AN ADENOSINETRIPHOSPHATE-ACTIVATED HEMOLYTIC SYSTEM

II. Utilization of Adenosinetriphosphate in the Reaction

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ABSTRACT

Failure to demonstrate ATP[1] utilization in an ATP-activated hemolytic system had been previously reported. In the present study ATP utilization is shown to be associated with the hemolytic reaction and also with the ATP-dependent inactivation of the hemolytic factor of the system by crude, washed, human red cell stroma. Using crude stroma, relatively large ATP utilization occurs and continues, but at a decreasing rate, after inactivation of the hemolytic factor is complete. With purified stroma there is very slight uptake of ATP by the stroma in the presence of hemolytic factor and Mg^{++}. This uptake can only be demonstrated by radioactive ATP. Both C^{14}- and P^{32}-labelled nucleotide were used for this purpose. In the presence of an excess of stroma the uptake seems to be dependent on the amount of hemolytic factor used. Evidence is given contraindicating the possibility that this uptake is non-specific.

In a previous report (1) we described some properties of an ATP-activated hemolytic system consisting of ATP, a soluble protein obtained from rabbit red blood cell hemolysate, $MgCl_2$, and human red cells which are lysed on addition of the other components. It was further shown that stroma prepared from human red cells, if preincubated with Mg^{++}, ATP, and the rabbit red cell hemolytic factor, inactivated the lytic power of the latter for subsequently added human red cells. This inhibition reaction was also ATP-dependent.

Despite the fact that the hemolytic reaction was ATP-dependent, we were nevertheless unable to demonstrate any utilization of ATP associated with hemolysis, either by measuring change in acid-labile P or by direct ATP analysis. One possible explanation of this discrepancy could be that there was so

[1] The following abbreviations are used: ATP, adenosinetriphosphate; AMP, adenosinemonophosphate; DCC, *N*, *N*[1]-dicyclohexyl carbodiimide; EDTA, ethylenediamine tetraacetate; GSH, glutathione. A.R.P.P.$^{32}P^{32}$, adenosinetriphosphate labelled with P^{32} in the two acid-labile phosphates.

little ATP utilized in the reaction that it was not detectable in the presence of the relatively large control endogenous utilization of ATP by the red cells and its destruction by rabbit factor and stroma.

Further attempts have been made, therefore, to demonstrate ATP utilization in both the hemolytic and inhibition reactions, using a 100-fold purified rabbit hemolytic factor, purified stroma, and both unlabelled and radioactive isotope-labelled ATP under various conditions of reaction. The results are reported below.

Materials and Methods

All glassware was carefully washed as previously described (1).

Adenosinetriphosphate (ATP).—Obtained as the crystalline sodium salt from Pabst.

ATP-8C^{14}.—Obtained as the crystalline disodium salt from Schwarz having a specific activity of 0.5 to 1.0 microcuries C^{14} per micromole.

A.R.P.P.$^{32}P^{32}$.—Synthesized by a modification of the procedure described by Lowenstein (2). $H_3P^{32}O_4$ (3 millicuries in 0.5 ml. of 0.27 N HCl) obtained from Oak Ridge National Laboratory was evaporated to dryness by an air stream at room temperature. To this were added 500 mg. (1 millimole) of ADP, 380 mg. (1 millimole P) of $Na_3PO_4 \cdot 12H_2O$, and 5 gm. of *N*, *N*^{1}-dicyclohexyl carbodiimide (DCC) dissolved in 25 ml. of pyridine–H_2O (10:1). The flask was shaken vigorously at room temperature for 6 hours and then 50 ml. of H_2O was added. The mixture was extracted five times with an equal volume of petroleum ether to remove the unreacted DCC.

The water layer was adsorbed on a Dowex-1 Cl^- column (1.5 cm. diameter, 10.0 cm. high) and the ATP eluted with 0.01 N HCl–0.2 M NaCl according to the method of Cohn and Carter (3). The ATP eluate was then adsorbed on 5 gm. of norit which had previously been washed with 1 N HCl, water to neutrality, 0.1 M EDTA, water to remove the EDTA, and then dried *in vacuo* before use. Following adsorption of the ATP, the norit, after a preliminary water wash, was stirred for one-half hour at room temperature with 50 ml. of 15 per cent pyridine. The pyridine was filtered off and the extraction process repeated three more times (4). The combined pyridine extracts were lyophilized to dryness.

