ERYTHROCYTE ATP LEVELS IN MICE INFECTED WITH *PLASMODIUM BERGHEI* MALARIA†

SUVIT AREEKUL, KALAYA THAIJONGRAK, RATANAPORN KASEMSUTH and DUANGMARN MATRAKUL

Department of Radioisotopes, Faculty of Tropical Medicine, Mahidol University, Bangkok 4, Thailand.

INTRODUCTION

It has been well established that adenosine triphosphate (ATP) plays a vital role in erythrocyte metabolism. A certain level of ATP is needed for the survival of the erythrocyte during its natural life span. The role of ATP in red cells of subjects infected with malaria has been studied extensively during the past 25 years. One principal finding was that the level of ATP in the erythrocyte of the host may influence the rate of increase of parasitaemia (Brewer and Powell, 1965; Eaton and Brewer, 1969). Another development was the hypothesis that the rigidity of red cells in malaria infection which obstruct the blood vessels in various host organs might be due to ATP depletion in these erythrocytes (Areekul, 1973). To test this hypothesis, the erythrocyte ATP content was estimated in normal and *Plasmodium berghei*-infected mice.

MATERIALS AND METHODS

The study was performed in both normal and *P. berghei*-infected mice at various stages of infection. Blood was taken by cardiac puncture and collected in a heparinized tube. Fifty microliters of the blood sample were precipitated with 0.15 ml of ice-cold 6% (w/v) perchloric acid. After thorough stirring, aliquots of the supernatant obtained by centrifugation at 1500 rpm for 5 minutes were used for the estimation of ATP by means of luciferase enzyme using the liquid scintillation counter, as described by Stanley and Williams (1969).

Studies on the effect of temperature and post collection storage on erythrocyte ATP levels : A blood sample pool from normal mice was divided into 2 portions. The first portion was extracted immediately with perchloric acid and divided into 3 tubes. One tube was assayed for the red cell ATP level and served as the control at the zero time. The other two tubes were kept at room temperature and in a refrigerator, respectively. The second portion of blood was divided into two tubes and also kept in the refrigerator and at room temperature. All of the samples were assayed for ATP content at 1, 2, 3, 4 and 27 hours after collection and the values were expressed as percentages of the "zero-time" value.

Separation of parasitized from non-parasitized red cells and calculation of ATP content in these cells : Blood was taken from infected mice in a heparinized tube. The haematocrit values and the ATP content in the red cells were determined. One ml of each of 2 blood samples was mixed with 0.7 and 0.8 M sucrose in a Krebs-glucose-saline solution (specific gravities of 1.096 and 1.104) to a a final volume of 3 ml. After centrifugation for 5 minutes at 1000 rpm, each layer of these suspensions was resuspended in isotonic saline solution and the haematocrit value, parasite count and ATP content in the red

† This work was supported by research grants No. T-73-03 from SEAMEO-TROPMED Project and from Mahidol University, Bangkok, Thailand.

cells were determined and compared with values in the control blood.

Let X and Y be the ATP content of the parasitized and non-parasitized red cells respectively, C_1 and C_2 and R_1 and R_2 are the ATP content and the parasitaemia of the upper and lower suspensions respectively.

Then $R_1X + (100 - R_1)Y = C_1$ .....(1)

$R_2X + (100 - R_2)Y = C_2$ .....(2)

Solve for X and Y

If C_o and C_t are the ATP content of the infected blood at the zero time and at the time of determination of the suspensions, and R_o is the parasitaemia of this sample, Replace X and Y values in :

$$R_oX + (100 - R_o)Y = C_t \text{ (or } C_o) \quad ..(3)$$

The ATP contents in parasitized and non-parasitized red cells were obtained and these values were corrected to the original values from the proportion of C_t to C_o.

Haematocrit values were obtained after centrifuging the blood at 10,000g for 5 minutes. Parasitaemia was counted per 1000 erythrocytes or 200 white blood cells in the thin and thick blood films respectively.

RESULTS

Reproducibility: A blood sample pool from normal mice was simultaneously analyzed 30 times for ATP levels. A mean value ± one standard deviation of red cell ATP levels was found to be 153.16 ± 3.30 μM/100 ml RBC (range 148.26-158.87).

