ESTIMATION OF NUCLEOTIDE CONTENT OF RED CELLS

BY

N. C. HUGHES JONES AND MARGARET A. ROBINSON

*From the Medical Research Council's Blood Transfusion Research Unit,
Postgraduate Medical School, London*

This paper describes a method of estimating the nucleotides in red blood cells. The technique used is based on that of Cohn and Carter (1950), who have shown that the nucleotides, adenosine-monophosphate (A.M.P.), adenosinediphosphate (A.D.P.), and adenosinetriphosphate (A.T.P.) can be separated by an anion exchange column. Bartlett, Savage, Hughes, and Marlow (1953) have successfully used this method for estimations on 100 ml. of whole blood ; however, this volume is inconveniently large, especially if serial estimates are to be carried out. The present method uses only 1 ml. of red cells. Quantitative measurements have been made using the characteristic ultra-violet light absorption of adenine.

In order to reduce the quantity of other substances also absorbing ultra-violet light, preliminary precipitation of the nucleotides as barium salts is carried out before placing them on the column. After elution of the nucleotides in separate fractions, the ultra-violet light absorption at three wavelengths is measured and correction for impurities made by the method of Morton and Stubbs (1946).

This paper contains a detailed account of the method and findings of the nucleotide content of fresh red cells from 12 normal subjects. In order to demonstrate the use of the method, the results obtained before and after incubating stored red cells with adenosine are described.

Methods

Fresh, heparinized venous blood was obtained and washed with ice-cold saline (0.9%). After centrifuging, duplicate samples of the packed cells were delivered from a 1 ml. pipette.

It is usual to express the nucleotide concentration as micromoles per unit volume of red cells. However, under many experimental conditions red cell volume changes considerably, due to shifts of electrolytes and water ; in such cases it is more satisfactory to refer estimates of nucleotide content to the haemoglobin concentration of the sample. Thus, for example, if the cells shrink and the nucleotide content remains unchanged, the concentration per unit volume of cells will increase but will remain constant in relation to the haemoglobin concentration. Estimates have therefore been related to haemoglobin concentration, but in order to compare these results with those previously published they have been converted to nucleotide content per 100 ml. of red cells, on the assumption that normal cells contain 34 g. of haemoglobin per 100 ml.

Extraction of Acid-soluble Phosphates.—The cell suspension (1 ml.) was lysed with 1 ml. of distilled water. The proteins were then precipitated and acid-soluble phosphorus extracted by adding 1 ml. of 20% (w/v) trichloro-acetic acid. A further extraction was made, using 2 ml. of 5% (w/v) trichloro-acetic acid and combined with the first. The mixtures were kept cool throughout by surrounding them with crushed ice.

Precipitation of Nucleotides as Barium Salts.—A quantity, 0.3 ml., of 2 M $BaCl_2$ was added, followed by two drops of 1% phenolphthalein. Ethanol at 4° C. was then added to a final concentration of 80% (v/v). The pH of the solution was adjusted with 10 N NaOH until a faint pink colour appeared (approximately pH 8.2). The solution was left at 4° C. for one hour ; further adjustment of pH was sometimes necessary during this time. The precipitate was centrifuged and the supernatant removed. It was found that the precipitate could be stored at −20° C. for three weeks without significant deterioration.

The barium salts were dissolved in N HCl, added drop by drop until solution was complete, then 4 ml. of 0.05 N H_2SO_4 was added and the precipitate of barium sulphate removed by centrifuging in the cold. The solution was made alkaline (pH 10) with 10 N NaOH and transferred quantitatively to the top of the anion exchange column.

Anion Exchange Column.—Amberlite IRA-400 (a strongly basic cross-linked polysterene anion exchange resin) in the chloride form was used (mesh smaller than 120). To facilitate the flow of eluate through the column, one part of resin was mixed with
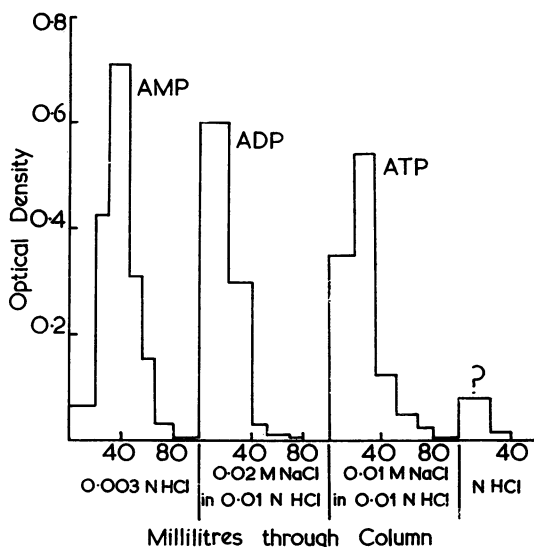
FIG. 1.—Elution of a mixture of commercial A.M.P., A.D.P., and A.T.P. from the anion-exchange column.

two parts of "celite" (Johns-Manville No. 545), a diatomaceous earth. The size of the column was 12 cm. ×0.2 sq. cm. Impurities in the resin absorbing ultra-violet light were removed with N HCl. Between 2 and 3 ml. of the eluting fluid was passed through the column per minute under pressure.