The A.R.P.P.$^{32}P^{32}$ thus obtained was chromatographically pure. Yield = 6 micromoles; specific activity P^{32} = 1,280,000 C.P.M. per micromole ATP.

ATP Assays:

The solutions to be assayed were deproteinized with an equal volume of ice cold 6 per cent perchloric acid and the perchloric acid was subsequently precipitated by neutralization to pH 7.4 with dilute potassium hydroxide using phenol red as an internal indicator. The protein- and perchlorate-free filtrates were then analyzed for ATP by either of the following two methods, depending upon whether or not glutathione was present in the medium to be assayed.

1. In the absence of glutathione the method of Kornberg (5) was used. Glucose, TPN, glucose-6-PO_4 dehydrogenase (prepared according to Kornberg (6)), and hexokinase (prepared according to Berger *et al.* (7) to step 3) were added and the reduction of TPN was followed at 340 mμ using a Beckman model DU quartz spectrophotometer.

2. When glutathione was present ATP was determined by addition of firefly luciferase as described by Strehler and Totter (8) using a Farrand model A fluorometer.

Human Erythrocytes:

These were washed and stored as previously described (1).

Partially Purified Hemolytic Factor from Rabbit Erythrocytes.—The entire procedure was carried out at 0 to 4°C. 200 ml. of rabbit blood obtained by heart puncture was diluted with an equal volume of sterile citrate–dextrose solution (sodium citrate, 0.073 M; citric acid, 0.0024 M; and glucose, 0.113 M) and then centrifuged. The plasma and buffy coat were discarded. The cells were washed three times with 200 ml. of isotonic sucrose containing 0.002 M histidine, pH 7. The washed, packed cells were lysed by the addition of 0.001 M EDTA pH 7, to a total volume of 700 ml. The lysate was adjusted to pH 6.5 with 1 N hydrochloric acid and centrifuged for 1 hour at 15,000 R.P.M. to sediment the stroma. The stroma precipitated far better at pH 6.5 than at 7.0 and the hemolytic activity of the hemolysate was not impaired at the lower pH for this length of time. The supernatant fluid was decanted, adjusted to pH 7.4 with 1 N sodium hydroxide, and 0.001 M EDTA was added to restore the volume to 700 ml.

The hemolytic factor was precipitated by the addition with stirring of 219.1 gm. of EDTA–recrystallized ammonium sulfate (9) to the stroma-free hemolysate to give a 50 per cent saturated solution. After centrifugation for 30 minutes at 15,000 R.P.M. the supernatant fluid, which contained none of the original hemolytic activity, was discarded.

The precipitate was dissolved in 0.001 M EDTA–0.002 M histidine, pH 7.0, the final volume being 100 ml. Solid ammonium sulfate (17.6 gm.) was added to 30 per cent saturation and the solution was centrifuged as before. The inactive precipitate was discarded.

Solid ammonium sulfate (9.4 gm.) was added to the supernatant fluid to 50 per cent saturation and after centrifugation the active precipitate was dissolved in 8 ml. of isotonic sucrose which contained 0.001 M EDTA and 0.002 M histidine, pH 7.0.

The solution was dialyzed against 2 liters of the isotonic sucrose–EDTA–histidine solution for 12 hours and stored at −20° until needed.

This partially purified enzyme preparation contained about 50 per cent of the original hemolytic activity of the stroma-free hemolysate and its specific activity was increased about a hundredfold. The average yield of seven enzyme preparations was 6,500 hemolytic units from 200 ml. of rabbit blood. Myokinase and ATP-ase were still present although the concentration of the latter was greatly diminished. Preliminary attempts to separate these activities completely from the hemolytic factor by adsorption and elution from $Ca_3(PO_4)_2$ or from Dowex-2 have been unsuccessful.

Human Stroma:

1. Crude Stroma.—Human blood (group O Rh^+) in ACD solution was centrifuged, the plasma and buffy coat were removed, and the cells were washed as described under Human erythrocytes above. The packed cells were lysed with 9 volumes of ice cold water, pH 7, and the lysate was centrifuged 1 hour at 15,000 *G* in a Lourdes centrifuge at 0–4°C. The supernatant fluid was discarded, the stroma was washed three times with water, and finally suspended in water to give a 20 per cent suspension based on

the original volume of cells from which the stroma was derived. The preparation was used immediately or after overnight storage at 0–4°C. This preparation contained a considerable amount of hemoglobin.

