AN ADENOSINETRIPHOSPHATE-ACTIVATED HEMOLYTIC SYSTEM

By BLANCHE ANN BOREK and MAX BOVARNICK

(From the Veterans Administration Hospital, Brooklyn, New York, and the Department of Medicine, College of Medicine, State University of New York at New York City)

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INTRODUCTION

In 1954 Synder and his coworkers (1) reported that water hemolysates of rabbit erythrocytes were capable of lysing whole rabbit cells if adenosinetriphosphate was also present in the system. Sheep cell hemolysate and adenosinetriphosphate did not have a corresponding effect on sheep erythrocytes. Subsequently, one of the group, (M.R.B.),[1] found that rabbit cell hemolysate also caused hemolysis of human erythrocytes in the presence of adenosinetriphosphate, whereas human hemolysate showed no lytic action on human cells under the same conditions. Since this system is unique in that a source of potential high energy phosphate appears to be involved in the hemolytic process, we thought it of interest to study it in some detail.

In this paper, studies on the activation and inhibition of the system and the preparation of a partially purified fraction obtained by fractional centrifugation of rabbit hemolysate are described. The concentration of adenosinetriphosphate is followed during the course of hemolysis and its possible role in the system is discussed.

Materials and Methods

Preparation of Diluents and Glassware.—Since the hemolytic system was found to be inhibited by distilled water as obtained from the usual metal still, all reagents were dissolved in water which had been passed through a Barnstead demineralizer and then distilled in an all-glass apparatus. All glassware was cleaned in sulfuric acid–dichromate solution and after preliminary rinsing in tap water was washed ten times with demineralized water and three times with glass-distilled water. When plastic tubes or dialysis casings were used they were soaked overnight in 0.1 M versene and then washed as above with demineralized and glass-distilled water.

Reagents.—All chemicals were prepared in concentrated stock solution which had been neutralized to pH 7 with dilute potassium hydroxide. Prior to use these were diluted to the required concentration with isotonic sucrose phosphate solution (9 parts of 0.25 M sucrose and 1 part of 0.1 M potassium phosphate buffer, pH 7.1).

[1] M. R. Bovarnick, personal communication.

The component solutions were sterilized by autoclaving and cooled before admixing). The same medium was used for the washing and final suspension of rabbit erythrocytes.

The diluent for human erythrocytes was sucrose–PO_4–Mg, prepared by adding 10 ml. of autoclaved 0.1 M $MgCl_2$ solution to 100 ml. of sucrose–PO_4 solution.

Adenosinetriphosphate (ATP)[2].—Pabst ATP–disodium salt was used. Both the crystalline and non-crystalline products proved equally effective in our system.

Hemolysate of Rabbit Erythrocytes.—Rabbit blood obtained by heart puncture was mixed with an equal volume of sterile citrate-dextrose solution (sodium citrate, 0.073 M; citric acid, 0.0024 M; and glucose, 0.113 M). After centrifugation and removal of the plasma and the buffy coat, the cells were washed three times with 4 volumes of sucrose–PO_4 and resuspended to a concentration of 8 per cent in the same diluent. These cells, stored at 0 to 4°C., produced active hemolysate for 3 weeks—the longest period tested.

Hemolysate was prepared as follows: 4 ml. of the above 8 per cent rabbit cells were centrifuged, the supernatant fluid discarded, and 4.8 ml. of water was added to lyse the packed erythrocytes. After 5 minutes 1.2 ml. of 42.5 per cent sucrose was added to render the solution isotonic and the hemolysate was centrifuged at 25,000 *G* at 4°C. for 10 minutes to bring down the greater part of the stroma. The supernatant fluid, which contained all the activity of the original hemolysate, was used in the hemolysin test. The hemolysate was prepared on the day of use although it retained some of its activity for as long as a week when kept at 4°C., and was fully active for 2 weeks when quickly frozen and stored at −70°.

Human Erythrocytes.—Human blood in ACD solution was obtained from a Blood Bank 1 or 2 days after drawing. Although all blood groups proved suitable, as routine group O blood was used. The blood was centrifuged and the plasma and buffy coat were discarded. The packed cells were then washed three times with 4 volumes of sucrose–PO_4–Mg solution and finally suspended to a concentration of 8 per cent in the same diluent. The cells were stored at 0 to 4° and discarded after 2 weeks.

