Simplified Determination of Blood Adenosine Triphosphate Using the Firefly System

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Adenosine triphosphate (ATP) appears to play a critical role in maintaining the integrity of the red cell. In the "storage lesion," the disappearance of high energy bonds represented largely by ATP correlates well with the loss of viability of erythrocytes upon reinfusion into the circulation.1,2 In early studies, erythrocyte ATP was estimated by determining "easily hydrolysable" phosphorus.1-4 ATP is not the only compound from which phosphorus may be split under the conditions employed, and such determinations must be considered, at best, as good approximations. More recently, chromatographic separation of red cell filtrates, either on paper or in columns, has been employed in estimating erythrocyte ATP.5-12 Trichloracetic acid or perchloric acid extracts of erythrocytes have been used and the quantity of ATP has been determined by phosphorus analysis and by measurement of ultraviolet absorption.

All of these technics are extremely time-consuming and their accuracy and precision is impaired by the many manipulations required. Several enzymatic technics have also been employed.13-21 Although these are less time-consuming than chromatographic technics, they are still quite complex.

The relatively recently introduced firefly lantern extract technic for assay of tissue ATP22 is probably the simplest and most sensitive method available. Simon et al.23 have used this method to determine ATP in perchloric acid filtrates made from erythrocytes, but no details were given in their publication. Other investigators may have also adapted this method for the determination of erythrocyte ATP, but we are not aware that any technic has been published. Under proper experimental conditions, we have found that firefly lantern extract may be employed as a basis for a relatively specific, highly reproducible and rapid technic for red cell ATP assay. In the present investigation the variables which influence determination of ATP by the use of firefly lantern extract have been studied and an attempt has been made to elucidate the optimal conditions for carrying out red cell ATP assays in this system.

MATERIALS AND METHODS

A. Reagents

1. Firefly extract was obtained commercially from Sigma Chemical Corp. The contents

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*This material is prepared by extracting firefly lanterns with a sodium arsenate buffer, 0.05 M, pH 7.4 containing MgSO₄ 0.02 M, removing insoluble material and drying.24
of each vial, containing extract from 50 mg. of firefly tails, were reconstituted with distilled water and thoroughly mixed to break up and evenly suspend particles. This produces a suspension with a volume of 5 cc. in 0.05 M sodium arsenate buffer, pH 7.4, containing 0.02 M MgSO4.

2. Dilution of firefly extract. Sufficient sodium arsenate (3.12 Gm. Na2HAsO4 .7 H2O per 100 ml.) to make a 0.1 M solution was weighed out and dissolved in slightly less than the calculated volume of distilled water. Its pH was adjusted with hydrochloric acid to 7.4 and the buffer was brought to volume with water. The pH of the magnesium chloride (0.81 Gm. MgCl2 .6 H2O per 100 ml.) or sulfate solution was similarly adjusted with dilute sodium hydroxide to give a final pH of 7.4 with a concentration of 0.04 M. The diluent for the firefly extract was prepared by mixing equal volumes of these two solutions on the day of use. The 1:5 dilution of firefly extract was prepared by adding 4 volumes of diluent to the firefly extract as reconstituted from the desiccated form. Once reconstituted, the diluted firefly extract was kept in an ice-bath. It was stable for several days.

3. Tris-borate buffer. A 0.04 M Tris (hydroxymethyl) aminomethane (Sigma) (0.49 Gm. per 100 ml.) solution was prepared and its pH was adjusted to 9.2 with a 0.04 M (0.25 Gm. H3BO4 per 100 ml.) boric acid solution.

4. Light emission measurements. Readings of light emission were made in a Turner Fluorometer Model #110, using a 12 x 75 mm. round cuvette. A piece of cardboard was placed between the instrument light source and the cuvette so that only light emitted from the firefly extract was measured. Unless otherwise indicated, readings were made exactly 1 minute following the addition of the sample to the firefly extract.

