A Comparison of Normal Red Cell ATP Levels as Measured by the Firefly System and the Hexokinase System

By Ernest Beutler and Claramma K. Mathai

The adenosine triphosphate (ATP) content of erythrocytes is of considerable interest from the viewpoint of red cell preservation, hereditary abnormalities of red cell metabolism, such as pyruvate kinase deficiency, and certain genetic disorders characterized by increased or decreased red cell ATP levels.

Several internally consistent methods are available for the measurement of red cell ATP. These methods include: (1) paper and column chromatography; (2) measuring glucose-6-phosphate formation in the hexokinase reaction with glucose-6-phosphate dehydrogenase (G-6-PD) and TPN; (3) employing the "backwards" glyceraldehyde phosphate dehydrogenase reaction to measure the oxidation of DPNH; and (4) the firefly technic. Each of these technics has been reported to give reproducible results with human blood, and adequate recoveries of ATP added to blood have been achieved. The simplest and most reproducible of these methods is the firefly luciferase method; yet, this system yields results which are considerably higher than those reported using other technics (Table 1). The firefly system differs from all other methods in use in that the blood is added directly to a buffer and boiled without washing of red cells or preparation of a deproteinized filtrate. The discrepancy between results obtained with the firefly technic and other methods could thus be due to lack of specificity of the firefly reaction, to destruction of ATP during processing by the other methods, or to incomplete extraction of ATP from red cells. It has also been suggested that the presence of hemoglobin may result in falsely high ATP levels when the firefly technic is employed.

It is the purpose of this report to investigate some of the possible causes of discrepancies between red cell ATP levels as determined by the firefly technic and red cell ATP level as estimated by other methods. The hexokinase technic has been employed for comparative purposes.

Materials and Methods

Blood Samples. Blood samples were obtained from normal human donors and collected into heparin, 20 units per ml. of blood, or ACD NIH formula B, 1 cc. per 4 ml. of blood.

Reagents. Firefly extract, ATP, hexokinase, and G-6-PD were obtained from Sigma.*

From the Division of Medicine, City of Hope Medical Center, Duarte, California.

This investigation was supported, in part, by Grants HE 07449 and HD 01974.

First submitted Jan. 10, 1967; accepted for publication March 1, 1967.

Ernest Beutler, M.D.: Chairman, Division of Medicine, and Director, Department of Hematology, City of Hope Medical Center, Duarte, Calif.; Clinical Professor of Medicine, California College of Medicine, Los Angeles, Calif. Claramma K. Mathai, Ph.D.: Assistant Research Scientist, Department of Hematology, City of Hope Medical Center, Duarte, Calif.

*Sigma Chemical Company, St. Louis, Mo.
<table>
<thead>
<tr>
<th>Author</th>
<th>Anticoagulant</th>
<th>Method of Extraction</th>
<th>Method of ATP Assay</th>
<th>Normal As Given</th>
<th>Calculated as Hm./Gm. Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minikami et al.</td>
<td>Heparin</td>
<td>Whole blood TCA extract</td>
<td>CAPD back reaction</td>
<td>1.13 ± .27 µM/ml RBC</td>
<td>3.32 ± .079</td>
</tr>
<tr>
<td>Gross et al.</td>
<td>Heparin</td>
<td>Whole blood perchloric acid extract</td>
<td>Hexokinase</td>
<td>3.86 µM/Gm. Hb S.E.</td>
<td>3.86 ± .13</td>
</tr>
<tr>
<td>Greenwalt &amp; Ayers</td>
<td>ACD</td>
<td>Perchloric acid extract of washed cells</td>
<td>Paper chromatography</td>
<td>106 µM/100 ml RBC</td>
<td>3.12</td>
</tr>
<tr>
<td>Brewer &amp; Powell</td>
<td>Heparin</td>
<td>TCA extract of washed cells</td>
<td>Hexokinase</td>
<td>2.75 µM/Gm. Hb</td>
<td>2.75</td>
</tr>
<tr>
<td>Mandell et al.</td>
<td>Heparin</td>
<td>PCA extract of washed cells</td>
<td>Column chromatography</td>
<td>82.4 µM/100 ml RBC</td>
<td>2.42</td>
</tr>
<tr>
<td>Gerlach et al.</td>
<td>3.5% Na Citrate</td>
<td>TCA extract of washed cells</td>
<td>Paper chromatography</td>
<td>.68 µM/Gm. RBC</td>
<td>2.19</td>
</tr>
<tr>
<td>DeLuca et al.</td>
<td>Heparin</td>
<td>PCA extract of washed cells</td>
<td>Column chromatography</td>
<td>69.2 µM/100 ml RBC</td>
<td>2.035</td>
</tr>
<tr>
<td>Bartlett</td>
<td>Heparin</td>
<td>TCA extract of washed red cell</td>
<td>Column chromatography</td>
<td>2.7-3.7 µM P/ml RBC</td>
<td>2.03 – 3.63</td>
</tr>
<tr>
<td>Beutler &amp; Bahuda</td>
<td>ACD Heparin</td>
<td>Whole Blood</td>
<td>Firefly</td>
<td>5.2 µM/Gm. Hb</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whole Blood</td>
<td>Firefly</td>
<td>5.45 ± 1.36 µM/Gm. Hb</td>
<td>5.45 ± 1.36</td>
</tr>
</tbody>
</table>
Fireflies were obtained from Sigma and extracted with a solution containing 0.05 M Na$_2$HAsO$_4$ and 0.02 M MgCl$_2$, pH 7.4, in a Potter-Elvehejm homogenizer, and were centrifuged at a low speed just before use. The preparations of G-6-PD and hexokinase employed were free of 6-phosphogluconate dehydrogenase activity. Preparations of bovine serum albumin were obtained from Calbiochem* or Sigma.