Eluting Solutions.—To determine the volume and composition of eluting solutions needed to remove the nucleotides separately, commercial A.M.P., A.D.P., and A.T.P. were used. A mixture of these was brought to pH 10 and added to the column. The following solutions were found to be satisfactory: for A.M.P., 80 ml. of 0.003 N HCl; for A.D.P., 80 ml. of 0.02 M NaCl in 0.01 N HCl, and for A.T.P., 80 ml. of 0.1 M NaCl in 0.01 N HCl. Finally, N HCl was used to regenerate the column. The identity of the nucleotide in each fraction was confirmed by determining the adenine-phosphorus ratio. Fig. 1 shows the optical density of the eluates which were collected in 20 ml. amounts to show the fall in successive samples. Fractionation of commercial muscle A.T.P. yielded a further purine compound in the N HCl fraction. This was probably adenosine tetraphosphate (Sacks, 1955) as the absorption spectrum was characteristic of adenine and the estimated adenine-phosphorus ratio was 1:4.2.

Absorption Spectrum of Adenine Compounds.— Optical density measurements on the eluates were carried out with a "unicam" quartz spectrophotometer, S.P. 500. The densities at 2570 Å were usually below 0.2. To determine the absorption spectrum of adenine compounds estimations were made on commercial adenosine and A.M.P. at pH 2.0. To determine the purity of the compounds, ascending paper chromatography was carried out by two different

methods; one using butanol saturated with an aqueous solution of 10% urea, the other using distilled water brought to pH 10 with NH_4OH. No other substances absorbing ultra-violet light were detected.

Two estimations of the optical density of each compound were carried out over the range 2300 Å to 2800 Å, in 20 Å steps. The compounds were dissolved in 0.01 N HCl and blank estimations were made on the solvent beforehand. The mean of all four estimations was then used. There was no significant difference between the absorption spectrum of adenosine and A.M.P.

At pH 2 adenosine has a maximum absorption at 2570 Å. At 2400 Å and 2743 Å the densities were found to be equal and were 42.5% of the density at 2570 Å (vide infra).

Volkin and Cohn (1954) have given the millimolar extinction coefficient at 2600 Å as 14.2. The density ratio of 2570 Å:2600 Å has been found to be 1.02:1, and thus an extinction coefficient of $14.2 \times 1.02 = 14.5$ at 2570 Å has been used.

Correction for impurities in the eluates also absorbing ultra-violet light was made by the method of Morton and Stubbs (1946), using the three wavelengths 2400 Å, 2570 Å, and 2743 Å.

The proportion of light absorbed by impurities at 2570 Å varied in different experiments on normal red cells, but was usually 2–10% of the total A.T.P. fraction and 10–20% in the A.D.P. fraction.

The smallest amount of purine that can be estimated has been taken as that which gives a reading on the spectrophotometer of 0.005 at 2570 Å, corresponding to approximately 3 micromoles adenine per 100 ml. of cells.

Estimation of 2 : 3-Diphosphoglyceric Acid (D.P.G.). —Diphosphoglyceric acid is also precipitated as a barium salt, and Bartlett et al. (1953) have shown that it is adsorbed by the anion-exchange column and eluted off with the A.T.P. fraction. The total P was estimated in this fraction and the D.P.G. content found by subtraction of P due to A.T.P. The latter was calculated from the known adenine content.

Estimation of Phosphorus.—The method used was a modification of that of King (1932). A sample of eluate was evaporated to dryness. Then 1.1 ml. 60% perchloric acid was used for digestion, which was carried out at 200°. Then 0.7 ml. 5% ammonium molybdate was added, followed by 0.6 ml. of 0.2% (w/v) aminonaphtholsulphonic reagent and the volume made up to 10 ml. Approximately sevenfold intensification of the blue colour was obtained by heating the final solution to 70° for 15 minutes. The density of the colour was determined at 8250 Å and was found to be stable for 24 hours (Hughes Jones and Robinson, to be published).

