for 15 min Curve A shows the spontaneous hydrolysis in 15 min of the hemolytic factor–erythrocyte complex at various times of its formation during the preincubation period Curve B indicates the simultaneous efflux of intracellular K^+ Curves C and D are similar to A and B, respectively, except that 1 mM *N*-ethylmaleimide was added to the sucrose medium in which the 1 0-ml aliquots of preincubation medium were suspended

hemolysis. Inhibition was probably at the enzyme rather than the red cell membrane level as demonstrated by the data of Table III

The interaction of the enzyme with the red cells probably involved a disruption of membrane permeability which resulted in equilibration of external and intracellular Na^+ and K^+ rather than active transport. The data in Table IV showed that cation movement was always down the concentration gradient. Since ATP was

TABLE III

THE EFFECT OF *N*-ETHYLMALEIMIDE ON THE ROLE OF HEMOLYTIC FACTOR, ERYTHROCYTES AND STROMA IN HEMOLYSIS

In Expt I, the enzyme was preincubated at 20 °C for 15 min with and without *N*-ethylmaleimide (NEM) before testing for hemolysis In Expt II, red cells were similarly treated then washed 3 times with 0 15 M NaCl to remove unbound *N*-ethylmaleimide In Expt III, stroma was similarly treated, washed 3 times with 0 15 M NaCl to remove unbound *N*-ethylmaleimide and then tested for its ability to inhibit hemolysis utilizing the stroma inhibition assay in Methods Thus, % inhibition refers to hemolysis inhibition in Expts I and II but indicates reduction of stroma inhibition n Expt III HF, hemolytic factor

Expt	*Preincubation mixture*	% *hemolysis*	% *inhibition*
I	HF + cofactors	25 4	—
	HF + cofactors + 1 mM NEM	0 8	97
II	Erythrocytes + cofactors	31 7	—
	Erythrocytes + 1 mM NEM + cofactors	30 4	4
III	Stroma + cofactors	28 0	—
	Stroma + cofactors + 1 mM NEM	27 5	2

TABLE IV

THE EQUILIBRATION OF Na^+ AND K^+ DURING HEMOLYTIC FACTOR-CATALYZED HEMOLYSIS

Hemolytic factor and all cofactors, except that 100 mM sucrose replaced 100 mM NaCl, were incubated with red cells for 5 min at 30 °C in media containing concentrations of Na^+ and K^+ given in Columns 1 and 2 The surviving cells were removed by centrifugation and the % hemolysis determined from aliquots of the supernatant fractions (Column 3) Differences in enzyme activity (Column 3) were due to inhibition by high Na^+ or K^+ concentrations After careful removal of the supernatant from the red cell pellet by suction, intracellular Na^+ and K^+ analysis of the pellets was carried out by flame photometry and recorded in Columns 5 and 7, respectively The initial intracellular Na^+ and K^+ concentrations given in Columns 4 and 6, respectively, were obtained from incubated control tubes containing similar cofactors and hemolytic factor but no ATP Under these conditions no interaction between hemolytic factor and red cells should have occurred

External cations (mequiv/l)		*Hemolysis* (%)	*Internal cations (μequiv/l cells)*			
			Initial	*Final*	*Initial*	*Final*
Na^+	K^+		Na^+	Na^+	K^+	K^+
15 0	0	70 0	23 2	21 2	56 0	8 2
150 0	0	41 8	43 2	133 6	58 0	27 0
0	15 0	67 0	19 6	12 4	54 0	25 2
0	150 0	42 0	15 8	10 4	72 0	132 0

unnecessary for cation equilibration and hemolysis, passive diffusion rather than energy, requiring active transport presumably occurred.