2. *Purified Stroma.*—All steps were carried out at 0–4°C.

A liter of human blood (group O Rh^+) in ACD solution was centrifuged and the plasma and buffy coat were removed. The cells were washed three times with 2 liters of isotonic saline. The packed cells were then frozen in a dry-ice acetone bath and thawed at 34°C. The process was repeated twice. Isotonic saline containing 0.001 M EDTA, pH 7, was added to a total volume of 1200 ml. and the mixture was centrifuged at 20,000 R.P.M. in a Spinco preparative centrifuge at 4°C. for 1 hour. The stroma precipitate was then washed as follows:—

(*a*) Six times with isotonic saline–0.001 M EDTA, pH 7 (810 ml.; centrifugation time = 30 minutes).

(*b*) Four times with 2 M sodium chloride–0.001 M EDTA, pH 7 (810 ml.; centrifugation time = 45 minutes).

(*c*) Three times with 0.001 M EDTA, pH 7 (480 ml.; centrifugation time = 1 hour).

The stroma was then lyophilized to dryness. The resultant white, fluffy powder was stored at room temperature in a desiccator and maintained its inhibitory potency for up to 3 months.

Yield = 1.5 gm.; 300,000 inhibitory units

Hemolysin Test:

The following components were added to Kahn tubes: 0.55 cc. of a mixture containing 8 parts of 0.5 M sucrose + 2 parts of 0.1 M potassium phosphate, pH 7.0, + 1 part of 0.001 M EDTA, pH 7.0, 0.05 ml. of 0.05 M $MgCl_2$, 0.1 ml. of 0.01 M ATP, pH 7.0, 0.2 ml. of 24 per cent human erythrocytes, and 0.1 ml. of rabbit hemolytic factor which had been serially diluted as required in a solution containing 5 mg. of glutathione per ml. of 0.001 M EDTA–0.002 M histidine or phosphate buffer, pH 7.0. The total volume was 1 ml. At the same time a tube containing all the above components except the hemolytic factor was set up and served as the instrument blank against which all other tubes were read.

The tubes were incubated for 30 minutes at 34°C., mixed with 2 ml. of ice cold saline, centrifuged for 10 minutes at 2000 R.P.M., and the optical density of the supernatant was measured at 545 mμ in a Coleman Junior spectrophotometer. All values above 0.700 were corrected for the non-linearity of the instrument.

One hemolytic unit of enzyme was defined as the amount which produced hemolysis corresponding to an optical density of 0.700 in this system.

Determination of Inhibitory Titer of Stroma:

Rabbit hemolytic factor was diluted in glutathione solution (10 mg. GSH per ml. of 0.001 M EDTA–0.002 M potassium phosphate buffer, final pH = 7.0) so that 0.15 ml. contained 6 to 9 hemolytic units. Stroma was suspended in water with the aid of a teflon homogenizer in a concentration of 1 per cent.

To Kahn tubes in an ice bath were added 0.075 ml. of 0.05 M $MgCl_2$, 0.55 ml. of diluent composed of 0.25 M sucrose–4×10^{-4} M EDTA–4×10^{-2} M phosphate, pH 7.0, 0.075 ml. of 0.01 M ATP, and the following: (1) Complete system: 0.15 ml. of serial

dilutions of stroma suspension. (2) Hemolytic factor control: 0.15 ml. of water. Finally, 0.15 ml. of rabbit hemolytic factor was added where indicated under the surface of the solution.

At the same time controls for (1) and (2), containing 0.075 ml. of water instead of ATP, were set up. The total volume in each tube was 0.6 ml.

