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CHANGES IN NUCLEOTIDE CONCENTRATIONS IN THE ERYTHROCYTES OF MAN, RABBIT AND RAT DURING SHORT-TERM STORAGE

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SUMMARY: The ATP/ADP ratio, measured by high performance liquid chromatography, has been used as an indicator of stability of erythrocyte nucleotides. The nucleotides from human, rabbit and rat whole blood, but not separated erythrocytes were stable for maximum periods of 40, 20 and 15 min respectively after venepuncture. The ratios then declined rapidly from 9 to 5, 12 to 4 and 9 to 1 respectively during 2h storage at room temperature. Similar changes occurred in GTP/GDP ratios. The relevance of these observations to metabolic studies in intact cells, nucleotide analyses in the clinical situation and comparative studies in other species is discussed.

It has long been accepted that, due to ischaemia, rapid changes in nucleotide levels and their relative concentrations can occur in intact tissues (e.g. liver, kidney) and therefore, in order to obtain accurate quantitation, elaborate freeze-clamping procedures have been developed. On the other hand, from studies based on specific methods for ATP analysis (e.g. enzymic, luciferase) it is commonly believed that erythrocyte nucleotides remain stable throughout not only lengthy washing procedures and timely incubations but also transportation between laboratories.

High performance liquid chromatograpy (HPLC) enables the simultaneous quantitation of all the nucleotides in any one sample. Using this improved technique we have re-investigated the stability of erythrocyte nucleotides *in vitro* for humans and two common laboratory animals.

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METHODS: Non-fasting blood was obtained by venepuncture from normal human male subjects; from the tail-vein of mature female Wistar rats (wt. 200-250 g) and from the ear-vein of male New Zealand white rabbits (wt. 2.5 - 3.5 Kg). The blood was collected into heparinised tubes and divided into two portions. 200 µl aliquots of whole blood were pipetted into separate tubes and kept stoppered at room temperature (25°C). At each timed interval up to 2h one aliquot was extracted with 20% w/v trichloracetic acid (100 µl). The remaining half of the blood was immediately centrifuged (1000 g, 3 min) and the plasma and buffy coat discarded. Erythrocyte aliquots (200 µl) were stored as above and similarly extracted employing 400 µl of 10% w/v trichloracetic acid per 200 µl packed cells. All acid extracts were washed with water-saturated diethyl ether, neutralised with solid Tris[1] and stored deep-frozen (-20° C) prior to analysis by HPLC. The chromatographic conditions were slightly modified from those previously reported[2]. Column, 18 x 0.4 cm; AS Pellionex SAX; Temp, 75°C. Flow 36 ml/h, Gradient 0.003M KH_2PO_4 (pH.6.5) to 0.18M KH_2PO_4 containing 0.13M KCl (pH.4.5). Chart speed 300 mm/h. Injection vol. 10 µl of extract.

RESULTS: Representative erythrocyte nucleotide profiles for man, rabbit and rat obtained by HPLC are compared in Fig. 1 (A, B, C) respectively. The changes effected by storing erythrocytes anaerobically for 2h at room temp. are shown in corresponding profiles D, E, F (Fig. 1). The initial values for the above examples agree well with values previously obtained in these laboratories, man (n=9): ATP 1278 S.D. $\pm$ 127; ADP, 114 $\pm$ 24; NADP, 48 $\pm$ 12; AMP 10 $\pm$ 3; rat (n=16): ATP, 682 $\pm$ 117; ADP, 84 $\pm$ 15; GTP, 131 $\pm$ 20, GDP, 25 $\pm$ 5 nmole/ml packed red blood cells (PRBC).

The chromatograms show that in all cases the triphosphate nucleotides ATP, GTP break down to the corresponding mono- and di-nucleotide. Over the 2h period some loss (<5%) of total nucleotides was observed due to nucleoside and base formation. In all cases only small amounts of IMP were formed.