The effect of pH on hemolytic factor stability, hemolytic factor-catalyzed hemolysis, hemolytic factor binding and spontaneous hemolysis of the hemolytic factor–erythrocyte complex

Curve A of Fig 6 indicated that free hemolytic factor was unstable at values below pH 5.6 and was also rapidly inactivated as the pH increased from a value of

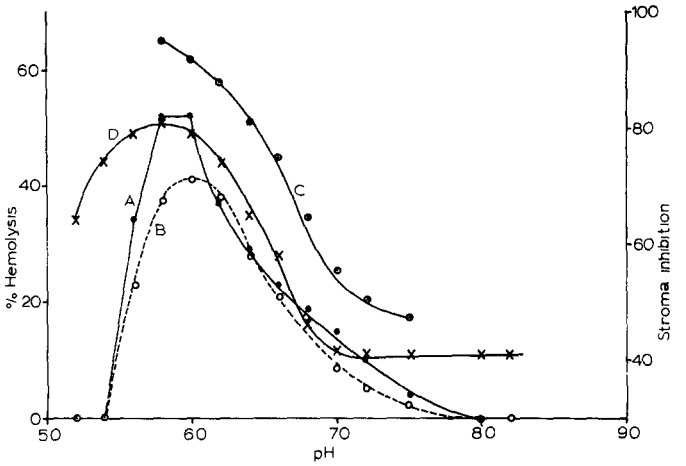

Fig 6 The effect of pH on hemolytic factor stability, binding, hemolysis and the spontaneous hemolysis of the hemolytic factor–erythrocyte complex To determine hemolytic factor stability (Curve A), 3 0 units of hemolytic factor were preincubated for 15 min at 20–21 °C in 1 0 ml of 5 mM sodium citrate–phosphate buffer at various pHs The enzyme solutions were diluted 10-fold to obtain the proper hemolysis range and 0 10-ml aliquots were incubated in 1 0 ml of 0 01 M sodium citrate–phosphate at pH 6 0 and all other cofactors necessary for the hemolysis assay as described in Methods Hemolytic factor-catalyzed hemolysis (Curve B), stroma inhibition of hemolytic factor (Curve C) and spontaneous hemolysis of the preformed hemolytic factor–erythrocyte complex (Curve D) were measured according to the procedures described in the appropriate section of Methods utilizing 10 mM sodium citrate–phosphate buffer with the necessary pH variations

6.0. This instability was reflected in Curve B for overall hemolysis by hemolytic factor as a function of pH. It might be pointed out that below pH 5.4, the hemolytic factor preparation tended to become cloudy and excessive spontaneous hemolysis of the red cells also occurred. The stroma inhibition curve (C) was a measure of the ability of hemolytic factor to bind to the red cell stroma. This curve demonstrated that the binding of hemolytic factor was also adversely affected by increasing pH as shown by a decrease in the ability of stroma to bind and thus inhibit hemolytic factor-catalyzed hemolysis At pH 7.5, hemolysis was virtually completely inhibited (Curve B), whereas the stroma inhibition (hemolytic factor binding) was still 50 % effective (Curve C) This difference in binding and hemolysis activity was utilized to isolate the unhemolyzed hemolytic factor–erythrocyte complex.

After hemolytic factor was bound to the red cell membrane, the hemolytic factor moiety became more stable at lower pH levels as indicated by the spontaneous hemolysis of the complex at pH values below which free hemolytic factor can function (Curve D) This phenomenon is analogous to the allotropic properties of several mitochondrial coupling factors as described by Bennum and Racker[9]. However, the subsequent membrane permeability disruption which resulted in spontaneous hemolysis was also inhibited by pH going from a high of 50 % at pH 5.8 to a low of 10 % hemolysis at pH 7.2. The latter is assumed to be the basic leakage of hemoglobin from the hemolytic factor–erythrocyte complex as determined by a control containing 1 mM *N*-ethylmaleimide inhibitor. In other words, no further disruption of membrane permeability occurred at pH 7.2 which was n't already present as a result of the formation and washing of the hemolytic factor–erythrocyte complex.