Hemolysin Test.—The procedure followed was essentially the one described by Synder *et al.* (1) except that the incubation period was reduced to 1 hour since maximal hemolysis was achieved in this time.

Into Kahn tubes kept in an ice bath were placed 0.05 ml. of 0.002 M versene, 0.05 ml. of 0.008 M ATP, and aliquots of rabbit cell hemolysate ranging from 0.02 to 0.14 ml. Sucrose–PO_4 was added to bring the total volume to 0.3 ml. Then 0.3 ml. of an 8 per cent suspension of human erythrocytes in sucrose–PO_4–Mg solution was added to each tube. Proper control tubes, lacking ATP and rabbit hemolysate respectively, were set up at the same time, the latter serving as an instrument blank against which all other tubes were read. The tubes were then placed in a 34° C. water bath for 1 hour. After incubation, 2 ml. of isotonic saline was added to each tube, the

[2] The following abbreviations are used: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosinemonophosphate; CTP, cytidine triphosphate; GSH, glutathione; GSSG, oxidized glutathione; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; FMN, flavine mononucleotide; FAD, flavine adenine dinucleotide; versene, ethylene-diaminetetraacetic acid; Tris, tris (hydroxymethyl) amino methane.

tube contents were well mixed, and then centrifuged at 2,500 R.P.M., for 10 minutes. The optical density at 545 mμ was measured in a Coleman Jr. spectrophotometer against the instrument blank described above.

The O.D. readings were corrected for the hemoglobin value contributed by various aliquots of the rabbit hemolysate by subtracting the optical density obtained in the control tube (lacking ATP) from that obtained in the corresponding complete system (with ATP). The per cent hemolysis was calculated by dividing the corrected O.D. readings by the optical density obtained by complete lysis of 0.3 ml. of 8 per cent human erythrocyte suspension with 2.3 ml. of water. Per cent hemolysis *vs.* rabbit hemolysate concentration is plotted in Fig. 1. In studies on the effect of various

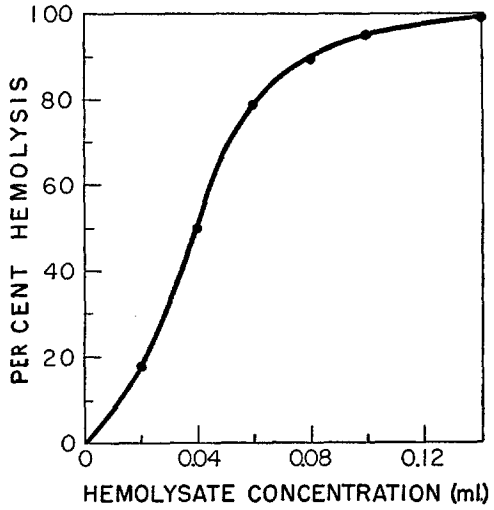

FIG. 1. Hemolysis of human erythrocytes by rabbit red cell hemolysate. Each tube contained 0.3 ml. of 8 per cent human erythrocytes in sucrose–PO_4–Mg; ATP (6.67×10^{-4}M); versene (1.67×10^{-4}M); and rabbit hemolysate as indicated. Diluent is sucrose–PO_4. Total volume = 0.6 ml.

additional agents on the system, that amount of rabbit hemolysate which resulted in the hemolysis of 50 per cent of the human erythrocytes without added agents was used.

Experimental and Results

Centrifugation of Rabbit Hemolysate.—When a hemolysate of rabbit red cells made isotonic with sucrose was subjected to centrifugation at 25,000 *G* for 30 minutes at 4° in the high speed attachment of the International refrigerated centrifuge, the resultant precipitate was inactive and the total hemolytic activity of the original hemolysate remained in the supernatant fluid. However, centrifugation even at low speed (146 *G*) of rabbit hemolysate rendered isotonic with saline resulted in the precipitation of 60 per cent of

the lytic factor as shown in Table I. The remainder of the activity was retained in the supernatant fluid and could not be brought down by further centrifugation at speeds of up to 25,000 *G* for 30 minutes.