After gentle mixing 0.1 ml. aliquots of each tube were assayed in duplicate for hemolytic activity as described under Hemolysin test. The remainder of the tube contents was incubated for 30 minutes at 34° and the hemolytic activity of 0.1 ml. aliquots was again determined. ATP-dependent inhibition was calculated from the titres, expressed as optical density read at 545 mμ, of these aliquots.

$$\text{Per cent inhibition} = 100 \left\{ 1 - \frac{\text{Titre of postincubation aliquot}}{\text{Titre of preincubation aliquot}} \right\}$$

ATP-independent inhibition was similarly calculated from the corresponding values obtained in the absence of ATP during the incubation period. The hemolytic factor controls, with and without ATP, served as a check on the stability of the hemolytic factor under the conditions of the experiment.

The amount of stroma which inhibited 1 hemolytic unit of enzyme by 100 per cent was defined as 1 inhibitory unit.

Under the conditions of this test stroma only inactivates the hemolytic factor after incubation with it in the presence of ATP and Mg^{++} prior to the addition of the red cells. Therefore, in the determination of pre- and postincubation hemolytic titres the presence of the stroma does not significantly affect the determination of these titres.

EXPERIMENTAL AND RESULTS

ATP Utilization Associated with Hemolysis:

A further attempt to demonstrate ATP utilization in the hemolytic reaction was made by lysing a greater concentration of red cells than had been previously used. The results of some typical experiments are shown in Table I. It can be seen that there appears to be a definite net utilization by the complete system. While this type of result was consistently obtained, the rather variable and complex situation with respect to the controls was such that the experiment did not lend itself to clear interpretation in relation to possible mechanisms. Thus there is ATP-ase action associated with both red cells and hemolysates but there is also some ATP synthesis associated with these and the net balance between the two can be variable depending on the mode of hemolysis (10), the state of the red cells, and many other unknown factors. The effect of the present method of hemolysis on ATP-ase activity is, for instance, unknown as is the length of time the cells in the experiment are present as whole cells or lysate. Furthermore, the reaction between hemolytic factor and human hemolysates is also associated with ATP utilization (Table I, Experiment 4).

ATP Utilization Associated with Inhibition of Hemolytic Factor by Stroma:

Because of these considerations attention was turned to further experiments concerning ATP utilization in the reaction between rabbit hemolytic factor and human red cell stroma. The washed stroma should, at least, be free of those complicating factors introduced by the liberation of the soluble components of red cells in the reaction and should have the added advantage of being more nearly in the same state both at the beginning and end of the incubation than is the case when whole red cells are the substrate.

TABLE I

ATP Utilization by Rabbit Hemolytic Factor and Human Red Cells

Experiment No.	ATP disappearance			
	Hemolytic factor + human red cells	Hemolytic factor control	Red cell control	Net utilization
	μM/15 min.	*μM/15 min.*	*μM/15 min.*	*μM/15 min.*
1	0.39	0	0	0.39
2	0.36	0.01	0.13	0.22
3	0.19	0.02	0	0.17
4*	0.41*	0.01	0.11*	0.19

All tubes contain a final concentration of 2×10^{-2} M histidine, pH 7, 7.5×10^{-3} M $MgCl_2$, 0.25 M sucrose, and an initial concentration of 0.5 μM of ATP. In addition the following are added to the respective tubes: (1) Complete system: 60 hemolytic units of rabbit hemolytic factor and 1.0 ml. of 40 per cent human red cells in 0.25 M sucrose. (2) Hemolytic factor controls: 60 hemolytic units of rabbit factor. (3) Red cell controls: 1.0 ml. of 40 per cent human red cells in 0.25 M sucrose. The total volume is 2.0 ml. Incubation is carried out at 34°C. for 15 minutes.

* Experiment 4 contains 1.0 ml. of water-lysed 40 per cent human red cells instead of whole cells.

The utilization of ATP in the presence of a constant amount of freshly prepared crude stroma and varying amounts of hemolytic factor is seen in Table II. A definite net utilization above the controls is evident after a 15 minute period of incubation. However, the amount of ATP utilized was not directly proportional to the concentration of hemolytic factor used when the stroma concentration was kept constant and in excess of the lytic enzyme.

In several experiments when crude stroma which had been stored at 4° for a few days was used it was observed that the amount of ATP utilized during reaction with hemolytic factor was very low. Therefore, the effect of the aging of a 20 per cent suspension of stroma at 4° on its inhibitory titre and ATP utilization in the presence of rabbit hemolytic factor was studied over a period of 16 days (Table III). Both factors were found to decrease with the age of the stroma and by the 16th day the inhibitory titre had dropped to 40 per cent and the ATP utilization to 22 per cent of the original values.