**Methods.** The determination of red cell ATP levels by the firefly technic was carried out as previously described unless otherwise indicated. In this method 0.1 ml of whole blood is added to 2.9 ml of tris-borate buffer, pH 9.2, the mixture boiled for 5 minutes, and 0.2 ml of the boiled hemolysate added to 4 ml of diluted ice-cold firefly extract. A reading was made in a Turner Model 110 Photofluorometer after 1 minute. Determination of red cell ATP levels by the hexokinase-linked technic was carried out as described by Greengard, modified, however, to measure TPNH generation by light absorption at 340 m$_\lambda$ in a Gilford Model 2000 recording spectrophotometer rather than by fluorometry. The final assay system contained triethanolamine buffer, pH 8.0, 100 mM; glucose, 100 mM; MgCl$_2$, 15 mM; EDTA, 0.4 mM; TPN, 0.5 mM; hexokinase, .165 units/ml; and G-6-PD, .039 units/ml.

**EXPERIMENTAL**

*The Effect of Protein on the Firefly System*

It has been suggested that hemoglobin greatly intensifies the light output in the firefly system, possibly through "secondary fluorescence" of heme or denatured proteins. Such an increase of light output in the presence of hemoglobin would give falsely high ATP values, since hemoglobin would be present in the blood specimens being evaluated, but no such enhancing effect would occur in the standard.

Incorporation of dialyzed hemoglobin at a concentration of 1.1 to 6.7 mg./ml into a system containing tris-borate buffer and ATP was sometimes found to produce an increase in light output. This was true only with the lyophilized firefly extract, and could not be demonstrated with freshly extracted firefly tails from three different lots of fireflies (Fig. 1). No effect was found when heme was used alone, but solutions of protein other than hemoglobin, including normal human serum and bovine serum albumin, produced an effect equivalent to that of hemoglobin. GSH was without appreciable effect. The protein effect was never observed when used, unfiltered firefly extract was employed for measurements, presumably because it was already high in protein content. The possibility that the time-course of the light-producing reaction might be retarded by protein solutions and therefore give a higher value after one minute's reaction was also investigated. The time-course of the reaction was identical with and without the addition of protein. Since maximum effect of protein is observed with protein concentrations of less than 1 per cent in the tris-borate buffer, it has been found convenient to add 0.4 ml of 7 per cent bovine albumin to the "standard" cuvette (i.e., 2.5 ml tris-borate, 0.4 ml. 7 per cent albumin, 0.1 ml. 0.5 mM ATP) in the determination of ATP levels by means of the firefly technic. Maximum stimulation of light output is obtained in this manner and the possible artificially high blood values obtained through the protein effect are obviated. In all studies subsequently reported, bovine albumin was added to the tris-borate solution in which the standard was prepared.

*Calbiochem, P.O. Box 54282, Terminal Annex, Los Angeles, Calif.*
The Specificity of the Firefly System

According to the studies of others, purified luciferin-luciferase from fireflies has virtually absolute specificity for ATP. However, it has been recognized that crude extracts may be contaminated with myokinase which will convert a small fraction of the ADP in red cell extract to ATP and that some reaction also takes place with guanosine triphosphate. The extent of this nonspecific reaction has previously been estimated to be well under 1 per cent when human blood ATP levels are measured. Nonetheless, the possibility that unknown materials in red cell extracts reacted with a firefly system required further study.