The effect of Na^+, K^+ and sucrose on hemolytic factor binding, spontaneous hemolysis of the hemolytic factor–erythrocyte complex and hemolytic factor-catalyzed hemolysis

The effects of Na^+ and K^+ on the overall system and its two-component reactions of binding and hemolysis are basically similar. Sucrose differs from these cations in its lack of effect on the initial binding step.

In the stroma inhibition reaction (hemolytic factor binding), Na^+ and K^+ concentrations up to 0.3 M were found to enhance the reaction whereas similar increases in sucrose concentration failed to increase hemolytic factor binding in parallel experiments containing no Na^+ or K^+ in the incubation medium (Fig. 7).

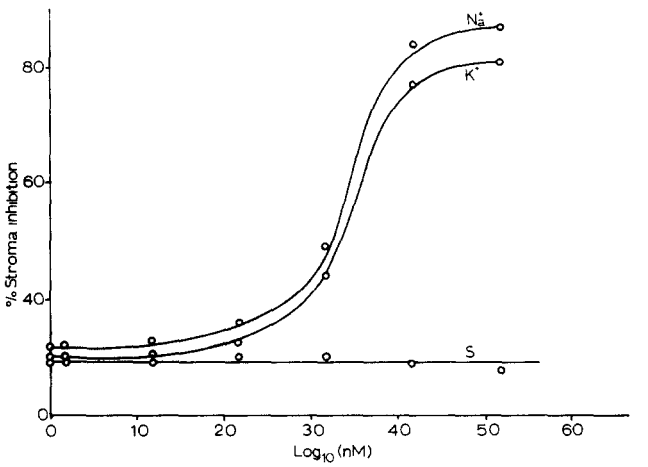

Fig 7 The effect of Na^+, K^+ and sucrose on the stroma inhibition of hemolytic factor. A series of tubes containing various concentrations of NaCl, KCl and sucrose were preincubated with 3.0 units of hemolytic factor and stroma (200 μg protein) along with a series of corresponding controls which contained no stroma according to the stroma inhibition assay procedure in Methods

A complete requirement for these cations could not be demonstrated as 25 to 30 % stroma inhibitions were produced on the average even when the preincubation medium contained less than 1 ppm of Na^+ or K^+ by flame photometry analysis. However, addition of either Na^+ or K^+ (but not sucrose) activated the binding process. Other monovalent cations such as Li^+ and NH_4^+ also had no stimulating effect on hemolytic factor binding at concentrations up to 0.3 M in the absence of Na^+ or K^+.

The curve in Fig. 8 showed the rate of enhancement of hemolytic factor binding at a fixed concentration of Na^+ and K^+. The "early" reversible stroma inhibition which invariably occurred (Fig. 8) is not yet understood. It is presumably related to the presence of stroma since no change occurred in the corresponding controls containing all cofactors except stroma whereas a pronounced fluctuation in hemolytic factor activity occurred during the "early" time interval in the stroma-containing tubes.

The effect of Na^+, K^+ and sucrose on the spontaneous hemolysis of the preformed hemolytic factor–erythrocyte complex appeared to be complicated. If the complex was resuspended in increasing concentrations of sucrose, an optimum rate