It was of interest to determine whether the ATP utilization paralleled the inactivation of rabbit hemolytic factor by crude human stroma. An excess of freshly prepared crude human stroma was incubated with hemolytic factor and ATP. The hemolytic titre and ATP concentrations were determined at various

TABLE II

ATP Utilization by Rabbit Hemolytic Factor and Crude Human Stroma

Hemolytic units	ATP disappearance			
	Hemolytic factor + stroma	Hemolytic factor control	Stroma control	Net utilization
	μM/15 min.	*μM/15 min.*	*μM/15 min.*	*μM/15 min.*
35	0.22	0	0.07	0.15
70	0.23	0.02	0.07	0.14
140	0.25	0.05	0.07	0.13
210	0.34	0.08	0.07	0.19
280	0.42	0.11	0.07	0.24
420	0.61	0.17	0.07	0.37

All tubes contain a final concentration of 2×10^{-2} M histidine, pH 6.5, 7.5×10^{-3} M $MgCl_2$, and an initial concentration of 0.88 μM of ATP.

In addition, the following are added to the respective tubes: (1) Complete system: 1.0 ml. of a 20 per cent suspension of crude human stroma and rabbit hemolytic factor as indicated. (2) Hemolytic factor controls: rabbit hemolytic factor. (3) Stroma controls: 1.0 ml. of 20 per cent stroma. The total volume is 2 ml. Incubation is carried out at 34°C. for 15 minutes.

TABLE III

Decrease in Inhibitory Activity and ATP Utilization with Age of Stroma

Age of stroma	Inhibitory activity	ATP utilized
days	*per cent of original*	*μM/20 min.*
1	100	0.152
3	86	0.104
6	68	0.096
16	40	0.032

All tubes contain a final concentration of 2×10^{-3} M histidine, pH 6.5, 3×10^{-4} M versene, 7.5×10^{-3} M $MgCl_2$, 1.0 mols ATP, 1 ml. of 20 per cent crude stroma of the specified age, and 20 hemolytic units of rabbit hemolytic factor. Total volume = 2 ml. Incubation is carried out at 34° for 20 minutes.

time intervals up to 25 minutes after mixing. The results of a typical experiment are shown in Table IV. There was only 2 per cent net utilization of the original ATP by the complete system in 5 minutes at which time complete inactivation of the hemolytic factor had been effected. The net utilization of ATP continued with time but the rate decreased after 10 minutes.

The continuation of net utilization of ATP in this system beyond the time required for development of complete inhibition of the hemolytic factor raised the question of whether the ATP utilization might be due in part or in whole to side reactions not related to the hemolytic reaction. It is quite evident that the crude stroma used was a complex mixture of many components and probably contained many enzyme systems. The inhibition reaction with purified stroma was therefore examined.

Using the very sensitive luciferase method of ATP analysis many attempts to demonstrate net ATP utilization under varying experimental conditions in the reaction between hemolytic factor, ATP, and purified stroma all failed despite the fact that inhibition of the hemolytic factor was always complete. We

TABLE IV

ATP Utilization Associated with Inhibition of Hemolytic Factor by Crude Stroma

Time	ATP utilized				Inhibition
	Stroma control	HF control	Stroma + HF	Net utilization	
min.	*μM*	*μM*	*μM*	*μM*	*per cent*
5	0.035	0.059	0.115	0.019	97
10	0.043	0.075	0.299	0.181	97
15	0.059	0.075	0.371	0.237	97
25	0.083	0.083	0.475	0.309	97

All tubes contain a final concentration of 2×10^{-3} M histidine, pH 6.5, 3×10^{-4} M EDTA, 7.5×10^{-3} M $MgCl_2$, and 0.92 mols ATP. In addition, the following are added to the respective tube: (1) Stroma control—1 ml. of 20 per cent crude human stroma. (2) Hemolytic factor control—150 hemolytic units of HF. (3) Stroma + hemolytic factor—1 ml. of 20 per cent crude human stroma and 150 hemolytic units of HF. Total volume = 2 ml. Incubation is carried out at 34°.

therefore turned to the use of isotopically labelled ATP in order to elucidate the role of ATP in this system.