If the active precipitate, P-1, was washed by suspension in saline—0.01 M PO_4 and centrifuged at 1929 *G* for 10 minutes only 25 per cent of the original activity of P-1 was precipitated. However, if P-1 was suspended in sucrose—PO_4 and centrifuged as above the resultant precipitate, P-2, contained 60 per cent of the original activity of P-1 (Table I). The final method evolved for obtaining the active precipitated fraction was as follows: 0.032 ml. of packed

TABLE I

Centrifugation of Lytic Factor

Fraction	Hemolytic activity
	per cent
1. Original hemolysate in isotonic saline	100
2. Centrifuged at 146 *G* for 10 min.	
(*a*) Supernatant fluid (S-1)	33
(*b*) Precipitate (P-1)	63
3. P-1 suspended in saline–0.01 M PO_4 and centrifuged at 1929 *G* for 10 min.	
(*a*) Supernatant fluid (S-2)	47
(*b*) Precipitate (P-2)	16
4. P-1 suspended in sucrose–0.01 M PO_4 and centrifuged at 1929 *G* for 10 min.	
(*a*) Supernatant fluid (S-2)	25
(*b*) Precipitate (P-2)	38

rabbit erythrocytes was lysed with 4.8 ml. water and 1.2 ml. of 4.5 per cent NaCl was then added to restore isotonicity. The hemolysate was centrifuged at 1929 *G* at 4°C. for 10 minutes, the supernatant fluid discarded, and the precipitate, P-1, was washed by suspension in 6 ml. of sucrose—PO_4 and recentrifugation at the same speed, 1929 *G*. The supernatant fluid was discarded and the second precipitate, P-2, was suspended in 3 ml. of sucrose—PO_4 (half the original volume) for use in the hemolysin studies. This preparation, hereafter referred to as rabbit P-2, was relatively free of hemoglobin and of the other soluble factors (glutathione, ergothionine, etc.) present in the original hemolysate, a decided advantage when studying the effect of these components on the hemolytic system. It could be preserved at −70°C. and was fully active after a storage period of 1 month. Further purification of the hemolytic factor is currently underway.

Absolute Requirements.—Rabbit red cell hemolysate did not lyse human erythrocytes unless both Mg^{++} and ATP were present. Mg^{++} at final concentration of 5×10^{-4} to 5×10^{-3} M gave maximal hemolysis in the presence of 1.67×10^{-4} M ATP. The response to increasing concentrations of ATP is shown in Fig. 2.

It can be seen that maximal hemolysis is obtained with 1.67×10^{-4} M ATP. A fourfold excess, namely 6.67×10^{-4} M, was used in all subsequent experiments to insure fulfillment of the ATP requirement. If more ATP was added

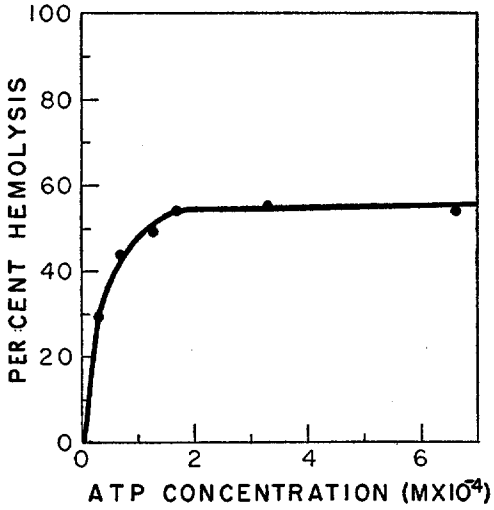

FIG. 2. Effect of ATP concentration on hemolysis. Each tube contained 0.04 ml. of rabbit hemolysate; 0.3 ml. of 8 per cent human erythrocytes in sucrose–PO–Mg; versene (1.67×10^{-4}M); and the given amount of ATP. Diluent is sucrose–PO_4. Total volume = 0.6 ml.

to a system which had been incubated for 1 hour with 6.67×10^{-4} M ATP, and the enriched system was then incubated for an additional hour, no further hemolysis occurred.

Adenine, adenosine, or inorganic pyrophosphate at a concentration of 3.3×10^{-4} M neither caused hemolysis in the absence of ATP nor inhibited the system in the presence of an equal concentration of ATP. Adenylic acid (AMP) was found to inhibit hemolysis in the presence of 6.67×10^{-4} M ATP, the inhibition being proportional to the AMP concentration, namely, 0 per cent inhibition at 3.34×10^{-4} M AMP, 20 per cent at 6.67×10^{-4} M AMP, and 50 per cent at 1.33×10^{-3} M AMP.