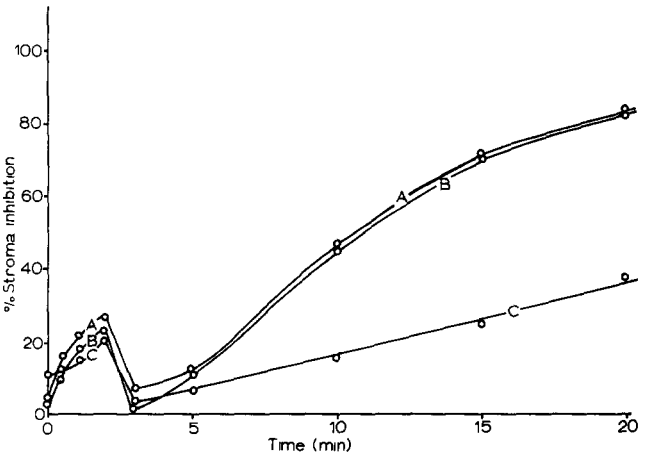

Fig 8 The rate of stroma inhibition of hemolytic factor in the presence and absence of Na^+ and K^+ Aliquots were withdrawn at various preincubation times and tested for hemolytic activity according to the procedure in Methods. Curve A, 15 mM Na_2SO_4, Curve B, 15 mM K_2SO_4; Curve C, no Na^+ or K^+ added The preincubation medium contained 3 units of hemolytic factor activity and 200 μg stroma protein

of hemolysis occurred at 0.3 M (Fig. 9). On the other hand, if 0.2 M sucrose was used to prevent hypotonic hemolysis of the complex, the addition of a low concentration of Na^+ or K^+ produced a sharp initial decrease in hemolysis. Further addition of these cations caused a rise in hemolysis reaching a maximum at about 0.3 M followed

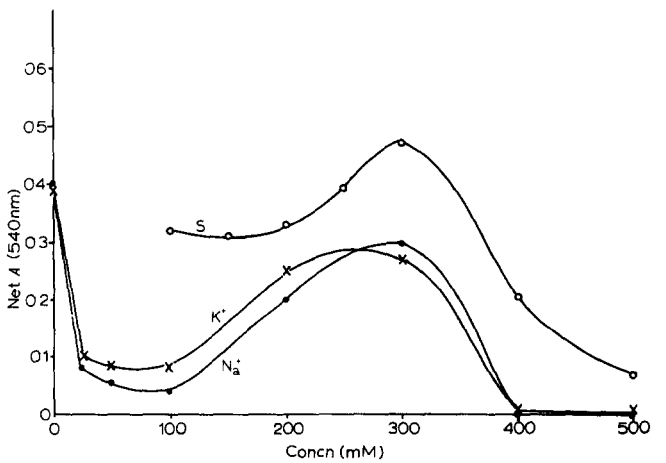

Fig 9. The effect of Na^+, K^+ and sucrose on the spontaneous hemolysis of the hemolytic factor–erythrocyte complex The hemolytic factor–erythrocyte complex, prepared as described in Methods, was resuspended in 1 0 ml of 0 20 M sucrose containing various concentrations of Na^+ and K^+ and incubated at 30 °C for 15 min The assay for spontaneous hemolysis was carried out as described in Methods The effect of sucrose concentration without Na^+ or K^+ (Curve S) was determined in a separate experiment Control tubes containing 1 mM *N*-ethylmaleimide inhibitor had basal hemolysis absorbance values of 0 120 and 0 200 for the cation and sucrose experiments, respectively, and were subtracted from the corresponding experimental values to obtain net absorbance values

by a decrease to the basal level of hemolysis of the *N*-ethylmaleimide-inhibited controls.

Curves showing the effect of Na^+ and K^+ concentrations on overall hemolysis (Fig. 10) was thought to be a composite of two competing actions. Thus, both Na^+ and K^+ have optimum concentrations of 0.03 M for hemolysis which is a result of an activation of hemolytic factor binding (see Fig 7) which enhances hemolysis and a simultaneous inhibitory effect on the subsequent hemolysis step. It should be pointed out that the inhibition of overall hemolysis by Na^+ and K^+ at concentrations above 0.03 M differed from their inhibition of spontaneous hemolysis by the hemolytic factor–erythrocyte complex