The chromatograms for whole blood and separated erythrocytes were essentially similar, except that for man and rabbit the levels in whole blood were 20%, 30% higher for ATP and 60%, 30% higher for ADP respectively. Only in human blood did these differences affect the ATP/ADP ratio, (9 in whole blood, 12.5

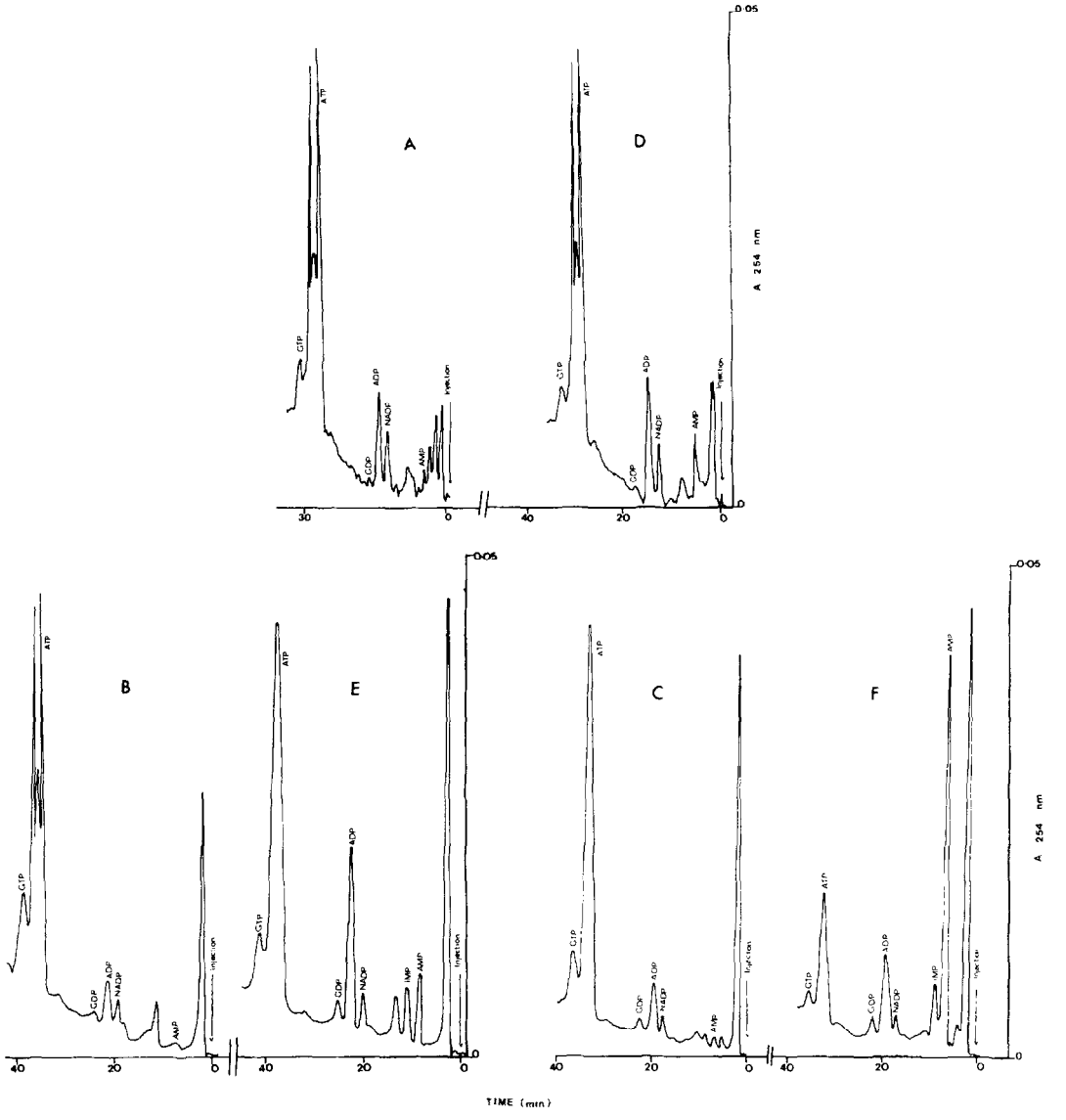

Fig. 1. Typical nucleotide chromatograms obtained from freshly separated erythrocytes of man (A), rabbit (B) and rat (C). The effects of storage in vitro at 25°C for 2h are shown in the corresponding figures (D, E, F) respectively. Chromatography as described in Methods. N.B. The ATP peak's maxima in A, B, D has been automatically reduced by 50%.
Quantitation nmol/ml PRBC, (2h storage values in parentheses):
man: A(D): GTP 60 (38): ATP 1280, (1054); GDP 15, (20); ADP 90, (154) NADP 47, (46); AMP 15, (88); IMP 15, (15).

rabbit; B(E): GTP 230, (148); ATP 1036 (805); GDP 20, (48); ADP 85, (200) NADP 22, (28); AMP 21, (88); IMP 15, (67).

rat; C(F): GTP 108, (38); ATP 651, (227); GDP 21, (28); ADP 87, (140): NADP 20, (20); AMP 15, IMP 15, (83).

in the separated erythrocyte, Fig. 2).