Recovery of added ATP: A standard ATP solution was mixed with the potassium phosphate buffer to the final concentrations of 50 to 200 μM/100 ml solution. One ml of a blood sample with known amount of erythrocyte ATP level was mixed with the different amount of a standard ATP solution to final concentrations of 57 to 307 μM/100 ml RBC. ATP contents in these samples were estimated and compared with the theoretical values. The percentage of recovery of this study is shown in Table 1.

The effect of temperature and post collection storage on erythrocyte ATP levels: The post collection storage and the temperature had a profound effect on the red cell ATP levels (see Fig 1). The ATP activity decreased to one half value at 2, 4 and 5½ hours in the blood sample kept in room temperature and at 4°C, and the extracted blood sample being kept at room temperature, respectively. The ratio of activity of extracted blood after storage for 1 hour and 4 hours at 4°C to the initial activity were 0.97 and 0.81, respectively. It is apparent, therefore, that approximately 3% and 19% of red cell ATP activity was lost under these circumstances and that accurate values can be obtained from blood samples only when the samples are extracted and assayed immediately.

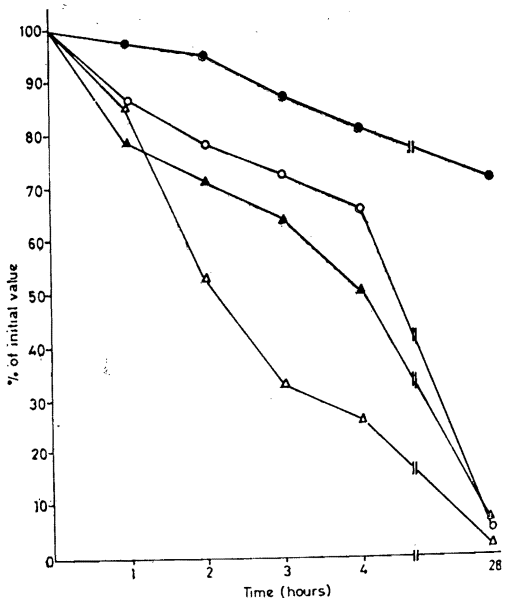

Fig. 1—The effect of temperature and post collection storage on erythrocyte ATP level. •——• = extracted blood in refrigerator (4°C), o——o = extracted blood in room temperature, ▲——▲ = blood in refrigerator, △——△ = blood in room temperature.

Table 1

Percentage recovery after adding the known amount of standard ATP solution.

	Theoretical value (μM/100 ml)	Duplicate determined values (μM/100 ml)	Percentage Recovery
Total volume of 8 ml of buffer with:-			
2.0 ml 200 μM/100 ml standard	50	50, 48	100, 96
3.0 ml 200 μM/100 ml standard	75	64, 64	86, 86
4.0 ml 200 μM/100 ml standard	100	92, 91	92, 91
5.0 ml 200 μM/100 ml standard	125	103, 104	82, 83
6.0 ml 200 μM/100 ml standard	150	158, 162	105, 108
7.0 ml 200 μM/100 ml standard	175	170, 182	97, 104
8.0 ml 200 μM/100 ml standard	200	195, 199	97, 100
Blood pool 1 ml with :-			
1.0 ml 500 μM/100 ml Std.	57	55, 55	97, 97
1.5 ml 500 μM/100 ml Std.	82	69, 69	84, 84
2.0 ml 500 μM/100 ml Std.	107	105, 102	98, 96
2.5 ml 500 μM/100 ml Std.	132	115, 112	87, 85
3.0 ml 500 μM/100 ml Std.	157	145, 138	92, 88
3.5 ml 500 μM/100 ml Std.	182	152, 142	83, 78
4.0 ml 500 μM/100 ml Std.	207	200, 209	100, 105
4.5 ml 500 μM/100 ml Std.	232	179, 225	77, 97
5.0 ml 500 μM/100 ml Std.	257	303, 297	118, 115
5.5 ml 500 μM/100 ml Std.	282	318, 325	113, 115
6.0 ml 500 μM/100 ml Std.	307	303, 303	99, 99

Erythrocyte ATP content in normal and the *P. berghei*-infected mice : An average value ± one standard deviation of ATP contents in red cells of 18 normal mice was found to be 114.60 ± 24.26 μM/100 ml RBC (range 70.5-152.5).

