The Nucleotides in Normal Human Blood*

CHARLES BISHOP, DAVID M. RANKINE AND JOHN H. TALBOTT

From the Department of Medicine, the University of Buffalo School of Medicine and the Buffalo General Hospital, Buffalo, New York

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Nucleotides are known to be present in blood. Some investigators (1, 2) have determined the amounts of certain nucleotides only. Others (3, 4) have been more interested in the relative amounts of the various nucleotides present. It was the purpose of this investigation to identify and quantitate all of the major nucleotides in normal human blood.

EXPERIMENTAL

Approximately 15 ml. of venous blood were withdrawn and expressed into a chilled tube containing 0.30 ml. (300 U.S.P. units) of sodium heparin solution. After thorough but gentle stirring, 10.0 ml. were transferred by Folin pipette to 10 ml. of ice cold 10 per cent trichloroacetic acid. The mixture was thoroughly homogenized with a heavy stirring rod and centrifuged in the cold. The supernatant was poured into a glass-stoppered graduate, and the residue was re-extracted twice with lo-ml. portions of cold 5 per cent trichloroacetic acid. The combined supernatant solutions were extracted with equal volumes of cold ether until neutral, aerated for 5 minutes to remove as much as possible of the remaining ether, and stored frozen. The extra blood was centrifuged for hematocrit determination and the plasma saved for other determinations.

The separation of blood nucleotides followed the method of Hurlbert et al. (5) with the use of 20 x 1-cm. columns of Dowex 1-formate resin, except that gradient elution was not employed. The trichloroacetic acid extract of blood was poured onto a column, and distilled water was allowed to flow through the column until no more ultraviolet-absorbing material came through into the effluent (approximately 1 hour). The eluting solutions ranged from 0.13 N formic acid to 4 N formic acid-0.8 M ammonium formate. Fractions of 5 ml. were collected. An ultraviolet monitor from Gileon Medical Electronics, Middleton, Wisconsin, was used to delineate the elution pattern. The absorbance of each fraction was read in the Bockman DU spectrophotometer at 260 mp.1

The material from each peak was evaporated to dryness on a steam bath and the free purines or pyrimidines liberated by hydrolysis for 1 hour at 100° in 72 per cent HCIO4. The neutralized solutions were spotted on Whatman No. 1 paper for descending chromatography in the isopropanol-HCl system of Wyatt (6) followed by ascending chromatography in the butanol-formic acid system of Markham and Smith (7). The bases were eluted with dilute HCl and their spectra determined.2

After identification of the bases in the various peaks of the column chromatograms, additional samples of the peaks were freeze-dried to avoid hydrolysis and chromatographed in one-dimension on paper, with one or more of the following solvent systems: 1, isobutyric acid-concentrated NH4OH-H2O (66:1:33); 2, 0.1 M phosphate buffer (pH 6.8)-solid ammonium sulfate-n-propanol (100:40:2); and 3, isobutyric acid-concentrated NH4OH-H2O (58:4:39).3 The difficulty of lyophilizing solutions containing ammonium formate was avoided by exchanging the NH₄⁺ for H⁺ with the use of columns of Dowex 50 in the acid form.4 Compounds from Pabst Laboratories, Nutritional Biochemicals Corporation, and Sigma Chemical Company were used as reference compounds.

The quantitation of the compounds in the column chromatograms was first attempted by determining total spectra of the pooled fractions. The results were unsatisfactory, probably because formate contributes greatly to the ultraviolet absorption at the lower wave lengths and because there is interference from other phosphorylated compounds which have nonspecific absorption at the lower wave lengths. Taking these readings against various blank solutions did not help. The most reliable quantitation of the compounds in the column chromatograms was achieved by integrating the areas under the peaks of the chromatograms (read at 260 m). Subtraction of background attributable to the factors mentioned above was quite simple in this nongradient system.5

1 A 10-ml. syringe with a 71 inch, 17 gauge needle tipped with a small piece of polyethylene tubing was used for transferring solutions. A spiral spring fitting closely around the plunger and thereby returning the plunger approximately to the 4-ml. graduation allows the transfer to be made with one hand.