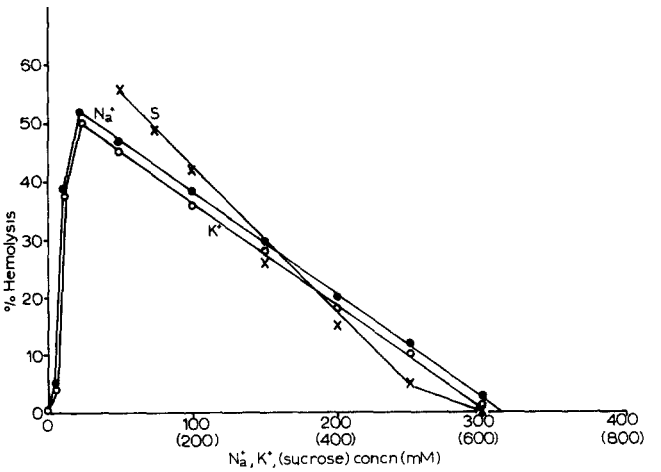

Fig 10 The effect of Na^+, K^+ and sucrose on hemolytic factor-catalyzed hemolysis The assay was conducted according to the procedure in Methods

In the case of sucrose, which had no effect on the hemolytic factor-binding step, inhibition was proportional to the sucrose concentration. Since it required twice the concentration of sucrose to inhibit hemolysis to the same extent relative to NaCl or KCl, a colligative or osmotic pressure effect rather than a specific type of inhibition may be preventing hemoglobin release.

The effect of ATP

ATP, a substrate in the hemolyte factor-binding step of the overall hemolysis reaction, was found to act as an allosteric inhibitor for overall hemolysis and its component reactions of hemolytic factor binding and spontaneous hemolysis of the complex at higher concentrations. The curves of Fig. 11 show that, at a constant 2 mM $MgSO_4$, increased ATP concentration produced an optimum activity (since it is required for binding) followed by inhibition On the other hand, increasing $MgSO_4$ concentration at a fixed concentration of ATP, caused no inhibition of hemolysis. Further experiments showed that variations in the ATP–$MgSO_4$ ratio did not influence the inhibition which depended only upon the concentration of ATP at a given enzyme.

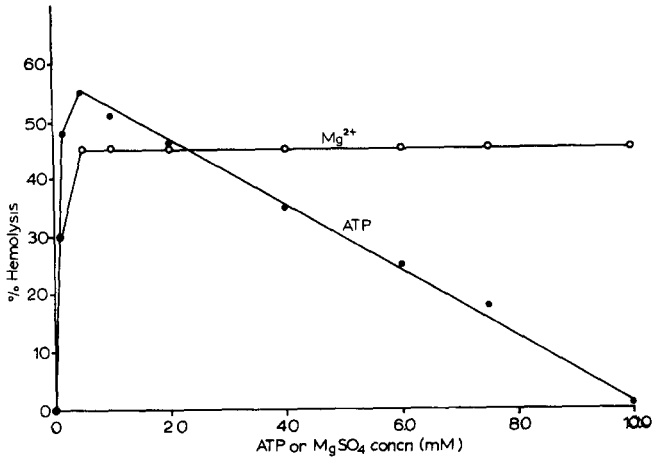

Fig 11. The effect of ATP and $MgSO_4$ on hemolytic factor-catalyzed hemolysis The hemolysis assay used is described in Methods with the exception that ATP concentration is varied while $MgSO_4$ is held at 2 mM (Curve ATP) and $MgSO_4$ concentration is varied while ATP is held at 2 mM (Curve Mg^{2+}).

Curve A in Fig. 12 indicated that ATP can also inhibit the hemolytic factor-binding step. The higher concentrations of ATP required for this inhibition were probably a reflection of the higher enzyme concentrations used in the preincubation step of the stroma inhibition assay which was taken as a measure of hemolytic factor binding. ATP can also effectively inhibit the spontaneous hemolysis of the preformed hemolytic factor–erythrocyte complex reaching the basal level of hemolysis at about 10 mM ATP. Although ATP is not necessary for the spontaneous hemolysis of the complex, concentrations up to 2 mM produced an enhancement of this type of hemolysis.