Trichloroacetic acid (TCA) extracts were made from whole blood and from three-times washed erythrocytes. The ATP content of aliquots of the extracts was then determined, using both the hexokinase technic and the firefly method. The ratio of the values obtained with hexokinase method to the value obtained with the firefly method on determinations carried out on 16 normal blood samples were found to average .995 with a standard error of .042. Clearly, the difference between the values obtained with the hexokinase method and the firefly method is not due to any red cell component which could be extracted with trichloroacetic acid.

The Effect of Postcollection Storage

Heparinized blood is frequently used in the determination of red cell ATP
COMPARISON OF NORMAL RED CELL ATP LEVELS

levels (see Table 1). It is standard practice in most biochemical analyses to cool samples rapidly to 4-5 C. in the interval between collection and analysis. For the purpose of this study, it was assumed that the average sample may have been held at this temperature for 1 hour. It has been shown that there is a rapid decline in red cell ATP levels when heparinized blood is stored at 4 C. Accordingly, the magnitude of this loss has been quantitated in a larger number of blood samples.

Samples of blood from 20 donors were collected in heparin, and ATP determinations were carried out immediately after collection and after 1 hour's storage in an ice bath. The ratio of activity after storage for 1 hour at 4 C. to initial activity averaged .912 with a standard error of .0090. It is apparent, therefore, that approximately 9 per cent of red cell ATP activity is lost under these circumstances and that accurate normal values of heparinized blood can be obtained only when the sample is pipetted into tris-borate solution immediately and the ATP stabilized by boiling. In contrast, as previously shown, the ATP levels of blood collected in ACD were somewhat less unstable, the ratio of poststorage to prestorage ATP level averaging .940 with a standard error of .010 after 1 hour's storage at 4 C. Lowering the pH of the ACD solution, as previously described, improved the stability of ATP. When six samples were stored with ACD, pH 4.0, the ratio of the ATP levels after 1 hour of storage to the initial level averaged 1.02 with a standard error of .017. The corresponding values at pH 3.5 were .991 with a standard error of .0048.

The Effect of Preparing the Sample for TCA Extraction

Most technics for the determination of red cell ATP levels include the preparation of the red cells by removal of buffy coat, washing, and finally extraction with TCA or perchloric acid (PCA) to produce a protein-free filtrate for further analysis (Table 1). In contrast, in the firefly method the whole blood is added to a tris-borate buffer and boiled. Accordingly, the possible losses of ATP at various stages of the washing and extraction procedure have been evaluated.

The Effect of Washing and Removal of Buffy Coat. It was noted that when ATP determinations were carried out on blood samples before and after washing, substantial losses of ATP were sometimes observed. In a series of determinations carried out on 9 normal blood samples, the ratio of ATP levels after washing to before washing of red cells was found to be .946 with a standard error of .026. In order to determine the point at which such loss of ATP occurred, determinations were done before centrifugation, after removal of the buffy coat, and after three saline washes. It was found that virtually the entire loss of ATP activity occurred during the centrifugation process itself. Centrifugation of heparinized blood samples from 13 donors resulted in a loss of 4.7 per cent of the ATP with a standard error of 1.4 per cent when the blood was remixed and assayed. This loss was of the same order of magnitude as observed with equivalent periods of storage of blood at 4 C. The time consumed in balancing blood samples, centrifugation of 15 minutes, removal from the centrifuge, etc., was equivalent to a second storage period sufficient to account
for the loss of ATP. Longer periods of centrifugation would result in correspondingly greater losses of ATP. When the buffy coat was removed together with the upper layer of the red cells, the average ratio of ATP after removal of buffy coat was .992 with a standard error of .0087; there was no significant loss of ATP.

Saline washing of red cells caused no further loss of ATP. The red cells from five subjects were washed three times in 6-8 volumes of cold saline. The ratio of ATP determinations after washing to those before washing in saline was 1.007, with a standard error of .008. This lack of further ATP loss in saline washing probably represents the combined effect of lower pH levels and glucose deprivation.12

Losses due to TCA or Perchloric Acid (PCA) Extraction. Whole blood and washed red cells from nine subjects were extracted with trichloroacetic acid according to the method of Bartlett.8 After removal of trichloroacetic acid with ether and evaporation with a stream of air of the remaining residual ether, 1 ml. of TCA extract was added to 2 ml. of tris-borate buffer containing 1 per cent bovine albumin. ATP determinations were then carried out on the boiled extract, using the firefly method as previously described. The average ratio of the quantity of ATP extracted by TCA to the quantity found after boiling the red cells without prior extraction was .954 with a standard error of .031. When whole blood was extracted with TCA, the ratio of the ATP content of the extract to the unextracted whole blood was .864 with a standard error of .027. Sham TCA extract had no effect on light output from the firefly system. Thus, it was apparent that there was a small but significant loss of ATP during the process of ATP extraction of washed cells, and that the losses were significantly larger when extraction of whole blood was carried out.