The erythrocyte ATP in *P. berghei*-infected mice at various stages of infection are illustrated in Fig 2. These values (149.68 ± 46.44 μM/100 ml red cells) were found to be significantly higher than those in the normal mice ($P < 0.01$). A wide variation of the ATP content in the infected mice at different levels of parasitaemia was observed and there was no correlation between these values and the parasitaemia.

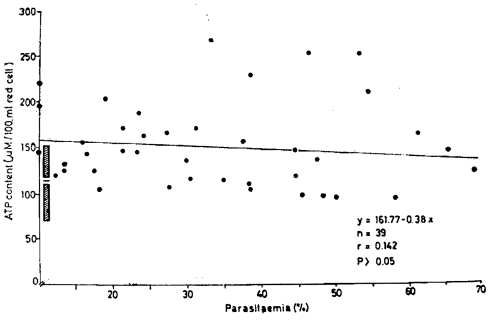

Fig. 2—Relationship between the parasitaemia (%) and the measured red cell ATP content of *P. berghei*-infected mice.

Nine samples of blood from the infected mice were mixed with 0.7 and 0.8 M sucrose in a Krebs-glucose-saline solution and centrifuged; two suspensions of erythrocytes

Table 2

The measured ATP content in red cells of mice infected with *P. berghei* and the calculated erythrocyte ATP content in the parasitized and non-parasitized red cells.

No.	Parasitaemia (%)	ATP content (μM/100 ml RBC)				
		Measured in RBC (a)*	Calculated ATP content from (a)*		Total ATP content	
			Parasitized RBC (b)*	Non-parasitized RBC (c)*	Parasitized RBC	Non-parasitized RBC
2	21.6	170.4	142.2	28.2	658.3	36.0
3	13.4	123.9	61.7	62.2	460.4	71.8
4	16.1	156.7	70.9	85.8	440.4	102.3
6	16.5	140.3	82.0	58.3	497.0	69.8
7	27.4	76.1	46.2	29.9	168.7	40.9
8	30.7	85.5	62.0	23.5	202.0	33.9
10_a	38.7	228.1	203.6	24.5	526.1	40.0
11_b	53.5	250.8	78.9	171.9	147.5	369.7
11_c	33.2	265.4	198.8	66.6	598.8	99.7
13	17.4	123.2	55.1	68.0	316.7	82.3
14	67.8	110.2	98.7	134.2	98.7	134.2
15	13.3	129.8	71.9	57.9	540.5	66.8
18	12.3	119.4	77.2	42.2	627.5	55.6
19	31.9	75.7	63.1	12.6	200.3	18.8
21	44.5	117.9	110.7	7.2	248.7	13.1
16	13.3	98.1	42.8	55.3	322.0	63.0
23_a	65.5	144.8	115.1	29.5	175.7	85.4
23_c	69.1	121.6	92.9	28.2	134.4	40.8

* (a) = (b) + (c).

from each solution were prepared and assayed for ATP content. The results (Table 2) showed that the ATP content of the parasitized red cells was significantly higher than that of the non-parasitized erythrocytes ($P < 0.05$). There was an inverse relationship between the parasitaemia and the ATP content in parasitized red cells as illustrated in Fig 3. From the proportions of parasitized and non-parasitized erythrocytes, it could be estimated that the level of ATP in non-parasitized red cells was not

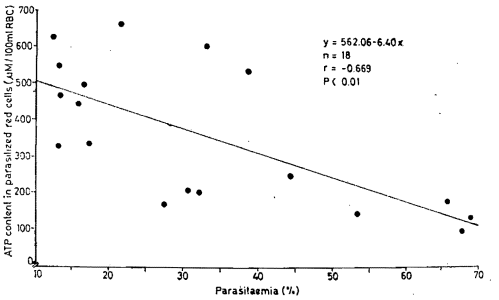

Fig. 3—Relationship between the parasitaemia (%) and the calculated ATP content in the parasitized red cells.

greater than that in erythrocytes before infection in these mice.