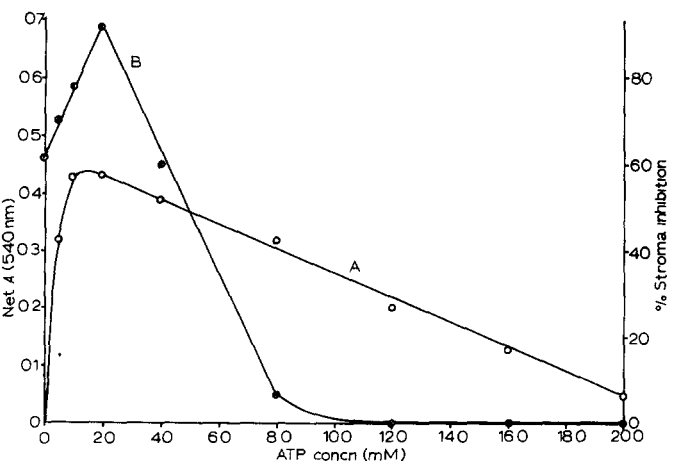

Fig 12 The effect of ATP on the stroma inhibition of hemolytic factor (Curve A) and the spontaneous hemolysis of the hemolytic factor–erythrocyte complex (Curve B) Assays were conducted according to the procedures described in Methods Controls, containing 1 mM *N*-ethylmaleimide, for the hemolytic factor–erythrocyte hemolysis assay had basal hemolysis absorbance readings of 0 160 which were subtracted from the experimental values to obtain net absorbance

The effect of ADP

Since ATP, but not ADP, was necessary for overall hemolysis, ATP was present when studying the ADP effect. In the case of spontaneous hemolysis of the hemolytic factor–erythrocyte complex where ATP was not required ADP was used in the absence of ATP. In Fig. 13, the effect of ADP on overall hemolysis is shown at three different concentrations of ATP. As expected from Fig. 11, hemolysis in the absence of ADP decreased as ATP increased At the lowest ATP concentration (Curve A) ADP up to 2 mM caused an initial increase in activity followed by a rapid inhibition which is complete at 6.0 mM. At 2 mM ATP concentration (Curve B) the activation

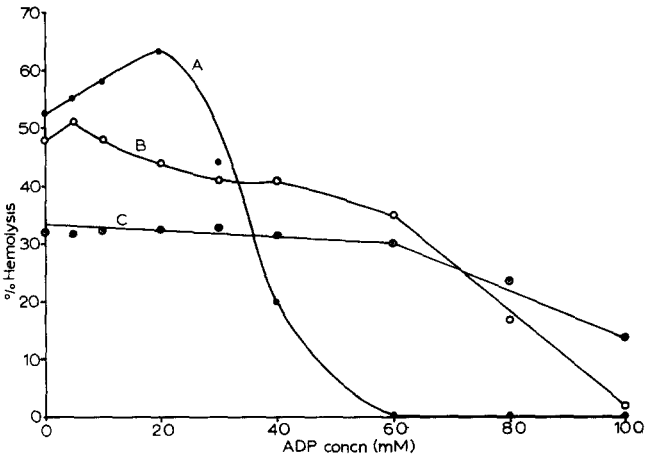

Fig 13 The effect of ADP concentration on hemolytic factor-catalyzed hemolysis at several concentrations of ATP The hemolysis assay used is described in Methods ATP concentrations of 0 5 mM (Curve A), 2 0 mM (Curve B) and 5 0 mM (Curve C) were used in determining the effect of ADP on overall hemolysis