2 This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Bethesda, Maryland.

3 If a 5-ml. fraction has an absorbancy of A₁ and the solute has a molar extinction coefficient (at 260 m) of ε, then its content of nucleotide will be (A₁/ε) x 5 x 1000 μmoles of Aₖ where k = 5000/ε. Hence total micromoles of the nucleotide will be A₁k + A₂k + ... or kA₀. Since a 10-ml. sample of blood was used and since the final results are expressed as micromoles of compound per liter of blood, the whole expression must be multiplied by 100. If the over-all constant is now called k₀ (i.e. 100k) the total micromoles per liter of blood becomes k₀A₀. The values for k₀ are as follows: DPN, 27.8; uric acid, 129; AMP, 34.4; TPN, 27.0; ADP, 34.5; ATP, 35.0; GTP, 42.4. The values of 87.7 for CDP-choline and 79.5 for UDP-glucose were used in the recovery studies only. Most of the extinction coefficients were obtained from Bock et al. (8) but a few were determined in this laboratory.

* This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Bethesda, Maryland.

4 This was work for support by grants from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Bethesda, Maryland.

5 A IO-ml. syringe with a 7f inch, 17 gauge needle tipped with a small piece of polyethylene tubing was used for transferring solutions. A spiral spring fitting closely around the plunger and thereby returning the plunger approximately to the 4-ml. graduation allows the transfer to be made with one hand.

6 A IO-ml. syringe with a 7f inch, 17 gauge needle tipped with a small piece of polyethylene tubing was used for transferring solutions. A spiral spring fitting closely around the plunger and thereby returning the plunger approximately to the 4-ml. graduation allows the transfer to be made with one hand.

7 A IO-ml. syringe with a 7f inch, 17 gauge needle tipped with a small piece of polyethylene tubing was used for transferring solutions. A spiral spring fitting closely around the plunger and thereby returning the plunger approximately to the 4-ml. graduation allows the transfer to be made with one hand.

8 This procedure was suggested by Dr. Robert B. Hurlbert.

9 If a 5-ml. fraction has an absorbancy of A₁ and the solute has a molar extinction coefficient (at 260 m) of ε, then its content of nucleotide will be (A₁/ε) x 5 x 1000 μmoles of Aₖ where k = 5000/ε. Hence total micromoles of the nucleotide will be A₁k + A₂k + ... or kA₀. Since a 10-ml. sample of blood was used and since the final results are expressed as micromoles of compound per liter of blood, the whole expression must be multiplied by 100. If the over-all constant is now called k₀ (i.e. 100k) the total micromoles per liter of blood becomes k₀A₀. The values for k₀ are as follows: DPN, 27.8; uric acid, 129; AMP, 34.4; TPN, 27.0; ADP, 34.5; ATP, 35.0; GTP, 42.4. The values of 87.7 for CDP-choline and 79.5 for UDP-glucose were used in the recovery studies only. Most of the extinction coefficients were obtained from Bock et al. (8) but a few were determined in this laboratory.
After the separation and quantitation had been adequately verified, the nucleotide content of 13 blood samples from normal males and 8 blood samples from normal females was determined.

RESULTS

Fig. 1A shows the chromatogram of the nucleotides in a sample of normal human blood. The peaks were tentatively identified by paper chromatography.

Peak A contained cytosine but because of the paucity of material could not be further identified. From previous work (5) it was surmised that this peak was CMP. Peak B contained adenine and behaved like DPN in Solvent System 1. Peak C was shown by its spectrum in acid and base to be identical to uric acid. The only solvent system found to move uric acid as a distinct spot was distilled water. Carter and Potter (9) reported finding uric acid as its ribosyl derivative but only in beef blood. Peak D contained adenine and behaved like AMP in Solvent System 1. Peak E contained adenine and behaved like TPN in Solvent System 2. Peak F contained adenine and behaved like ADP in Solvent System 2. Peak G contained adenine and behaved like ADP in Solvent System 2. Peak Gz contained uracil and behaved like UDP in Solvent System 2. Peak H contained adenine and gave two spots corresponding to ADP and ATP in Solvent System 1. It was assumed that the ADP had arisen by breakdown of the ATP. Peak L appeared to contain adenine and could not be further characterized. Peak I contained guanine and gave two spots corresponding to GDP and GTP in Solvent System 3. The GDP was assumed to be a breakdown product.

A synthetic mixture containing appropriate amounts of CMP, DPN, uric acid, AMP, TPN, ADP, UDP, ATP, and GTP was then prepared and resolved according to the method used for blood extracts. The pattern closely resembled that from normal blood except that Peaks A (CMP), Gz (UDP), and L (unknown) were missing.