Adenosinediphosphate (ADP) was quite effective in promoting hemolysis. Maximal activity, which was reached at 6.67×10^{-4} M ADP, averaged 60

per cent of the control maximal hemolysis value obtained with ATP (6.67×10^{-4} M), and the same rabbit hemolysate preparation. When both ADP and ATP were added simultaneously to the system, the resultant hemolysis was equal to that obtained with ATP alone.

Cytidine triphosphate (CTP), and uridine triphosphate (UTP) caused slight hemolysis (about 25 per cent of the maximal value obtained with ATP) when tested at a concentration of 4×10^{-4} M. CTP and UTP added together were about 50 per cent as effective as ATP. The presence of 1.33×10^{-5} M ATP, which in itself produced no hemolysis, almost doubled the activity of UTP and CTP when used singly but had very little effect on hemolysis produced by simultaneous addition of UTP and CTP. When the system contained maximal concentrations of ATP (6.67×10^{-4} M) hemolysis was not enhanced by the addition of either UTP or CTP or both.

Effect of Dialysis and Heating.—The hemolytic activity of rabbit hemolysate was not affected by dialysis against an isotonic saline solution containing 0.01 M PO_4 buffer, pH 7, and 0.001 M versene, for 18 hours, at 4°C. Moreover, no lysis of human erythrocytes occurred when a dialysis bag containing rabbit hemolysate, versene (2×10^{-3} M), and ATP (3.4×10^{-4} M) was placed in a test tube containing human red cells in versene and ATP (also at 2×10^{-3} M and 3.4×10^{-4} M, respectively) and the system was incubated for 3½ hours at 34°C. These observations, coupled with the fact that the hemolysate is completely inactivated by heating at 56°C. for 1 hour, suggest that the active principle is a large molecule and probably a protein.

Inhibitions and Activators.—(1) Metals. All metals were tested in a final concentration of 5×10^{-4} M, at which level Mg^{++} produced maximal hemolysis. In the absence of Mg^{++}, both Mn^{++} and Co^{++} showed some activity, amounting to 21 and 41 per cent respectively, of the hemolysis produced by Mg^{++}, Ca^{++}, Fe^{++}, and Fe^{+++}, could not substitute for Mg as activators. The following metals proved inhibitory in the presence of a tenfold excess of Mg^{++} (5×10^{-3} M); Ca^{++} (21 per cent inhibition), Mn^{++} (30 per cent), Fe^{++}, (16 per cent), Zn^{++} (100 per cent), Hg^{++} (100 per cent). Cu^{++} caused hemagglutination of the human erythrocytes.

In this connection it is interesting to note that human urine inhibited hemolysis, apparently by virtue of its metallic cations, since the inhibitory factor was removed by exhaustive dialysis of the urine and was overcome by versene. Moreover, the inhibitor was not destroyed by sulfuric acid digestion of the urine.

The hemolysin test is carried out in the presence of 1.67×10^{-4} M versene in order to chelate any trace of metal contaminants. A higher versene concentration was not employed since it in itself tended to lyse human red cells.

(2) Organic compounds. Eserine (physostigmine) in a concentration of 1×10^{-4} M neither inhibited nor activated the system.

Cysteine (1.67×10^{-4} M) caused a 64 per cent inhibition of hemolysis by rabbit hemolysate but glutathione (GSH) in high concentrations, (1.67×10^{-3} to 1.67×10^{-2} M), increased the hemolytic activity by about 20 per cent. Oxidized glutathione (GSSG) at 1.67×10^{-3} M caused 84 per cent inhibition and this was almost completely overcome by the simultaneous addition of a tenfold excess of GSH.

The hemolytic activity of freshly prepared, highly active, rabbit P-2 was enhanced only to a slight extent by GSH when versene (1.67×10^{-4} M) was present. In the absence of versene, however, 1.7×10^{-2} M GSH increased hemolysis by about 30 per cent and this was equal to the activation obtained

TABLE II

Activation of Rabbit P-2 by Glutathione

GSH concentration	Hemolysis	Activation
moles	*per cent*	*per cent*
0	6	—
1.7×10^{-6}	6	0
1.7×10^{-5}	7	0
1.7×10^{-4}	6	0
1.7×10^{-3}	21	350
1.7×10^{-2}	41	683

Each tube contained 0.1 ml. of rabbit P-2 which had been aged for 24 hours at 4° before use; versene (1.67×10^{-4}M); ATP (6.67×10^{-4}M); 0.3 ml. of 8 per cent human erythrocytes in sucrose–PO_4–Mg; and sucrose–PO_4 or GSH as indicated. Total volume = 0.6 ml.

by the addition of versene alone. In this case glutathione may have merely served to combine with trace metal inhibitors in the system, as did versene, and this resulted in an increased hemolytic effect.