ATP-8C^{14} Uptake by Stroma Incubated with Hemolytic Factor:

Several preparations of purified stroma and of hemolytic factor were incubated together with ATP-8C^{14} for 15 minutes at 34°C. and after incubation the stroma was isolated by centrifugation for 20 minutes at 25,000 G and washed three times with approximately 40 times its volume of non-isotopic 0.001 M ATP to remove any non-specifically absorbed ATP-8C^{14}. The stroma was plated quantitatively on nickel planchets with 0.3 ml. of 10 per cent human serum and was counted in an automatic nuclear D-47 gas flow counter. All values were corrected for background and self-absorbency. Stroma controls, from which hemolytic factor was omitted, were subjected to the same procedure and their counts, which averaged about 30 per cent of the complete system, were subtracted from those obtained in the latter.

There was a very small but consistent net uptake of ATP–8C^{14} by the stroma (Table V) amounting to 0.75–2.0 $\times$ 10^{-6} μmols of ATP per hemolytic unit of rabbit enzyme used in the reaction. This uptake did not depend on the ratio of stroma to hemolytic factor, since the values per hemolytic unit obtained when excess stroma was used (Experiments 4 to 8) were essentially the same as those found when an equivalent amount of stroma was present (Experiments 1 to 3). The C^{14} uptake calculated per inhibitory unit of stroma decreased steadily from an average value of 1.9 $\times$ 10^{-6} μmols ATP when equivalent amounts of stroma and hemolytic factor were used (Experiments 1 to 3) to 0.22 $\times$ 10^{-6} μmols ATP when the ratio of stroma to hemolytic factor was 5:1

TABLE V

ATP 8C^{14} Uptake by Stroma Incubated with Hemolytic Factor

Experiment No.	Hemolytic factor	Purified stroma		ATP 8C^{14} added		Isolated stroma C^{14}	
	Hemolytic units	Inhibitory units	Mg.	μM	C.P.M.	Total C.P.M.	μM ATP per 1 hemolytic unit
1	200	200	1.0	1.0	92,000	36.0	2.0 $\times$ 10^{-6}
2	200	200	1.0	1.0	46,000	14.6	1.6 $\times$ 10^{-6}
3	400	400	6.2	2.0	209,200	81.3	2.0 $\times$ 10^{-6}
4	2400	3500	20.0	6.0	261,500	168.0	1.7 $\times$ 10^{-6}
5	400	800	12.4	2.0	209,200	78.2	1.9 $\times$ 10^{-6}
6	400	1200	18.6	2.0	209,200	31.3	0.75 $\times$ 10^{-6}
7	400	1600	24.8	2.0	209,200	33.9	0.80 $\times$ 10^{-6}
8	400	2000	31.0	2.0	209,200	43.1	1.1 $\times$ 10^{-6}

Each tube contains a final concentration of 1 mg./ml. GSH, 2 $\times$ 10^{-2} M histidine, pH 7, and 6 $\times$ 10^{-3} M $MgCl_2$. The total volume is 4 ml. Incubation is carried out at 34°C. for 15 minutes.

(Experiment 8). This seems to rule out the possibility of non-specific adsorption of ATP to stroma, for if this were the case the C^{14} uptake would be proportional to the amount of stroma used.

Uptake of A.R.P.P.^{32}P^{32} by Purified Stroma Incubated with Hemolytic Factor:

A similar series of experiments carried out in the presence of ATP labelled in the two terminal phosphates with P^{32} is summarized in Table VI. The P^{32} uptake by stroma, calculated as micromoles of ATP per 1 hemolytic unit, is of the same order of magnitude as the 8C^{14}–ATP uptake (Table V) but is consistently higher. This may indicate that stroma is phosphorylated, either in addition to or consequent to the attachment of the nucleotide. The fact that the stroma P^{32} counts increase with incubation time (Experiment 6 Table VI) lends some support to this theory.

While it is apparent that ATP uptake and possibly phosphorylation occur in the reaction between stroma, rabbit red cell factor, and ATP, the possibility

exists that this merely represents non-specific absorption of ATP into some of the rabbit cell factor proteins plus non-specific uptake of the labelled protein complexes by the stroma.