DISCUSSION

Several methods for measurement of red cell ATP levels have been developed recently. The simplest and most reproducible of these methods is the firefly luciferase enzyme and liquid scintillation counter method. It is a rapid and economical method which can be used to determine ATP levels over a wide range of 10^{-9} to 10^{-12} mole. The reproducibility and the recovery of this method in our laboratory (see Table 1) were quite satisfactory, especially in view of the minute quantities involved.

A number of factors are involved in the determination of the erythrocyte ATP level. The results from the present study showed a rapid decline in red cell ATP levels when heparinized blood was stored at 4°C. These levels decreased progressively and reached 26 per cent and 50 per cent of their original values when the heparinized blood was stored at room temperature and at 4°C for 4 hours, respectively. The corresponding figures for the extracted blood kept under these conditions were 66 and 81 per cent, respectively. It was, therefore, necessary to precipitate and extract the heparinized blood immediately as changes occurred in the ATP level if there was any delay even when the extracted blood was stored at 4°C.

For the eighteen normal mice used in these experiments, a mean figure for ATP of 114.60 μM/100 ml erythrocytes (S.D. ± 24.26) was obtained, a figure which was in accordance with those quoted for human and monkey red cells (Areekul and Chantachum, 1973; Areekul and Thaijongrak, 1974).

In the present study there was a rise in red cell ATP levels during *P. berghei* infection and these levels showed no correlation with the proportion of red cells infected. It was possible that younger erythrocytes which were preferentially infected by *P. berghei* had somewhat higher ATP levels than the older red cells. However, the effect of age on levels of ATP in red cells in the present study was not sufficiently pronounced to explain this result. Furthermore, Brewer (1969) concluded from the available evidence that there was no difference in ATP contents of young and old cells in normal human erythrocyte populations. Findings of increased erythrocyte-ATP levels in *P. berghei*-infected mice was in accordance with results reported earlier in *P. knowlesi*-infected monkeys (Ball *et al.*, 1948; Dunn, 1969; Eaton and Brewer, 1969; Fletcher *et al.*, 1970). However, these levels were found to fall during malarial infection in rats infected with *P. berghei* and *P. vinckei* and in ducks infected with *P. lophurae* (Brewer and Coan, 1969; Trager, 1967).

ATP is by far the most important donor of energy for metabolic reactions in biological systems. In the entire chain of glucose utilization, ATP plays an important part starting with its role in the phosphorylation of glucose by hexokinase. Malaria parasites require ATP for survival, growth and multiplication as illustrated by Trager (1950, 1963). This led to an assumption that the malaria parasite could possibly not synthesize ATP due to a lack of at least one enzyme for the formation of this substance (Trager, 1967). The malaria parasite may therefore have to utilize the energy-producing mechanisms of the host erythrocyte, especially *P. berghei* which was found to be dependent upon the host cell reserves to fulfill ATP requirements (Nagarajan, 1968). Furthermore, the human erythrocyte can synthesize adenine nucleotides from the free purine, i.e. adenine; failure of the parasite to do so would be an additional factor leading to the parasitic dependence on an external source of ATP (Trager, 1967).

Findings that the increase in erythrocyte-ATP levels in *P. berghei*-infected mice occurred mostly in the parasitized erythrocytes was in accordance with the results reported previously by many workers. It has been shown that monkey erythrocytes parasitized by *P. knowlesi* contained a somewhat higher concentration of ATP than unparasitized erythrocytes (Ball *et al.*, 1948). Fletcher *et al.*, (1970) found that although increase in ATP levels took place mainly in erythrocytes of *P. berghei*-infected monkeys as a result of the metabolic activities of the parasites, smaller but appreciable increase also occurred in the non-parasitized cells as well. Since malaria parasites could not synthesize ATP *per se* and had to depend upon the host-cell reserves to fulfill this requirement (Trager 1967; Nagarajan, 1968), the increment of ATP levels in the parasitized erythrocytes probable indicated that these cells were better able than non-parasitized red cells to pick up purine nucleotides needed for ATP synthesis. The ATP content in the parasitized erythrocytes was used not only for maintaining the structural and functional integrity of the erythrocyte host but also for supplying the energy and nucleotide requirement for the variety of synthesizing processes taking place in a rapidly growing and dividing parasite (Fletcher *et al.*, 1970). The mechaism that initiates the increased ATP content in the parasitized erythrocytes needs further study.