One part whole blood was extracted with one part of 6 per cent perchloric acid (PCA) and the supernatant neutralized to a phenolphthalein end point with potassium hydroxide. In 9 experiments an average yield of .993 with a standard error of .031 was obtained when water content of ACD blood was assumed to be 80 per cent. In 5 experiments in which no assumption was made regarding water content of blood but the blood was extracted twice with PCA, the average yield was .980 with a standard error of .062. Sham PCA extract did not interfere with light output from the firefly system.

Revised Normal Values

Using a standard to which protein had been added we have redetermined with the firefly method the red cell ATP levels of 13 normal Caucasian and 9 normal Negro subjects. Average values of 4.05 ± .105 μmoles/Gm. Hb and 3.65 ± .19 μmoles/Gm. Hb, respectively, were obtained. These results confirm the previously reported difference between ATP levels of Caucasians and Negroes.11

DISCUSSION

As shown in Table 1, normal red cell ATP levels, as previously reported in studies using the firefly technic, are considerably higher than levels obtained when other methods are used. This discrepancy has now been clarified. The
COMPARISON OF NORMAL RED CELL ATP LEVELS

Fig. 2.—Average loss of ATP from blood samples processed by storing in heparin for one hour, centrifuging, removing buffy coat, washing in saline, and extracting the red cells in trichloroacetic acid.

firefly method overestimates the concentration of ATP when fresh firefly extract is used, because of the stimulating effect of small amounts of protein on the reaction. This effect is not observed when firefly extract is reused, and is present only with certain lots of firefly extract. It is not possible to demonstrate the effect in extract freshly prepared from fireflies, and it is possible that it may be related to slight loss of activity on storage with subsequent reactivation by protein. In any case, the effect is easily overcome by adding serum, dialyzed hemoglobin solutions, or serum bovine albumin to the standard.

A major portion of the discrepancy between the values observed with the firefly technic and other technics can be accounted for through losses in storage and processing the blood prior to carrying out the analyses. The loss of ATP from a heparinized blood sample stored for 1 hour at 4 C., in which the red cells have been prepared by washing and extracted with trichloroacetic acid, is shown diagramatically in Figure 2. The accumulative loss averaged 17.2 per cent. This loss and the protein effect fully account for difference between results reported with the firefly method and other technics. The results now obtained on normal subjects are in close agreement with the results of Gross (3.86 μM/Gm. Hb), who extracted whole blood with perchloric acid immediately after obtaining the sample. Perchloric acid extraction of whole blood results in complete recovery of ATP, in contrast to the 14 per cent loss obtained with TCA. These normal values are somewhat higher, as may be expected, than data obtained in the methods in which blood samples
were processed by washing, and possibly stored for brief periods of time prior to extraction and assay.

These studies suggest that if protein is added to the ATP standard before boiling, the firefly technic correctly reflects the ATP level of red blood cells. The effect of white cells and platelets appears to be relatively small, and the use of whole blood avoids losses which may occur when red cells are washed prior to ATP determination. It also avoids small but significant losses during extraction. For the most accurate determination of red cell ATP levels, the blood sample should be hemolyzed and boiled immediately after it is obtained. When blood must be stored at 4 C. prior to determination of ATP levels, as in certain types of field studies, an ACD solution with a pH of 3.5 to 4.0 is an adequate anticoagulant for at least 1 hour. When it is necessary to free red cells of buffy coat prior to ATP determination, such an anticoagulant should also be used. If a method is employed in which a protein-free filtrate is made, extraction of the whole blood with PCA seems preferable to TCA because of higher yield obtained. The variability of losses during storage, removal of buffy coat, and TCA extraction of heparinized blood is sufficiently great so as to render relatively unreliable measurements made by technics in which these processes are employed.