It was therefore of interest to determine the extent to which the soluble protein components of the system became labelled. In several experiments after removal of stroma from the incubated mixture by centrifugation the protein in the supernatant was isolated by the addition of ammonium sulfate to 100 per cent saturation. The protein precipitate was dialyzed for 20 hours against several changes of water and assayed for P^{32}.

TABLE VI

Uptake of P^{32} by Stroma on Incubation with Hemolytic Factor and A.R.P.P^{32}.P^{32}

Experiment No.	Hemolytic factor	Purified stroma		Incubation time	ATP^{32} added		Isolated stroma P^{32}	
	Hemolytic units	Inhibitory units	Mg.		μM	P^{32} C.P.M.	Total P^{32} C.P.M.	μM ATP/1 hemolytic unit
				min.				
1	824	830	3.8	15	4.70	39,397	118	9.4×10^{-6}
2	400	600	3.0	15	2.00	710,000	856	6.0×10^{-6}
3	400	600	3.0	15	2.00	280,000	464	8.2×10^{-6}
4	600	1200	7.2	15	2.13	144,000	303	6.7×10^{-6}
5	600	1200	7.2	15	2.20	109,500	124	4.2×10^{-6}
6	360	1920	11.5	15	3.20	123,800	171	7.0×10^{-6}
				30	3.20	123,800	215	9.3×10^{-6}
				60	3.20	123,800	269	12.7×10^{-6}

Each tube contains a final concentration of 1 mg./ml. GSH, 2×10^{-2} M histidine, pH 7, and 6×10^{-3} M $MgCl_2$. The total volume is 4 ml. Incubation is carried out at 34°C. for 15 minutes.

In a typical experiment, in which the stroma P^{32} was 303 C.P.M. (see Experiment 4, Table VI), the supernatant protein P^{32} was 2,402 C.P.M. (26.0 mg. protein) and the supernatant protein P^{32} of a control lacking stroma was 2,415 C.P.M. (25.5 mg. protein). Thus ATP^{32} was absorbed by the protein supernatant independently of the inhibitory reaction since this uptake was the same whether or not stroma was present. Similar results were obtained using ATP-$8C^{14}$.

The important question then became whether or not the observed ATP^{32} uptake by stroma in the presence of hemolytic factor was due to contamination of the isolated stroma by a small amount of highly labelled rabbit protein. Since the stroma controls of the various experiments lacked rabbit protein they would not be subject to such contamination.

Several types of experiments were performed to rule out this possibility. In the first, stroma was incubated in the usual manner with ATP^{32} (109,500 C.P.M.) and (1) 25.0 mg. of bovine serum albumin; (2) 21.4 mg. of hemolytic factor protein; and (3) without protein. After 15 minutes the stroma and protein

(when present) were isolated, washed, and assayed for P^{32}. The albumin protein P^{32} was 2,489 C.P.M. and the hemolytic factor protein P^{32} was 753 C.P.M. The stroma P^{32}, however, was 28 C.P.M. in case (1); 162 C.P.M. in case (2); and 25 C.P.M. in case (3). Therefore, although the albumin was over three times as heavily labelled as the hemolytic factor, the stroma which had been incubated with albumin had the same count as the protein-less control and only in the presence of rabbit enzyme did the stroma take up P^{32} above that of the control value.

In another experiment, hemolytic factor (600 hemolytic units) was reacted with an excess of purified stroma (7.2 mg.; 1200 inhibitory units) and ATP^{32} (2 μM; 85,000 C.P.M.) for 15 minutes, at which time the enzyme was completely inhibited. The stroma was removed by centrifugation and assayed for P^{32}. The supernatant fluid, which now contained no active hemolytic factor but did have all the other necessary components of the system plus the same amount of labelled non-specific protein as before, was then incubated with additional stroma (1200 inhibitory units) for 15 minutes and the stroma was isolated and counted. The first stroma, which had reacted with *active* hemolytic factor, had 126 C.P.M. P^{32} whereas the second stroma, which had been incubated with inactivated rabbit hemolytic factor, had 34 C.P.M. P^{32} the latter figure being equal to the control lacking enzyme (38 C.P.M. P^{32}).

A repetition of this experiment gave the same results.