Considering that the ATP content was higher in red cells of *P. berghei*-infected mice than in those of normal mice, the hypothesis that the rigidity of red cells in this species of malaria might be due to ATP depletion was not supported.

SUMMARY

ATP content was determined in erythrocytes of normal and *P. berghei*-infected mice by the luciferase enzyme and the liquid scintillation counter. The erythrocyte-ATP level in *P. berghei*-infected mice was found to be significantly higher than in normal mice, and there was no relationship between these values and the level of parasitaemia. An inverse relationship between parasitaemia and the ATP content in parasitized red cells was observed while the non-parasitized red cells showed the same content of ATP as in the normal erythrocytes.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Denise C. Reynolds and Dr. Curt R. Schneider for reading and criticism of the manuscript and Professor Chamlong Harinasuta, Dean of the Faculty of Tropical Medicine, for his encouragement and support.

REFERENCES

AREEKUL, S., (1973). Rigidity of red cell during malarial infection *J. Med. Ass. Thailand, 56* : 163.

AREEKUL, S. and CHANTACHUM, Y., (1973). Measurement of the red cell ATP levels in Thai blood donors using the luciferase enzyme and the liquid scintillation counter. *Southeast Asian J. Trop. Med. Pub. Hlth., 4* : 440.

AREEKUL, S. and THAIJONGRAK, K., (1974). ATP content in erythrocytes of *Plasmodium knowlesi*-infected monkeys. *J. Med. Ass. Thailand.* (In press)

BALL, E.G., MCKEE, R.W., ANFINSEN, C.B., CRUZ, W.O. and GEIMAN, Q. M., (1948). Studies on malarial parasites. IX. Chemical and metabolic changes during growth and multiplication *in vivo* and *in vitro. J. Biol. Chem., 175* : 547.

BREWER, G.J. and POWELL, R.D., (1965). A study of the relationship between the content of adenosine triphosphate in

human red cells and the course of falciparum malaria. A new system that may confer protection against malaria. *Proc. Nat. Acad. Sci., 54* : 741.

BREWER, G.J., (1969). Adenosine triphosphate. *In* Biochemical Methods in Red Cell Genetics. P.201. Academic Press, N.Y.

BREWER, G.J. and COAN, C.C., (1969). Interaction of red cell ATP and malaria and the treatment of malaria with hyperoxia. *Milit. Med., 134* : 1056.

DUNN, M.J., (1969). Alterations of red blood cell metabolism in simian malaria: Evidence for abnormalities of non-parasitized cells. *Milit. Med., 134* : 1100.

EATON, J.W. and BREWER, G.J., (1969). Red cell ATP and malaria infections. *Nature, 222* : 389.

FLETCHER, K.A., FIELDING, C.M. and MAEGRAITH, B.G., (1970). Studies on the role of adenosine phosphates in erythrocytes of *Plasmodium knowlesi*-infected monkeys. *Ann. Trop. Med. Parasit., 64* : 487.

NAGARAJAN, K., (1968). Metabolism of *Plasmodium berghei*. II. P_i incorporation into high energy phosphates. *Exp. Parasit., 22* : 27.

STANLEY, P.E. and WILLIAMS, S.G., (1969). Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anal. Biochem., 29* : 381.

TRAGER, W., (1950). Studies on the extracellular cultivation of an intracellular parasite (avian malaria) I. Development of the organisms in erythrocyte extracts and the favouring effects of adenosine triphosphate. *J. Exp. Med., 92* : 349.

TRAGER, W., (1963). The biochemistry of intracellular parasitism. *Seventh Int. Congr. Trop. Med. Malaria* (abstract), p. 467.

TRAGER, W., (1967). Adenosine triphosphate and the pyruvate and phosphoglyceric kinase of the malaria parasite *Plasmodium lophurae. J. Protozool., 14* : 110.