Since UDP was not eluted in the position of Gz, it could be assumed that Gz was not UDP but some other compound similar to it. Subsequent experiments indicated that UDP-glucose would be eluted from the column in the position of Peak Gz. Insufficient material from this peak was obtained for unequivocal identification of it as UDP-glucose.

The absence of an A peak with the synthetic mixture indicated that A could not be CMP. This was confirmed by demonstrating that CMP follows DPN when eluted from Dowex 1-formate columns by 0.13 N formic acid.

When several A peaks were combined and lyophilized, it was possible, with the use of 0.01 N formic acid instead of the 0.13 N acid, to resolve this material into two small but distinct peaks. Neither peak gave a definite spectrum. From the work of Kennedy (10) it was thought that one of these compounds might be CDP-choline. When this compound was eluted from a resin...
May 1959

C. Bishop, D. M. Rankine, and J. H. Taibott

Table I

Recovery of nucleotides from synthetic mixture*

<table>
<thead>
<tr>
<th>Peak</th>
<th>Compound</th>
<th>Amount added</th>
<th>Amount recovered</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>A</td>
<td>CDP-choline†</td>
<td>13.5</td>
<td>19.1</td>
</tr>
<tr>
<td>B</td>
<td>DPN</td>
<td>33.9</td>
<td>26.0</td>
</tr>
<tr>
<td>C</td>
<td>Uric acid</td>
<td>171</td>
<td>102</td>
</tr>
<tr>
<td>D</td>
<td>AMP</td>
<td>7.0</td>
<td>14.7</td>
</tr>
<tr>
<td>E</td>
<td>TPN</td>
<td>12.9</td>
<td>12.6</td>
</tr>
<tr>
<td>G</td>
<td>ADP</td>
<td>54.5</td>
<td>49.7</td>
</tr>
<tr>
<td>G₂</td>
<td>UDP-glucose†</td>
<td>11.0</td>
<td>2.3</td>
</tr>
<tr>
<td>H</td>
<td>ATP</td>
<td>463</td>
<td>487</td>
</tr>
<tr>
<td>L</td>
<td>Unknown</td>
<td>26.2</td>
<td>25.0</td>
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</table>

* Calculated as μmoles per l. Actual amounts of nucleotides added and determined have been multiplied by 100 to make the values directly comparable with those in blood, as determined in this method.
† Tentative identification. Consult text.

The identity of Peak L is unknown. It does not appear to be adenine tetraphosphate since this compound elutes slightly later and can be resolved from L in our system.

To test the ability of this fractionation system to resolve and determine quantitatively the nucleotides shown to be present in blood extracts, a synthetic mixture was prepared as shown in Table I. The recoveries in replicate analyses are indicated. The poorest recoveries are with Peaks A, G₂, and L, the ones least well characterized. The recovery of AMP is too high, suggesting that some might have arisen from breakdown of, for example, ADP. In evaluating these recovery data it should be borne in mind that the amounts are actually 0.1 ml. of the figures indicated since all data are calculated in terms of 1 l. of blood when in fact only a 0.1-ml. sample is used.

It has been generally assumed that nucleotides do not appear in the plasma. Willoughby and Waisman (4) found small amounts of three compounds in plasma but did not characterize them. We have found only two components in plasma. A typical chromatogram is shown in Fig. 1B. The second peak is uric acid as can be established by its spectrum and its position in the elution pattern. The first peak appears not to be a purine or pyrimidine on the basis of its spectrum. This would confirm the hypothesis that the blood nucleotides are confined within the cells and do not appear in the plasma.

Since the bulk of the blood nucleotides is contained in the red cells (assuming a negligible contribution from other formed elements), we have corrected all our values to standard hematocrit values of 42 per cent (female) and 47 per cent (male). The concentration of each compound is expressed as micromoles per liter of whole blood.

Uric acid, which is found in both cells and plasma, cannot be corrected for hematocrit. Furthermore, it is worthy of note that the concentration of uric acid in whole blood by the above procedure is usually less than the concentration of uric acid in plasma as determined by the ordinary colorimetric procedure.

There are many factors which preclude a simple comparison here. It does appear, however, that the recovery of uric acid from the synthetic mixture (Table I) is reasonably good.