However, weakly active rabbit P-2 preparations, obtained by storage for several days at 4° or by preincubation for 1 hour at 34°, showed a pronounced response to GSH which was independent of the presence of versene as shown in Table II.

In Fig. 3 the inhibition of freshly prepared rabbit P-2 by GSSG in a concentration of from 3.3×10^{-4} M to 1.67×10^{-3} M is graphically presented. As in the case of rabbit hemolysate, inhibition of rabbit P-2 by GSSG (1.67×10^{-3} M) was almost completely overcome by ten times the concentration of GSH (1.67×10^{-2} M).

L-Ergothionine (1.7×10^{-3} M to 1.7×10^{-2} M) also completely inhibited the system, showing no effect at lower concentrations. Here again GSH was effective in partially negating the inhibition. In the presence of 1.67×10^{-2} M GSH an equal concentration of L-ergothionine reduced hemolysis by only 50 per cent and 1.67×10^{-3} M ergothionine caused only 23 per cent inhibition.

DPN, TPN, FMN (all at 1×10^{-4} M), FAD (2×10^{-5} M), and coenzyme-A (9×10^{-5} M) were without effect on the hemolytic end-point. When coenzyme-A was added in combination with 1.7×10^{-3} M GSH no activation above that of the GSH alone was observed.

Metabolic Inhibitors.—When various metabolic inhibitors at a final concentration of 0.002 M were added to our system it was found that hemolysis was reduced by sodium cyanide (54 per cent inhibition), α-dinitrophenol (22 to 40 per cent), and sodium azide (10 per cent). Potassium fluoride inhibited the

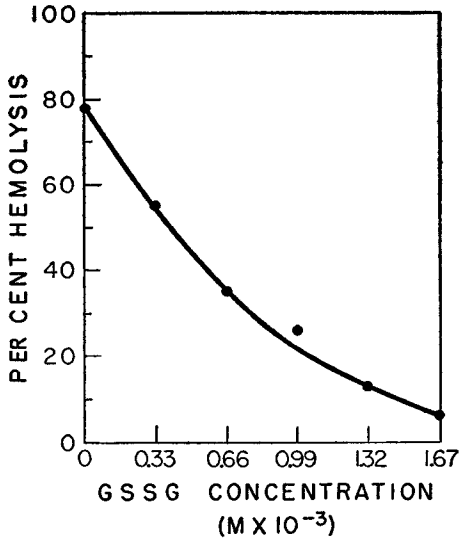

FIG. 3. Inhibition of hemolysis by oxidized glutathione. Each tube contained 0.15 ml. of freshly prepared rabbit P-2; versene (1.67×10^{-4}M); ATP (6.67×10^{-4}M); 0.3 ml. of 8 per cent human erythrocytes in sucrose–PO_4–Mg; and GSSG as indicated. Diluent is sucrose–PO_4. Total volume = 0.6 ml.

system completely at 0.04 M but had no effect at 0.004 M. Iodoacetate tested at 0.005 M and 0.001 M resulted in 85 and 53 per cent inhibition, respectively.

Inhibition by Human Stroma.—Since it appeared plausible that in our system the lysis of human erythrocytes by rabbit hemolysate was mediated by some reaction involving the membrane of the human red cell, the effect of human stroma on the hemolytic end-point was investigated.

Stroma was prepared as follows: 1.2 ml. of packed, saline-washed, human erythrocytes were lysed by the addition of 12 ml. of water and the stroma was separated by centrifugation at 5000 R.P.M. for 15 minutes at 4°. The supernatant fluid was discarded and the stroma was washed twice with 4 volumes of saline and was then suspended in saline to a final volume of 5 ml.

Thus, 0.1 ml. of this preparation contained the stroma derived from 0.024 ml. of packed cells which was equivalent to the concentration of human erythrocytes used in the hemolysin test (0.3 ml. of 8 per cent cells). In all cases, the stroma was prepared from the same batch of cells as were subsequently used in the hemolysin test.