5. Standard solutions. A standard ATP solution containing 0.5 μmoles ATP (sodium salt, 98 per cent pure, Sigma) per ml. in Tris-borate buffer was prepared and kept at -20 C. The purity of the ATP was confirmed by measuring its optical density at 260, 280, and 290 μm as recommended by the Committee on Biochemistry of the National Research Council. It was found to be stable for at least 16 days. In order to prepare a standard curve, 0.1 ml. of standard was added to 2.9 ml. of Tris-borate buffer solution and 0.2 ml. of standard were added to 2.8 ml. of Tris-borate buffer solution. These samples were treated in a manner identical to that used for the blood samples.

6. Blood samples. Unless otherwise indicated, blood samples were heparinized or collected in approximately 1 part of acid-citrate-dextrose, N.I.H. solution "B" (ACD), per 4 parts of blood.

Procedure

One-tenth ml. of blood is added to 2.9 ml. of Tris-borate buffer in a test tube marked at the 3.00 level. The hemolysate is immediately placed in a boiling water bath for 5 minutes and then transferred to an ice-bath. If, for any reason, the boiling process must be delayed for more than 1 or 2 minutes, iced Tris-borate buffer should be employed. The volume is restored, if necessary, to 3.0 ml. with distilled water. Two-tenths ml. of the turbid, mixed, boiled hemolysate is transferred to 4 ml. of ice-cold diluted firefly extract, and a stopwatch is started. The tube is mixed by inverting it 4 times. In exactly 1 minute a reading of the light emission is made in the fluorometer.

Experimental and Results

A. The effect of time and of dilution of firefly extract. Maximum readings were obtained 30 seconds after addition of ATP-containing extracts to firefly extracts with all dilutions tested. It was not technically feasible to obtain readings prior to 30 seconds. Following this, there was a progressive decrease in the quantity of light emitted. This decrease was more rapid when concentrated firefly extract was employed than when diluted firefly extract was used. This finding, as well as the effect of dilution of firefly extract, is illustrated in figure 1.
Fig. 1.—The effect of time and of dilution of firefly extract on light emitted from the firefly extract-ATP mixture. The slightly diminished light output when undiluted firefly extract was used may be due to internal light absorption because of turbidity. Although maximum light output was achieved by using a 1:2 dilution of firefly extract, the 1:5 dilution has been chosen because of the more gradual decrease of light output at this dilution and because of the lower cost.

B. Recoveries. Excellent recoveries of ATP added to blood were achieved. In three experiments, recoveries ranged from 99.2 to 103 per cent.

C. Extraction of ATP from erythrocytes. It was found that different methods of extraction produced marked variation in the results of ATP determination. It was of interest that even methods which resulted in low ATP values gave excellent recovery of added ATP in the instances studied. Studies were made of extraction of ATP by perchloric acid according to the method described by Greenwalt and Ayers,\textsuperscript{25} by trichloracetic acid (TCA) according to the method of Bartlett,\textsuperscript{5} and by boiling. A neutralized perchloric acid extract of blood gave only 72 per cent of the reading obtained from the boiled sample using the firefly luciferase technic. On the other hand, an ATP standard treated with perchloric acid in identical fashion gave 81 per cent of the reading. It was demonstrated that neutralized perchloric acid extracts interfered with the production of light by the firefly extract to an extent which fully accounted for the decreased reading obtained with the standard, but not with that obtained from blood. Accordingly, it appeared that the larger part of the effect of perchloric acid was due to interference of light production in the reaction system; a small part of the effect seemed to be due to inefficient ATP extraction. A trichloracetic acid (TCA) extracted blood sample gave 90 per cent of the ATP reading as was obtained with the boiling technic. “Extraction” of an ATP standard with ice-cold TCA under identical conditions as used with blood also resulted in a 10 per cent loss of activity. It could be shown that the loss of ATP was not due to interference of the ether-extracted trichloracetic acid extract with the firefly system, but rather due to destruction of ATP during the extraction process.
Fig. 2.—The effect of pH on the extraction of ATP from blood samples. All samples were diluted 1:30 in a .04 M buffer solution, as indicated. Optimal extraction was achieved at a pH of 9.2.