The concentration of the blood nucleotides in 13 samples of blood from normal male subjects and 6 samples of blood from normal female subjects is presented in Tables II and III. Means and standard deviations (as percentage of means) are indicated. Peaks A, G₂, and L have been omitted because their identity is uncertain. It does appear, however, that the recovery of uric acid from the plasma as determined by the ordinary calorimetric procedure is reasonably good.

Table II

Nucleotides in normal male blood

Concentration expressed as micromoles of compound per liter of whole blood and corrected to hematocrit of 47 per cent red cells.

<table>
<thead>
<tr>
<th>Subject</th>
<th>DPN</th>
<th>Uric acid</th>
<th>AMP</th>
<th>TPN</th>
<th>ADP</th>
<th>ATP</th>
<th>GTP</th>
<th>Hematocrit</th>
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<td>142</td>
<td>9</td>
<td>60</td>
<td>456</td>
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<td>28</td>
<td>148</td>
<td>14</td>
<td>62</td>
<td>411</td>
<td>26</td>
<td></td>
<td>47</td>
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<tr>
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<td>40</td>
<td>166</td>
<td>8</td>
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<td></td>
<td>117</td>
<td>452</td>
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<td></td>
<td>47</td>
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<tr>
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<td></td>
<td>113</td>
<td>442</td>
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<td>10</td>
<td>63</td>
<td>549</td>
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<td></td>
<td>47</td>
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<tr>
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<td>47</td>
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<td>2</td>
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<td>499</td>
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<td>34</td>
<td>131</td>
<td>6</td>
<td>50</td>
<td>405</td>
<td>51</td>
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<td>28</td>
<td>149</td>
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<td>40</td>
<td>376</td>
<td>22</td>
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<td>47</td>
</tr>
</tbody>
</table>

Mean    | 29.8| 140.9     | 6.2 | 11.6| 47.8| 433.2| 24.5|

<table>
<thead>
<tr>
<th>Subject</th>
<th>DPN</th>
<th>Uric acid*</th>
<th>AMP</th>
<th>TPN</th>
<th>ADP</th>
<th>ATP</th>
<th>GTP</th>
<th>Hematocrit</th>
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<td>PY</td>
<td>35</td>
<td>183</td>
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<td>28</td>
<td>42</td>
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<td>146</td>
<td>5</td>
<td>11</td>
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<td>10</td>
<td>46</td>
<td>384</td>
<td>24</td>
<td>42</td>
</tr>
</tbody>
</table>

Mean    | 32.4| 135.1      | 5.1 | 11.2| 46.6| 424.8| 26.4|

* Not corrected for hematocrit, since it is distributed in the plasma as well as the cells.
† Not measured, assumed to be 47 per cent.
‡ Poor resolution.

Table III

Nucleotides in normal female blood

Concentration expressed as micromoles of compound per liter of whole blood and corrected to hematocrit of 42 per cent red cells.

<table>
<thead>
<tr>
<th>Subject</th>
<th>DPN</th>
<th>Uric acid*</th>
<th>AMP</th>
<th>TPN</th>
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</table>

Mean    | 32.4| 135.1      | 5.1 | 11.2| 46.6| 424.8| 26.4|

* Not corrected for hematocrit, since it is distributed in the plasma as well as the cells.
not certain. Table II includes some replicate determinations in two subjects. Some of the variability may be physiological and some analytical.

At one stage of this investigation it was thought that time could be saved by pipetting the blood sample into the trichloroacetic acid and freezing the mixture. The rest of the extraction was then performed when convenient. Every sample run in this way showed a depressed ATP peak and an elevated ADP peak. This procedure was therefore abandoned. In similar vein, Willoughby and Waisman (4) noted that when blood was lysed before extraction, the ADP rose and the ATP tended to fall.

In none of the chromatograms to date has there been evidence of inosinic acid. This compound has been reported (3) as occurring in stored blood although not in fresh normal blood. This compound is eluted between TPN and ADP and can be quite easily resolved in either the gradient elution system (3) or our system. To detect the presence of inosinic acid in another way, two blood extracts were poured onto Dowex 1-formate columns and washed with water in the usual manner. The adsorbed nucleotides were then eluted with 4 M formic acid-1 M ammonium formate. This eluate was taken to dryness and hydrolyzed with HClOd. After neutralization, the supernatant was poured onto a column of Dowex 50, H+. Elution was started with 1.5 M HCl. There was a small peak in the position for hypoxanthine, which gave an ultraviolet spectrum characteristic of that compound. For this reason one would not like to state that inosinic acid (or other hypoxanthine nucleotides) are completely absent from the blood, although certainly the amount in freshly drawn blood must be negligible.