In these experiments the effect of 0.1 ml. of freshly prepared human stroma on rabbit hemolysate which had been titrated to a hemolytic end-point of approximately 50 per cent was tested with five different human stroma preparations as follows:—

1. Rabbit hemolysate and human erythrocytes, in the presence of 6.67×10^{-4} M ATP, were incubated according to the usual procedure with and without added stroma.

2. Rabbit hemolysate alone, and hemolysate plus human stroma, were preincubated for 1 hour at 34°. At the end of this time, ATP (6.67×10^{-4} M) and human erythrocytes in sucrose—PO_4—Mg were added and the complete systems were then incubated for an additional hour.

3. Rabbit hemolysate alone, and hemolysate plus human stroma, were preincubated for 1 hour in the presence of 6.67×10^{-4} M ATP. After preincubation, human erythrocytes in Su—PO_4—Mg, and ATP to readjust the final concentration to 6.67×10^{-4} M were added and incubation was continued as in (2).

In Table III the data obtained from five separate experiments using different preparations of stroma, red cells, and rabbit hemolysate are recorded.

Hemolysis is almost always inhibited (by about 20 per cent) by human stroma prepared from the same batch of cells as are used in the hemolysin test itself. Preincubation of rabbit hemolysate and stroma prior to the addition of human erythrocytes and ATP to the system, enhances the effect. Preincubation in the presence of ATP results in almost complete inhibition of hemolysis. Although we have found that rabbit hemolysate contains ATPase, the reduced lysis cannot be attributed to a low ATP concentration resulting from destruction during preincubation since, after preincubation, an amount of ATP equal to at least twice the concentration necessary for full hemolysis is supplied to the system at the same time that erythrocytes are added.

Adenosinetriphosphate Balance Studies.—In an attempt to elucidate the role of ATP in the hemolytic process the concentration of labile phosphate was followed during the course of hemolysis.

Freshly prepared rabbit P-2 was incubated with human red cells in the presence of versene, Mg^{++}, and ATP, and aliquots of the incubation mixture were assayed for the extent of hemolysis and for labile phosphate immediately before and after 30 and 60 minutes' incubation at 34°. At the same time, controls containing (1) human erythrocytes, (2) rabbit P-2, and (3) human he-

molysate were set up. In this experiment sucrose-0.03 M Tris, pH 7.2, was substituted for the usual sucrose—PO_4, both in the washing and preparation of human cells and of rabbit P-2, and as the diluent for the hemolysin test.

At the desired time the reaction was arrested by rapid chilling in an ice bath and the mixture was centrifuged for 10 minutes at 4°. The supernatant fluid was carefully separated from the cellular residue, the cells were resus-

TABLE III

Inhibitory Effect of Human Stroma on Hemolysis

Experiment No.	Per cent hemolysis					
	No Preincubation		Preincubation — ATP		Preincubation + ATP	
	Hemolysate alone	Stroma + hemolysate	Hemolysate alone	Stroma + hemolysate	Hemolysate alone	Stroma + hemolysate
1*	66	54	65	37	65	3
2*	52	37	54	36	56	13
3‡	55	44	37	31	32	4
4§	57	40	57	21	52	9
5§	63	54	69	30	69	2
Average per cent inhibition..		19		46		93

* Experiments 1 and 2. The final ATP concentration in the hemolysin test and in preincubation with ATP is 6.67×10^{-4} M. Mg^{++} is not present during the preincubation period but is added with the red cells (0.3 ml. of 8 per cent cells in $SuPO_4$–0.01 M $MgCl_2$) after preincubation.

‡ Experiment 3. The conditions are the same as above but, in addition, versene (1.67×10^{-4} M final concentration) is present during both the preincubation period and the hemolysin test.

§ Experiment 4 and 5. The final ATP concentration in the hemolysin test and in preincubation with ATP is 1.33×10^{-3} M. Mg^{++} (5×10^{-3} M final concentration) and versene (1.67×10^{-4} M final concentration) are present during the preincubation period and in the hemolysin test.

pended to the original volume with ice cold saline, and both fractions were deproteinized by the addition of ice cold trichloracetic acid (10 per cent final concentration). In the rabbit P-2 control, the centrifugation step prior to deproteinization was omitted since the resulting precipitate was very small and difficult to separate and the mixture was analyzed as a whole. The concentrations of labile phosphate in the protein-free filtrates were calculated as the difference between the inorganic phosphate values before and after hydrolysis in 1 N HCl for 7 minutes, using the procedure of Fiske and SubbarRow (2). The results of a typical experiment are shown in Table IV.