Extraction of ATP by boiling in an isotonic solution of sodium chloride or in distilled water was found to be incomplete. If the coagulum was removed from this boiled hemolysate, ATP was lost. In the Tris-borate system, a finely dispersed precipitate was formed on heating. Four hundredths molar phosphate buffers, pH 5.6, 6.4, 7.2, 7.8, or of Tris-borate buffer, pH 8.0, 8.6, and 9.8 gave rise to suboptimal extraction (fig. 2). The presence or absence of disodium ethylenediaminetetraacetate (EDTA) in the buffer system did not affect the ATP yield significantly. In dilutions of 1:30 or greater, the quantity of blood used showed a linear relationship to the amount of light emitted by the diluted firefly extract. However, at dilutions of less than 1:30, or in some cases 1:20, there was departure from linearity which was shown to be due to incomplete extraction (fig. 3). Once the red cells were lysed in the Tris-borate buffer, there was gradual loss of ATP. At 0 to 4 C. the ATP loss was negligible for at least 15 minutes, and even at 2½ hours it amounted to less than 15 per cent of the ATP initially present. At room temperature the loss of ATP in the hemolyzed red cells was more rapid. During the first 15 minutes, slightly under 15 per cent of the ATP was gradually lost; the rate approximated 1 per cent per minute. Boiling of the hemolysate stabilized the ATP, presumably because of the destruction of ATPase during boiling.

D. Relationship between quantity of ATP and light emitted. The use of varying quantities of either ATP solution or of boiled hemolysate demon-
Fig. 3.—The effect of dilution of blood in Tris-borate buffer pH 9.2 on extraction of ATP. At dilutions of blood of greater than 1:20 or 1:30, a linear relationship between the quantity of blood present and the light emitted was obtained. At higher concentrations of blood, less light was emitted than expected. This departure of linearity exceeded considerably that found in standard curves and represents sub-optimal extraction of ATP from red cells in more concentrated hemolysates.

strated that a linear relationship existed between quantity of ATP and light emitted up to a quantity of $6.7 \times 10^{-3}$ μmoles (fig. 4) of ATP. With the firefly preparation used, quantities of ATP greater than this gave rise to light emission less than that expected.

E. Anticoagulants. The use of acid-citrate-dextrose (ACD), heparin or EDTA as an anticoagulant did not appear to influence significantly the results of ATP determination. An apparent decrease of ATP readings in the ACD solution was observed and has been reported previously by Bartlett and Shafer. However, this decrease is due to an increase in the mean corpuscular volume of the erythrocytes in this anticoagulant so that values expressed as micromoles per milliliter of erythrocytes give artificially low values. No effect of ACD was observed when ATP values were expressed as micromoles/Gm. hemoglobin. Oxalate interfered markedly; this resulted in low ATP readings.

Of special interest was the finding that there was a considerable decline in the ATP content of blood samples collected in heparin, EDTA or by defibrination after storage for several hours at 4 C. The ATP content of blood collected in ACD solution was, in contrast, quite stable. The stabilizing effect of ACD solution on red cell ATP has been found to be due to the effect of ACD on the pH of blood, and not to be related to either the presence of the citrate ion or the presence of glucose.

F. Specificity. The nucleotides of erythrocytes have been carefully in-
Fig. 4.—The relationship between quantity of ATP added to a cuvette and the amount of light emitted. Triplicate determinations were carried out at each dilution of ATP and the individual values and means are presented. Studies involving quantities of ATP in excess of $7 \times 10^{-3}$ μmoles/cuvette departed from linearity, giving rise to slightly less light emission than expected.