Since there are fairly large quantities of nucleotides in the blood, we wondered whether free purines or pyrimidines or their nucleotides might also be present in the blood. The presence of such compounds would suggest possible metabolic pathways. Since these compounds are not adsorbed by Dowex 1-formate it was decided to fractionate the material from blood extracts which was not adsorbed by such columns. The water wash from two columns was combined and freeze-dried. This nonsorbing material was fractionated on 20 × 1-cm. columns of Dowex 50, H+ form, by the method of Wall (11). Only two very small peaks could be seen. The first peak had no distinct spectrum of a purine or pyrimidine; the second could have been hypoxanthine or inosine. No cytosine, adenine, or guanine was found.

It might be of interest to point out that although Wall reported that uracil could be eluted from this type of column with 1.5 M HCl, we have found that uracil will not adsorb to this column if the column is thoroughly washed with water before elution with acid is begun. Therefore we cannot be certain that uracil (or uridine) is absent from the blood extract, although this is probably the case. The behavior of the various ribonucleosides is quite similar to that of the free bases except that they are not held as tightly by the column. Both the free bases and their ribonucleosides elute in the order given by Wall, but at lower acid concentration than he reports.

From these fractionations one can conclude that virtually all of the acid-soluble purines and pyrimidines of whole blood are present as their nucleotides. Of course uric acid must be excepted from this statement.

6 In all blood samples processed to date, the ratio, ADP/(ADP + ATP), has been approximately 0.1. In the blood samples frozen with trichloroacetic acid this ratio was at least doubled.

**DISCUSSION**

From the data in Tables II and III it can be seen that the following compounds occur in whole human blood in relatively constant amounts: DPN, TPN, AMP, ADP, ATP, GTP, and uric acid. It is perhaps of interest to point out that the variability of DPN, TPN, and ATP is nearly identical in both the male and female subjects, whereas the variability of most of the other constituents differs more widely.

It has been generally accepted that nucleotides do not cross cell membranes and therefore are not found in the plasma. Fig. 1B indicates that with the exception of uric acid, virtually none of the compounds studied here is to be found in the plasma. This is in agreement with the data of Willoughby and Waisman (4). Uric acid is of course not a nucleotide.

In 1952, Rottino et al. (2) reported that the amount of ATP in whole human blood was 6.0 mg. of adenine per 100 ml. of blood, and that the amount of AMP plus ADP adenine was 0.78 mg. Those figures recalculated as micromoles of compound per liter of whole blood (but without hematocrit correction) are 444 and 57, respectively. These values, procured by enzymatic methods, are remarkably similar to our values of 433 and 54 (male) and 425 and 52 (female), respectively.

The establishment of the average concentration and variability of the nucleotides in human blood is a necessary prerequisite to the study of these compounds in pathological situations. The exact role of each compound cannot be completely elucidated at present but the mature, non-nucleated, mammalian red cell has certain peculiar functions as a cell. Most important, it must maintain hemoglobin in the reduced state. The citric acid cycle is essentially inactive (12), and the hexose monophosphate shunt assumes greater importance than is usually the case with the other cells.

The presence of adenine compounds in blood has been known for many years, but the presence of GTP has not been well established. No other guanine compounds were found in this study. Guanine nucleotides have been reported in other tissues such as blood platelets (18), bovine lens and cornea (14), barley and oat plants (15), and cerebral tissue (16). Cytosine and uracil nucleotides such as CDP-choline and UDP-glucose should not be unexpected since their metabolic importance is already recognized. The absence of appreciable amounts of inosinic acid in freshly drawn blood is interesting.

**SUMMARY**

By the use of Dowex 1-formate columns and a system of non-gradient elution the following nucleotides have been found in normal human blood: diphenophosphoryl nucleotide, triphosphoryl nucleotide, adenosine mono-, di-, and triphosphate, and guanosine triphosphate. Their mean concentrations and variability have been calculated for a series of ten male and eight female subjects.

Inosinic acid was not found in freshly drawn blood.

With the exception of uric acid, all of the purines and pyrimidines in whole human blood seem to be present as nucleotides, with none appearing as free bases or ribonucleosides.

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Charles Bishop, David M. Rankine and John H. Talbott


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