Incubation of Stored Blood with Adenosine.— Blood from a healthy donor was stored at 4° C. in acid-citrate-dextrose (A.C.D.) solution. After 30 days, half the blood was incubated with 2.5 millimoles of adenosine per 100 ml. of red cells for one hour at

37° C. This was the quantity found by Gabrio *et al.* (1955) to be optimal. The other half was used as a control and incubated without adenosine. Both samples were then washed twice with 0.9% saline, extracted, and fractionated.

Results

Normal Nucleotide Content of Red Cells.— Table I gives the nucleotide and diphosphoglyceric acid content of red cells in 15 samples obtained from 12 normal subjects.

TABLE I

RANGE OF A.M.P., A.D.P., A.T.P., AND D.P.G. VALUES IN 15 SAMPLES OF BLOOD FROM 12 NORMALS

A.M.P.	A.D.P.	A.T.P.	N HCl Fraction	D.P.G.
<3–5	12–25	63–116	<3–8	190–420

Each of the 15 samples was estimated in duplicate, and results are expressed as micromoles per 100 ml. red cells.

In the fraction in which A.M.P. is eluted some absorption of ultra-violet light was found, with a maximum density at 2570 Å. In only two of the samples was the density sufficient to give an accurate estimate: in these two the A.M.P. was estimated to be 4.0 and 5.0 micromoles per 100 ml. of red cells.

All the A.D.P. estimations fell within the range 12–25 micromoles per 100 ml. of red cells.

The A.T.P. estimations showed a wider range, namely from 63 to 116 micromoles per 100 ml. of red cells. These values were evenly distributed throughout this range, eight being above and seven below 90 micromoles per 100 ml. of red cells.

Four of the N HCl eluates contained adenine compounds in sufficient quantity for accurate estimation, but in no case did the total exceed eight micromoles per 100 ml.

Only nine estimates of D.P.G. concentration were made, and these varied from 190 to 420 micromoles per 100 ml. of red cells. Seven of the estimates lay between 300 and 420 micromoles per 100 ml.

To obtain an estimate of the accuracy of the method the standard deviation of a single determination was calculated from the results of duplicates and found to be 2.3 micromoles per 100 ml. for A.D.P., 4.5 micromoles per 100 ml. for A.T.P., and 25 micromoles per 100 ml. for D.P.G.
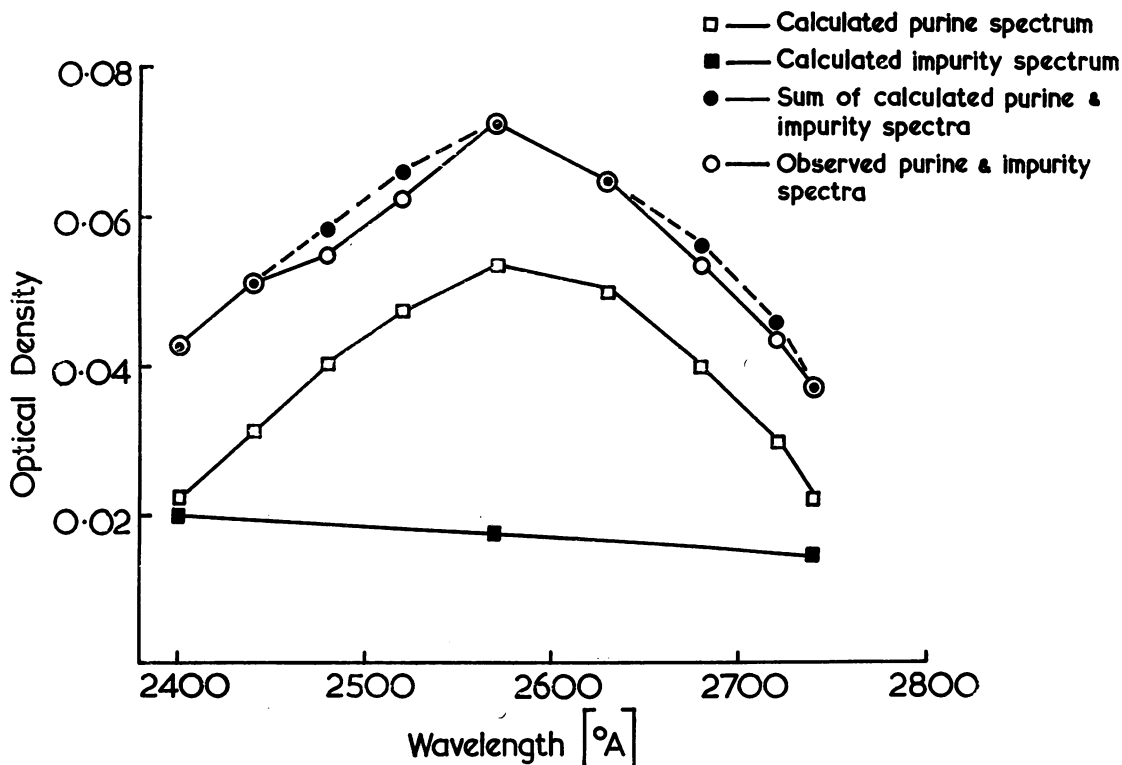

FIG. 2.—Observed and calculated purine spectra. For explanation, see text.