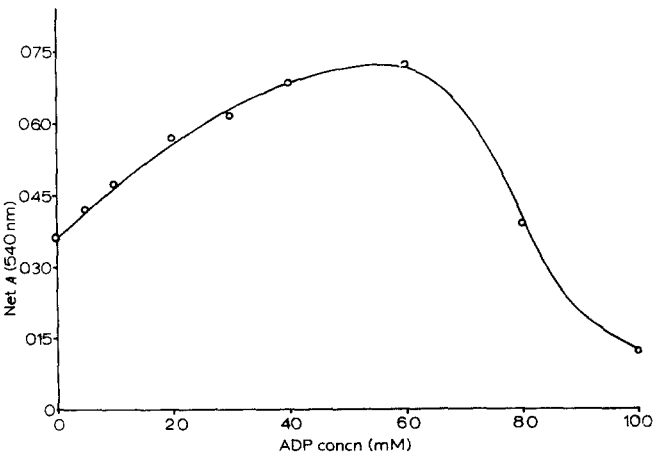

Fig 14 The effect of ADP on the spontaneous hemolysis of the hemolytic factor–erythrocyte complex The assay for spontaneous hemolysis of the complex was carried out according to the procedure in Methods Controls containing 1 mM *N*-ethylmaleimide had basal hemolysis values of 0 180 were subtracted from the corresponding experimental values to obtain the net absorbance.

of hemolysis is much less pronounced but complete inhibition requires 10 mM of ADP. In Curve C, at 5 mM ATP, the activation of hemolysis by low ADP concentrations has disappeared and inhibition by ADP is about 50 % at 10 mM.

As in the case of ATP, ADP enhances spontaneous hemolysis by the preformed hemolytic factor–erythrocyte complex at lower concentrations but becomes inhibitory as the ADP concentration increases (Fig. 14).

DISCUSSION

This system for red cell hemolysis can be divided into two distinct steps. The binding of the hemolytic factor to the red cell membrane and secondly, the disruption of membrane permeability which resulted in an equilibration of cations and subsequently, efflux of large molecules such as hemoglobin, leading to hemolysis.

Evidence for an initial binding step came from the lag period for hemolysis coupled with the fact that hemolytic factor binding preceded hemolysis. Furthermore, the isolation of a hemolytically active hemolytic factor–erythrocyte complex confirmed that binding occurred prior to hemolysis.

The ability of hemolytic factor to hydrolyze ATP and also catalyze ADP–ATP exchange indicated that a high-energy phosphorylated intermediate of the enzyme was formed before a binding to the membrane occurred. Other ATPases such as the F_1 coupling factor of mitochondria[7] and the (Na^+–K^+)-ATPase[8] of various plasma membranes have the capability of mediating this type of exchange, *in situ*. The hydrolysis of ATP and ADP–ATP exchange of the hemolytic factor–stroma complex may be extraneous to the hemolysis reaction except in terms of allosteric control since there is no further requirement of ATP or other cofactors for hemolysis after the hemolytic factor-binding step. No explanation for the site and function of the hemolytic factor–stroma ATPase reaction is presently available.

The hemolytic factor is probably linked to the membrane by means of covalent bonding or at least one which cannot be disrupted by washing the complex in NaCl or sucrose solutions at various concentrations. Whatever caused the subsequent disruption of membrane permeability resulting in cation equilibration and hemoglobin leakage, it is independent of the binding step and can be inhibited by the use of 1 mM *N*-ethylmaleimide.

The fact that overall hemolysis was completely inhibited by lack of external Na^+ and K^+ while 25 to 30 % hemolytic factor binding occurred appeared to be incongruous since this amount of binding should result in hemolysis which was not prevented by lack of Na^+ or K^+ once the hemolytic factor–erythrocyte complex was formed. This apparent discrepancy could be explained by assuming that the hemolytic factor binding which occurred in the abensce of Na^+ or K^+ was not involved in the subsequent hemolysis step.

The hemolytic process probably involved a disruption of membrane permeability which allowed cation equilibration and efflux of hemoglobin rather than an active transport process for two reasons. ATP was not required and secondly, the process of cation movement was one of diffusion down a concentration gradient. The cation curves of Fig. 1 which indicated an apparent active transport of intracellular cations can be explained by the fact that during resuspension and washing

of the surviving red cells in 0.25 M sucrose, a rapid equilibration of internal Na^+ and K^+ occurred, with the external medium resulting in low Na^+_i and K^+_i concentrations. Thus it appeared that intracellular cations, particularly Na^+, were actively translocated against a 6- or 7-fold external concentration in the incubation medium. Actually, Na^+_i and K^+_i were lost during resuspension of the red cells in a sucrose medium by passive diffusion.