Finally, hemolytic factor (400 hemolytic units), excess purified stroma (600 inhibitory units), and ATP^{32} (2 μM; 7.1×10^5 C.P.M.) were incubated (1) in the presence of and (2) in the absence of Mg^{++} which is an absolute requirement of the system, both for the lysis of red blood cells and for inhibition of the lytic factor by stroma. After incubation at 34° for 15 minutes both the stroma and rabbit protein were isolated as previously described and assayed for P^{32}. Two stroma controls, with and without Mg^{++}, were treated in the same manner.

In the presence of Mg^{++} (1), the stroma P^{32} count was 856 C.P.M. above the Mg^{++} plus stroma control which was 405 C.P.M. and the protein P^{32} count was 9624 C.P.M. In the absence of Mg^{++} (2), no P^{32} above that of the stroma minus Mg^{++} control value, which was 416 C.P.M., was taken up by the stroma although the protein P^{32} (9643 C.P.M.) was virtually the same as that found in the case in which Mg^{++} was present.

The results of these three different experiments make it seem highly unlikely that the labelling of stroma in the presence of labelled ATP, rabbit red cell factor, and Mg is not related to the specific inhibition process but is merely a consequence of non-specific absorption by the stroma of protein carrying the labelled ATP.

DISCUSSION

Our previously reported results had left unexplained the discrepancy of apparent non-utilization of ATP in the hemolytic reaction despite its being an absolute requirement for both the hemolytic and inhibitory reactions.

It is apparent that the experimental conditions used in our previous work were highly unfavorable for demonstrating ATP utilization in hemolysis. The concentration of red cells and the amount of hemolytic factor required for hemolysis were low and therefore the ATP utilized was less than the ATP disappearance from the controls, which was quite large due to the high ATP-ase and myokinase content of the crude rabbit hemolytic factor. These conditions have been improved in the present report with the result that ATP utilization in association with hemolysis has been demonstrated. Still other questions, however, have arisen which remain to be answered in connection with the utilization of ATP by these systems.

At the present time we believe the following to be the most reasonable general picture that can be presented. The results of the experiments using C^{14}–labelled ATP and purified stroma indicate a small but consistent uptake of nucleotide by the stroma in the presence of rabbit factor and Mg^{++}. There is a slightly larger uptake of P^{32} from ATP labelled with this isotope in the complete system and this uptake increases with time over a period of 1 hour. The inhibition of rabbit red cell factor by stroma, however, is complete within 5 minutes. In both instances, the 15 minute uptake is so small that quite obviously the net amounts of ATP utilized by the inhibition reaction with purified stroma could not have been detected with the usual non-isotopic analytic method. The requirement of far greater concentrations of ATP for maximum hemolysis and for inhibition by stroma is most probably explained by some non-specific absorption by other proteins in the rabbit cell factor preparation and by the presence of ATP-ase and myokinase in the enzyme preparation which, in addition to lowering the ATP available to the hemolytic system, mediates the formation of AMP which is a potent inhibitor of lysis (1). Further purification of the hemolytic factor is essential to a final solution of this anomaly.

The demonstration of a relatively large net continuing utilization up to 1 hour after initiation of the reaction using crude stroma may represent either unrelated reactions involving ATP and factors present as impurities in both the crude stroma and the partially purified rabbit cell preparations, or it may represent the end result of a chain of reactions, the first step of which is a reaction between rabbit red cell factor, ATP, and one component of stroma, still present in our purified stroma, leading to a complex between the three. In the purified stroma there is present very little or none of the components required for the other steps in the chain of reactions so that very small amounts of ATP disappear from solution although inhibition is complete and the reaction fluid obtained after spinning down the stroma shows loss of hemolytic power. Preliminary experiments using S^{35}–labelled rabbit factor which will be subsequently reported, indicate that the rabbit factor does attach to the stroma. The experiments with effect of age of crude stroma on net ATP uptake might be interpreted to also lend support to this theory if it is assumed that decrease in the

ability of the older stroma to enter into the initial reaction, as manifested by loss of inhibitory reaction, also curtails the occurrence of the subsequent steps as evidenced by decrease in net utilization.

These same considerations, if indeed actually valid in explaining the differerences in ATP utilization between crude and purified stroma, probably also pertain with respect to the demonstrated utilization of ATP by the hemolysis of large amounts of red cells. Further work with more highly purified components of the system and with reconstitution experiments should help answer these questions and this is under way.

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