Investigated by column chromatography by others. Normal erythrocytes contain, in addition to ATP, measurable quantities of adenosine diphosphate (ADP), adenosine monophosphate (AMP), guanosine triphosphate (GTP), an unidentified adenosine-containing compound which has been called AXP, diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN). According to Strehler, GTP is totally inactive with the purified luciferin-luciferase system. In our system, a slight reaction was found with GTP (Sigma). This amounted to less than 2 per cent of the reading given by an equimolar quantity of ATP. It was not ascertained whether this was due to GTP itself or a contaminant. Even if due to GTP itself, this would be insignificant, since the quantity of GTP in red cells is only of the order of 6 per cent of ATP. No reaction was found with TPN, DPN, or AMP in our system. "AXP," the unidentified adenosine compound, amounts to only about 10 per cent of the quantity of ATP, and even if reactive would produce a relatively small error. The type of crude firefly extract used appears to contain sufficient amounts of myokinase so that ADP (Sigma) gives rise to
a small reading. However, the effect of ADP is less than 5 per cent of that given by an equimolar quantity of ATP. These results are in agreement with those published by others. Even the 5 per cent value may be reduced to less than 3 per cent when readings are taken 30 seconds after the boiled hemolysate as added to the firefly extract; this procedure gives rise to higher readings with ATP and lower readings with ADP. The use of the 30-second time interval, although requiring somewhat greater speed and dexterity on the part of the individual performing the determinations, is otherwise entirely satisfactory. The concentration of ADP in normal erythrocytes is only approximately 15 per cent of that of ATP. Therefore, the error introduced by this nucleotide is very small.

G. The effect of white blood cells, platelets, and plasma. White blood cells and platelets contain appreciable quantities of ATP, while plasma does not. Since the number of these formed elements is normally much less than erythrocytes, ATP determinations may be carried out on normal whole blood in order to estimate red cell ATP without introducing much error. The ATP content of the white cells of normal blood has been found in preliminary studies by Scott to contribute only approximately 1 per cent of the activity of red cells in whole blood. Similarly, calculations from the data of Fantl and Ward suggest that the platelets of normal whole blood would account for less than 1 per cent of the ATP. If greater accuracy is desired, however, the determinations may be carried out on washed erythrocytes. In studying the blood of patients with very high white blood counts or platelet counts, it would be imperative to remove the buffy coat.

H. Normal values. Venous blood was drawn from 10 healthy adult male and female subjects. In seven instances, heparinized blood, as well as blood collected in ACD solution, was studied. Determinations were carried out within a few minutes of obtaining the blood sample. The average ATP content of seven heparinized blood samples was found to be 5.45 μmoles/Gm. hemoglobin with a standard error of 0.51 μmoles/Gm. hemoglobin. This represents 1.83 μmoles of ATP/ml. red cells with a standard error of 0.06 μmoles/ml. red cells. Blood samples collected in ACD were found to contain 5.28 μmoles of ATP/Gm. hemoglobin. In the case of the blood collected in ACD, results expressed in terms of ATP content/ml. of red cells are meaningless because of the swelling of cells induced by ACD.

I. Reproducibility. Eight replicate samples of blood from each of two normal donors were studied. In the case of one sample, the mean was 4.66 μmoles/Gm. hemoglobin with a standard deviation of 0.11 μmoles/Gm. hemoglobin, and the other was 5.73 μmoles/Gm. hemoglobin with a standard deviation of 0.071 μmoles/Gm. hemoglobin.

Discussion

The firefly luciferin-luciferase method for the determination of ATP is generally acknowledged to be a highly specific, sensitive and reproducible method for ATP assay. In the present series of investigations, we have found that this technic can be adapted to the determination of red cell ATP.
The method described is more rapid, more reproducible and more accurate than any previously described method of ATP determination. One technician should be able to carry out between 50 and 75 blood ATP determinations in a working day without any difficulty. The only disadvantages of the technic are the relatively high cost of the reagents, approximately $20 per test, the requirement for a fluorometer and the interfering effect of ADP if large quantities of this nucleotide are present. The latter difficulty could be overcome by further purification of the firefly extract to eliminate the myokinase.