Since human erythrocytes contain labile phosphate compounds, the flasks to which human cells or hemolysate had been added were found to contain

about 16 per cent more than the theoretical amount of labile phosphate which would arise from the added ATP alone. After 30 minutes of incubation the complete system (flask 4) showed that 19 per cent of the erythrocytes had been hemolyzed and that the total labile phosphate content of the combined supernate and the precipitate had decreased by a total of 18 per cent of the original value. The human hemolysate control decreased by only 3 per cent

TABLE IV

Change in Labile Phosphate during Hemolysis

Flask	Contents	Total labile phosphate								
		0 min.			30 min.			60 min.		
		Super.	Ppt.	Total	Super.	Ppt.	Total change	Super.	Ppt.	Total change
		μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols
1	Human erythrocyte control	15.44	3.46	18.90	16.09	3.46	+0.65	15.23	3.02	−0.65
2	Rabbit P-2 control	15.01	—	15.01	11.50	—	−3.51	11.07	—	−3.94
3	Human hemolysate control	17.66	1.30	18.96	17.82	0.65	−0.49	17.28	0.65	−1.03
4	Human erythrocytes + rabbit P-2	14.42	3.19	17.61	12.74	2.11	−2.76	12.20	1.13	−4.28
		0 per cent hemolysis			19 per cent hemolysis			61 per cent hemolysis		

All flasks contain a final concentration of 1.67×10^{-4} M versene, 5×10^{-3} M $MgCl_2$, and 2.96×10^{-4} M ATP. In addition, the following are added to the respective flask:

1. 7.25 ml. of 16 per cent human erythrocytes.
2. 11.25 ml. of rabbit P-2.
3. 7.25 ml. of hemolyzed 16 per cent human erythrocytes restored to isotonicity with sucrose.
4. 7.25 ml. of 16 per cent human erythrocytes and 11.25 ml. of rabbit P-2.

The diluent throughout is isotonic sucrose–0.03 M-Tris, pH 7.2.

Total volume = 27 ml. of which 5 ml. aliquots are analyzed at the stated time.

while the erythrocyte control actually increased by 3 per cent. However, in the case of rabbit P-2 alone, 23 per cent of the labile phosphate had disappeared.

Similarly, after 60 minutes when the complete system showed 61 per cent hemolysis, the total decrease in labile phosphate (24 per cent) was about the same as that in the rabbit P-2 control (26 per cent), the human erythrocyte and human hemolysate control values being decreased by 3 and 5 per cent, respectively, of the original value. It appeared, therefore, that the decrease in labile phosphate during hemolysis of human erythrocytes by rabbit P-2 was due to the activity of the rabbit preparation alone and that there was essentially no net change in total labile phosphorus in the hemolyzing process.

These results were duplicated in another experiment in which the concentration of ATP alone, instead of total labile phosphate, was followed. The incubation procedure was the same except that sucrose—PO_4 was the diluent throughout and deproteinization was effected by ice cold perchloric acid (2 per cent final concentration). The resulting protein-free filtrates were neutralized with potassium hydroxide to pH 7.4 (phenol red used as internal

TABLE V

Change in ATP Concentration during Hemolysis

Flask	Contents	Total ATP								
		0 min.			30 min.			60 min.		
		Super.	Ppt.	Total	Super.	Ppt.	Total change	Super.	Ppt.	Total change
		μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols	μ mols
1	Human erythrocytes control	6.62	0.86	7.48	6.34	0.61	−0.53	5.94	0.58	−0.96
2	Rabbit P-2 control	5.29	—	5.29	4.21	—	−1.08	3.02	—	−2.27
3	Human hemolysate control	6.19	0.25	6.44	6.01	0.18	−0.25	6.19	0.18	−0.07
4	Human erythrocytes + rabbit P-2	5.94	0.65	6.59	4.97	0.58	−1.04	3.99	0.36	−2.24
		0 per cent hemolysis			22 per cent hemolysis			51 per cent hemolysis		

All flasks contain a final concentration of 1.67×10^{-4} M versene, 5×10^{-3} M $MgCl_2$, and 3.33×10^{-4} M ATP. In addition, the following are added to the respective flask:

1. 4.50 ml. of 16 per cent human erythrocytes.
2. 8.00 ml. of rabbit P-2.
3. 4.50 ml. of hemolyzed 16 per cent human erythrocytes restored to isotonicity with sucrose.
4. 4.50 ml. of 16 per cent human erythrocytes and 8.00 of rabbit P-2.