TABLE II

INCREASE IN NUCLEOTIDES IN STORED BLOOD

	A.M.P.	A.D.P.	A.T.P.	Total
	(Micromoles/100 ml. Red Cells)			
Control	10·6	25·2	18·9	54·7
Adenosine	0·8	19·9	95·0	115·7

Control. Mean of two estimates of A.M.P., A.D.P., and A.T.P. content of 30-day-old blood stored in A.C.D. at 4° and incubated for 1 hour at 37°.
Adenosine. Similar estimates on another sample of the same blood incubated with adenosine for 1 hour at 37°.

Incubation of Stored Cells with Adenosine.— Table II shows the increase in nucleotide content of stored blood after incubation with adenosine for one hour at 37° C. After 30 days' storage these cells had a low nucleotide content, and the relative concentration of A.M.P., A.D.P., and A.T.P. was altered compared with normal fresh cells. Adenosinemonophosphate comprised 20% of the total. After incubation with adenosine the total content and distribution returned to within normal limits. The quantity of A.M.P. in the eluates from stored cells was far greater than that found in normal fresh cells and could not have been accounted for by breakdown of A.T.P. during the estimations.

Discussion

The formula of Morton and Stubbs (1946) is based on the assumption that the absorption spectrum of the impurity is linear throughout the range of 2400 Å to 2743 Å. The presence of an impurity whose spectrum is curvilinear would cause the adenine compounds to be under- or over-estimated, depending on the shape of the curve. The following is evidence that the impurity spectrum probably is slightly curvilinear:

Fig. 2 shows the observed spectrum in an A.T.P. eluate. The density at 2570 Å due to nucleotide and impurity has been obtained from the formula, assuming the absorption spectrum of the impurity to be linear. The nucleotide curve has then been drawn from the known relative densities at different wavelengths. The sum of the calculated nucleotide and impurity is shown by the dotted line and does not completely coincide with the observed densities between the wavelengths 2400 Å, 2570 Å and 2743 Å. This is the result that would be obtained if the impurity spectrum were non-linear.

If the same impurity were present in the duplicate samples, then there would be a systematic error in both. This degree of error cannot be accurately estimated, but the reasonably close fit between the predicted and observed curves in the above example suggests that it is small. It is clearly desirable to have the eluant and resin completely free from impurities.

Rapoport and Guest (1941) gave an estimation of A.T.P. of red cells based on the measurement of easily hydrolysable phosphorus. They found 13.5 mg. P (145 micromoles A.T.P.) per 100 ml. red cells, compared with 60–100 micromoles per 100 ml. found by the present method. The higher figures derived from the estimates of labile phosphorus is probably due to the presence of P from compounds other than A.T.P.

Prankerd and Altman (1954), using two-way paper chromatography, found a range of 27 to 80 micromoles A.T.P.

Gabrio *et al.* (1955) found 30-day-old blood stored in A.C.D. at 4° C. to have a low A.T.P. content (represented by a low labile P content). The present investigation shows that the distribution of nucleotides is also altered, 20% of the nucleotide being present as A.M.P. After incubation with adenosine the nucleotide content and distribution are restored to within normal limits.

Summary

The use of an anion-exchange column for the determination of the organic phosphate compounds in 1 ml. of red cells is described and the results obtained from 15 normal fresh red cell samples are given.

REFERENCES

Bartlett, G. R., Savage, E., Hughes, L., and Marlow, A. A. (1953). *J. appl. Physiol.*, **6**, 51.
Cohn, W. E., and Carter, C. E. (1950). *J. Amer. chem. Soc.*, **72**, 4273.
Gabrio, B. W., Hennessey, M., Thomasson, J., and Finch, C. A. (1955). *J. biol. Chem.*, **215**, 357.
King, E. J. (1932). *Biochem. J.*, **26**, 292.
Morton, R. A., and Stubbs, A. L. (1946). *Analyst*, **71**, 348.
Prankerd, T. A. J., and Altman, K. I. (1954). *Biochem. J.*, **58**, 622.
Rapoport, S., and Guest, G. M. (1941). *J. biol. Chem.*, **138**, 269.
Sacks, J. (1955). *Biochim. Biophys. Acta*, **16**, 436.
Volkin, E., and Cohn, W. E. (1954). *Methods of Biochemical Analysis*, ed. Glick, D., Vol. 1, p. 287. Interscience Publishers, New York.