Previously published methods for the determination of human red cell ATP have given widely divergent results. Using paper chromatography, results as low as 0.3 μmoles/ml. red cells and as high as 1.2 μmoles/ml. red cells have been described. Using column chromatography, results ranging from 0.63 μmoles/ml. red cells to 1.2 μmoles/ml. red cells have been described. One study employing the phosphoglycerate kinase method has given average values of 2.08 μmoles/ml. of red cells; another investigation utilizing the hexokinase reaction, gave a mean value of 3.86 μmoles/Gm. hemoglobin (= 1.3 μmoles/ml. RBC). In other studies employing various enzymatic methods for ATP assay, only whole blood values are given; based on a hematocrit of 45 per cent, average values of 1.05 μmoles/ml., 2.67 μmoles/ml. and 1.12 μmoles/ml. were obtained. The normal values which we have obtained, using the firefly technic, agree more closely with the higher values which have been reported with the enzymatic technics. Since substances other than ATP are not believed to react in the firefly system, we are inclined to the view that the values found with the firefly method approximate the true ATP content of erythrocytes. Since the entire boiled hemolysate is used, bound ATP which might be precipitated during treatment with PCA is measured. An overestimation of approximately 3 per cent is possible with the firefly method as described here because of the measurement of platelet and white cell ATP and the conversion of some ADP to ATP by firefly myokinase. Some of the lower values reported in the literature may be the result of failure to recognize the extreme lability of ATP in refrigerated heparinized blood. It is also of interest that we have found that the complete recovery of exogenously added ATP from erythrocyte suspensions does not insure adequate extraction of intracellular ATP. For this reason, the highest ATP values obtained using a specific technic for ATP determination are likely to reflect most accurately the true ATP content of erythrocytes.

**Summary**

A simplified method is described for the determination of red cell ATP using the firefly lantern extract method. Variables investigated include the effect of the time of reading, dilution of firefly extract and the effective range of the method. Excellent recoveries were obtained. Optimal extraction of ATP from red cells was achieved with a hypotonic buffer at pH 9.2. The method could be used with acid-citrate-dextrose, heparin or EDTA as an anticoagu-
lant. The method was found to be highly specific when the nucleotides found in normal human blood were investigated; only adenosine diphosphate and guanosine triphosphate gave slight readings, neither of which would significantly affect ATP determinations of human blood. Normal human values were found to be 5.45 \( \mu \)moles of ATP/Gm. of hemoglobin or 1.83 \( \mu \)moles/ml of red cells in heparinized blood samples. This method is believed to be more rapid, more reproducible and more accurate than any previously described method of ATP determination.

**SUMMARIO IN INTERLINGUA**

Es desribite un simplificate methodo pro le determination de adenosino-triphosphato (ATP) con le utilisation de extracto de musca a foco. Le variabiles investigate include le effecto del tempore del lectura, le dilation del extracto de musca a foco, e le region de efficacia del methodo. Excellente valores esseva obtenite. Le extraction optimal de ATP ab erythrocytos essecquare con un tampon hypotonic a pH 9.2. Le methodo poterea esser usate con acido-citrato-dextrosa, con heparina, o con EDTA como anticoagulante. Esseva constatate que le methodo es altemente specific quando le nucleotidas incontrate in normal sanguine human esseva investigate. Solmente adenosino-diposphato e guanosino-triphosphato produceva hasse lecturas, sed isto non pote afficer de maniera significative le determination de ATP in sanguine human. Esseva trovate que le valores normal pro humanos es 5.45 \( \mu \)mol de ATP per g de hemoglobina o 1.83 \( \mu \)mol per ml de erythrocvtos in heparinisate specimens de sanguine. Es opinate que iste methodo pro le determination de ATP es plus rapide, plus reproducihile, e plus accurate que non importa qual altere previemente describite.

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