Fig. 2 compares changes in ATP/ADP ratios for whole blood and separated erythrocytes during 2h storage. For human whole blood this ratio did not change significantly over the first 40 min and then fell by 20% over the next hour. Rabbit and rat whole blood was stable for 20 and 15 min respectively. In the separated erythrocytes of all three species no initial stable period was observed and ATP/ADP ratios declined rapidly, the rate of fall being marginally less for the human erythrocyte.

Alterations in GTP/GDP ratios are shown for the rabbit and rat in Fig. 3. (GTP and GDP levels in human blood were low and, because quantitation was accordingly less accurate, are not shown). For both species the fall in GTP/GDP ratios parallels the decline of ATP/ADP ratios and these ratios were also better maintained in whole blood than in the separated erythrocyte. Changes occurred also in the overall relationships between the nucleotides. For human erythrocytes the initial ATP:ADP:AMP relationship was 14.2:1:0.2 becoming 6.8:1:0.6 after 2h. Corresponding values for the rabbit were initially 12.2:1:0.3, becoming 4.0: 1:0.4 at 2h, and for the rat initially 7.5:1:0.2 becoming 1.6:1:2.5 at 2h.

DISCUSSION: A knowledge of cellular nucleotide concentrations in certain pathological states (e.g. adenosine deaminase deficiency, Lesch Nyhan Syndrome, chronic renal disease) is becoming increasingly important. Normally the red cell, being readily obtainable, is employed for this purpose and, at least, in man, its nucleotide concentrations were believed to be reasonably stable after collection.

Chromatographic techniques, in particular HPLC, currently

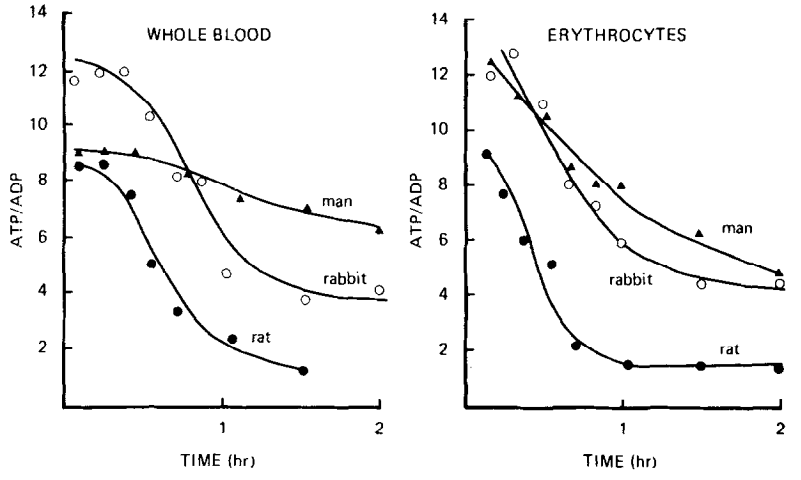

Fig . 2. The effect of in vitro storage at 25°C on the ATP/ADP ratio in whole blood (A) and separated erythrocytes (B). (▲ - ▲), (o - o) and (● - ●) represent the mean values obtained from 2 humans, 2 rabbits and 2 rats respectively.

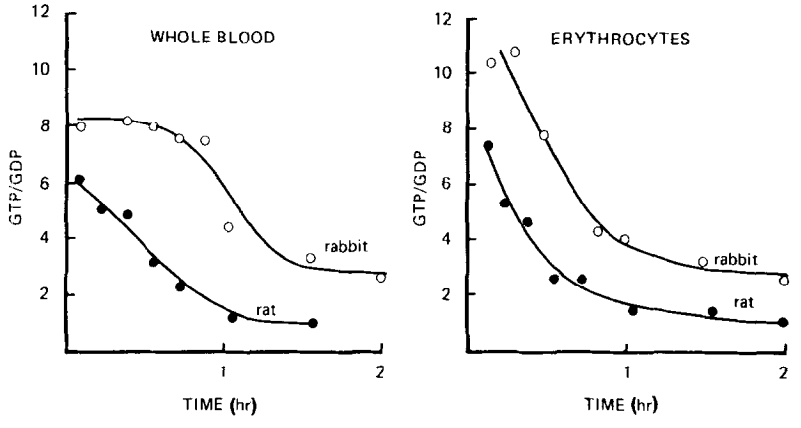

Fig. 3. The effects of in vitro storage at 25°C on the GTP/GDP ratios in whole blood (A) and separated erythrocytes (B), (o - o) and (● - ●) represent the mean values obtained for 2 rabbits and 2 rats respectively.