The diluent throughout is sucrose–PO_4.

Total volume = 18 ml. of which 5 ml. aliquots are analyzed at the stated time.

indicator) and the insoluble potassium perchlorate was removed by centrifugation at 4°. Aliquots of the perchlorate-free supernatant fluids were then analyzed enzymatically for ATP by the method of Kornberg (3) in which glucose, TPN, glucose—6—PO_4 dehydrogenase, and hexokinase are added to the system and the reduction of TPN is followed spectrophotometrically at 340 mμ using a Beckman model DU quartz spectrophotometer. Glucose—6—PO_4 dehydrogenase[3] was prepared according to Kornberg (4) and hexokinase[3] was made following the method of Berger *et al.* (5) to step 3. The data are given in Table V.

[3] We wish to thank Dr. Marianna R. Bovarnick for her kindness in providing these enzymes.

It can be seen that the loss of ATP after 30 and 60 minutes of incubation of combined rabbit P-2 and human erythrocytes when 22 and 51 per cent hemolysis, respectively, had been achieved, was equal to the decrease obtained in the rabbit P-2 control. These results show that the action of the rabbit factor on human red cells, which leads to hemolysis of the latter, is associated with no net decrease of ATP in the system but that the observed loss is equal to the endogenous ATP-ase activity of the rabbit preparation. Further studies to gain insight into the chemical reactions whereby ATP is utilized in the hemolytic process are currently under way employing isotopically labelled ATP.

DISCUSSION

The requirement for ATP, normally an intracellular source of energy for synthetic processes, in a hemolytic reaction, generally considered a degradative process, seems somewhat paradoxical. The precise nature of the reactions involved remains to be determined. One interesting possibility is that ATP is involved as an intermediary in a reaction between rabbit hemolysate and a receptor site on the red cell membrane. The presence of reactive sites for ATP has been postulated on red cell and other cell surfaces as part of the mechanisms involved in the transfer of both cations (6, 7) and phosphate (8) across the cell membrane. In both instances it has been hypothesized that the reactive transfer site on the cell surface utilized high energy phosphate which had been generated inside the cell as the driving force for the directional transfer of the ions under consideration.

In the case described in this paper an additional potential source of energy in the form of extracellular ATP is brought to bear on the system. If ATP does function here as a source of energy, the possibility arises that the reactive site in this system might be the same as that involved in normal ion transfer processes, but it is conceivable that the new condition might upset the normal balance and direction of ion transport in such fashion as to produce osmotic disturbances leading to hemolysis. Similar considerations might apply to the functioning of a "water pump" if this actually exists. Another possibility is that phosphorylation of the membrane destroys its integrity. The possibility that the acetylcholine–choline esterase system mechanism, known to be present in human red cells and to influence ion transport (9–11), is involved in our system seems to be ruled out by the failure of physostigmine, a choline esterase inhibitor, to affect our system.

The lytic factor present in rabbit hemolysate appears to be an enzyme since it is stable to dialysis and is destroyed at 56°C. Moreover, the marked activation of the system by glutathione, in conjunction with its sensitivity to oxidized glutathione, iodoacetate, and heavy metals, suggests the involvement of a sulfhydryl enzyme.

These various aspects, as well as the further interesting one of the relation of this phenomenon to "ATP shock" (12) are under investigation.

SUMMARY

1. Hemolyzed rabbit cells contain a factor which lyses human erythrocytes *in vitro* when Mg^{++} and certain nucleotides are supplied to the system. Of the nucleotides tested ATP is the most active, although no net loss of ATP or of labile phosphate seems to be associated with the hemolytic process.

2. The lytic factor appears to be a sulfhydryl enzyme which attacks the membrane of the human red cell, its hemolytic activity being inhibited by human stroma.

3. The system is activated by glutathione and is inhibited by heavy metals, oxidized glutathione, cysteine, ergothionine, and a number of metabolic inhibitors. Physostigmine has no effect.

4. Partial purification of the lytic factor has been achieved by fractional centrifugation.

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