**SUMMARY**

Widely divergent normal red cell ATP levels have been reported by investigators using different methods. In order to clarify the cause of these discrepancies and to establish correct normal values for red cell ATP, the firefly technic for measuring ATP levels was compared with other methods. The ATP content of TCA filtrates of red cells was the same when determined by the firefly method as by the hexokinase-linked technic. Relatively low concentrations of protein were found to stimulate light output when lyophylized firefly extract, but not freshly prepared firefly extract, was used. Thus, falsely, high values were obtained when red cell extracts were examined, unless protein was also added to the standard. Storage of heparinized blood for as little as 1 hour resulted in a substantial decrease in red cell ATP levels. The loss with ACD blood was less, and could be obviated entirely by using an ACD solution with a pH adjusted to between 3.5 and 4.0. Removing the buffy coat or washing cells in saline resulted in no further loss of red cell ATP. Extraction of washed red cells with TCA resulted in an average loss of 4.6 per cent of ATP, while extraction of whole blood with TCA resulted in a 14 per cent loss of ATP. In contrast, perchloric acid extraction resulted in no ATP loss. If ATP determinations are carried out using the firefly method, protein should be added to the standard. If red cells must be stored for any period of time prior to extraction of ATP, an ACD solution with a pH of 3.5 to 4.0 should be used. If extracts of red cells are made, perchloric acid appears significantly superior to trichloroacetic acid.

**SUMMARIO IN INTERLINGUA**

Diverse investigatores, utilisante diverse methodos de investigation, ha reportate extensemente diverse normal valores de triphosphato de adenosina in erythrocytos. Pro
clarificar le causa de iste discrepancias e pro establir correcte valores normal pro le contento erythrocytic de triphosphato de adenosina, le technica a muscas a foco pro le mesuretion de triphosphato de adenosina esseva comparate con altere methodos. Le contento de triphosphato de adenosina in filtratos per acido trichloroacetic ab erythrocytos esseva le mesme quando illo esseva determinate per le methodo a muscas a foco como quando illo esseva determinate per le methodo a ligation a hexokinase. Esseva trovate que relativamente basse concentrationes de proteina sufficeva a stimular le emanation de lumine quando lyophilisate extractos ab muscas a foco esseva usate sed non quando le extractos esseva frescamente preparate. Assi, falsamente alte valores esseva obtenite quando extractos erythrocytic esseva examine, excepte quando proteina esseva etiam addite al standard.

Le magasinage de sanguine heparinisate durante solmente un hora resultava in un declino substantial del nivellos de triphosphato de adenosina in le erythrocytos. Un minus marcate perdita resultava ab tractar le sanguine con acido citric, citrato trisodic, e dextrosa. Illo poteva esser evitate completemente quando le solution de acido citric, citrato trisodic, e dextrosa esseva adjustate a un pH de inter 3,5 e 4,0. Le elimination del strato de leucocytes o le lavage del cellulas con solution salin resultava in nulle perdita adicional de triphosphato de adenosina ab le erythrocytos. Le extraction de lavate cellulas con acido citric, citrato trisodic, e dextrosa resultava in un perdita medie de 4,6 pro cento del triphosphato de adenosina, durante que le extraction de sanguine total con acido trichloroacetic resulted in un perdita de 14 pro cento de triphosphato de adenosina.

Quando le determination de triphosphato de adenosina es effectuate con le methodo a muscas a foco, proteina deberea esser addite al standard. Quando le erythrocytos debe esser magasinate durante prolongate periodos de tempore ante le extraction de triphosphato de adenosina, un solution de acido citric, citrato trisodic, e dextrosa con un pH de inter 3,5 e 4,0 deberea esser usate. Quando extractos de erythrocytos es facite, acido perchloric pare esser significativamente superior a acido trichloroacetic.

ADDENDUM

Since this paper was submitted for publication two additional studies regarding the firefly method for the measurement of red cell ATP levels have appeared. An interesting investigation by Aledort et al. deals with the effect of ionic strength on light output by the firefly system. Because of the extremely high dilution of the blood sample used in our system, the final concentration of ions in the reaction mixture would be essentially independent of their concentration in the blood sample. Aledort et al. also observed considerable losses of ATP when red cells were extracted with TCA. Some of these apparent losses may have been due to removal of protein from the mixture by TCA. Brewer and Knutsen have also published a more detailed report regarding the stimulating effect of hemoglobin solutions on red cell ATP measurements made by the firefly luciferase technique.

REFERENCES


A Comparison of Normal Red Cell ATP Levels as Measured by the Firefly System and the Hexokinase System

ERNEST BEUTLER and CLARAMMA K. MATHAI