provide the most complete knowledge about the nucleotide content of tissues. Scholar et al[3], using HPLC, quantitated the nucleotide concentrations in the different human blood cells. Comparative studies of red cell nucleotides of different species

e.g. mammals, birds and amphibia have also recently been performed[4,5,6]. It would appear that these comparative reports have assumed that erythrocyte nucleotides from various species have the same apparent stability in vitro as those of human origin. When ATP levels alone are measured short term changes in human erythrocytes are not apparent. Because the energy state of the cells is finely balanced, breakdown of ATP leads to increases in ADP and AMP levels. Changes in the ATP/ADP ratio give a more sensitive indication of the metabolic state of the cell than can be obtained by measurement of the level of an individual nucleotide. For the human erythrocyte (ATP, ADP = 1280, 90 n mole/ml PRBC, respectively) it can be calculated that a 2% conversion of ATP to ADP changes the ATP/ADP ratio from 14.2 to 10.8 (24% decrease). Ratios determined by multiple analyses of the same blood sample gave 1 coefficient of variation $<3.5\%$ (n=8) which is approx. half that reported[2] for ATP determination. Such good reproducibility results from this ratio being effectively independent of many of the errors of measurement viz. variations in detector response, sampling, extraction and loading errors. Thus ATP/ADP ratios, when determined by HPLC are highly sensitive and reproducible. The results reported here, based on ATP/ADP and GTP/GDP ratios, show that red cell nucleotides are more labile than previously believed. Although the degradation with time of rabbit and rat nucleotides was most marked, the separated human erythrocyte was, at best, only slightly more stable (Fig. 2B). Maintaining the erythrocyte in its natural environment in vitro effectively stabilised the nucleotides for short periods only (40 min) and for the rat in particular this stable period was exceedingly short-lived (Figs. 2A, 3A).

In one experiment we found that maintaining human whole

blood at 4°C did not significantly delay the onset of degradation. It would appear from published values[4,5,6] for ATP, ADP and their calculated ratios that similar rapid degradations have occurred in samples studied from other species. It appears unsatisfactory to assume that red cell nucleotides, regardless of species, are stable and in order to obtain meaningful basal values, nucleotides should be extracted immediately after venepunture. In the clinical situation where this is not always possible, a reasonable amount of leaway is permissible if whole blood is employed. Unfortunately, due to salt interferance, acid extracts prepared directly from whole blood do not chromatograph totally satisfactorily. Additionally the result is affected by the overall blood composition, e.g. the relatively high concentration of ADP in platelets. Thus clinical samples should be transported to the laboratory on ice as whole blood and the erythrocytes separated rapidly with minimal washing so that acid extraction can be performed within 40 min of venepuncture.

Innumerable metabolic pathways are utilised to maintain the energy balance of the cell. Many of these rely on an adequate supply of glucose and phosphate, requirements that are readily met by whole blood, at least in the short term. Because of the interrelationships of their pathways, changes in ATP/ADP are paralleled by GTP/GDP changes. The lower ratio of ATP:GTP in rabbit and rat erythrocytes (5:1) as compared to human values (20:1) may well reflect their greater instability. Previously we reported[7] that short-term incubation in Earle's buffer at 37°C resulted in the degradation of ATP and the accumulation of IMP by the human erythrocyte whereas in the present study (at 25°C) AMP was the major mono-nucleotide formed. These observations may imply a temperature dependence

for one or more of the pathways involved in IMP formation.

The results reported here relate directly to metabolic studies performed on erythrocytes in vitro. Clearly changes in the energy charge of the erythrocyte could affect the activity of related metabolic pathways. To avoid erroneous results it would appear that metabolic studies are best performed over relatively short time periods. In the light of the present observations it is apparent that many literature values for erythrocyte nucleotides should be treated with some caution.

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