The basic effect of Na^+ and K^+ on hemolytic factor binding to the red cell membrane was one of enhancement whereas sucrose and other monovalent cations such as Li^+ and NH_4^+ have no effect. On the other hand, the effect of Na^+, K^+ and sucrose on overall hemolysis was clearly inhibitory. It is proposed that the attached hemolytic factor acted on a membrane substrate causing a flow of Na^+ and K^+ in either direction down a concentration gradient. At low external sucrose or cation concentrations, the efflux of ions and water carried hemoglobin through the membrane at a faster rate. When extracellular exceeded intracellular concentrations, however, influx of these ions or sucrose and water inhibited hemoglobin efflux.

ATP not only acted as a substrate in the hemolytic factor-binding reaction but exerted allosteric effects as well. In the overall reaction and its component binding and hemolysis steps, ATP produced an optimum activity followed by inhibition as the concentration increased.

This effect occurred for both ATP and ADP in the spontaneous hemolysis of the complex where neither was required indicating that the binding of ATP or ADP produced initial conformational changes which were activating. As ATP or ADP concentrations increased, allosteric binding sites may have been utilized which produced inhibitory conformational changes. The inhibition of phosphofructokinase by ATP[10], one of its substrates, appears to be analogous to the ATP and ADP inhibition of hemolytic factor and has an ATP *versus* activity curve which is strikingly similar to that in Fig. 11. The curves of Fig 13 indicated that ATP might compete with ADP for allosteric binding sites and thus modify the potent allosteric inhibition by ADP.

The physiological role of the hemolytic factor still remains to be elucidated This protein complex has been extracted from rabbit red cells where its natural function was exerted and was utilized in a system involving the external rather than the internal surface of the red cell membrane. Under the latter conditions it was capable of producing hemolysis It has not been extracted from other types of red cell including human although it is capable of hemolyzing a large number of different red cell types.

Preliminary experiments indicated that hemolytic factor exerts an effect on other membrane systems. For example, when hemolytic factor and its required cofactors were added to a system consisting of a single trabecule from frog heart auricle, there was an initial and irreversible loss of electrical excitability. The membrane was then depolarized by a solution of KCl and a contraction could again be produced. Approx 30 min after hemolytic factor addition, the muscle fibres no longer responded to high KCl perfusion but could still be relaxed by the use of 5 mM EDTA The subsequent addition of Ca^{2+} in excess of EDTA caused a contraction suggesting that the membrane was no longer a permeability barrier to outside cations.

The preliminary mechanism can be summarized as follows:

$$\text{hemolytic factor} + \text{ATP} \underset{}{\overset{Mg^{2+};\,[Na^+ \text{ or } K^+]?}{\rightleftharpoons}} \text{hemolytic factor} \sim \text{P} + \text{ADP} \quad (1)$$

$$\text{hemolytic factor} \sim \text{P} + \text{erythrocyte} \xrightarrow{Mg^{2+};\,[Na^+ \text{ or } K^+]?} \text{hemolytic factor–erythrocyte} + \text{P}_i \quad (2)$$

$$\text{hemolytic factor–erythrocyte} + Na_i^+ + K_i^+ \rightleftharpoons \text{hemolytic factor–erythrocyte}_x + Na_o^+ + K_o^+ \quad (3)$$

$$\text{hemolytic factor–erythrocyte}_x + \text{Hb}_i \rightarrow \text{hemolytic factor–erythrocyte}_H + \text{Hb}_o \quad (4)$$

where subscripts i and o refer to intracellular and extracellular, respectively and x and H refer to cation-equilibrated and hemolytic states of the complex, respectively.

ACKNOWLEDGEMENTS

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