Acid-soluble Nucleotides in Bovine Thrombocytes*

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In connection with our studies on bovine aplastic anemia (2-4), it became important to determine the nature and approximate amounts of the major acid-soluble nucleotides in blood cells. A survey of the literature revealed only meager information on nucleotides in human thrombocytes (5-8) and only one report dealing (6) with another species. The presence of adenosine diphosphate and adenosine triphosphate in human thrombocytes has been recorded (5-8). Pantl and Ward (7) found, in addition, adenine, guanine, cytosine, and uracil in hydrolysates of acid-soluble components of human thrombocytes in which these compounds were said to be present as ribose phosphates in undetermined combinations. Born (6) used the firefly test to establish the presence of adenosine triphosphate in the thrombocytes of pigs' blood.

This paper presents the results of an ion exchange analysis of the acid-soluble nucleotides of bovine thrombocytes.

EXPERIMENTAL PROCEDURE

Preparation of Acid-soluble Extracts—Blood was obtained at slaughter from 10 Holstein calves judged to be normal by clinical and hematological examinations. Thrombocyte concentrates were prepared, under refrigeration, from 2 liters of blood according to the method previously described (3). The average recovery of the thrombocytes in the concentrates was 59%. An arbitrary value of not more than one erythrocyte per 5000 thrombocytes was chosen as the minimal standard of purity of the concentrates. Contamination from the leukocytes was always less than this, and presumably negligible in these preparations.

Acid-soluble extracts were prepared by treating 1 volume of thrombocyte concentrate with 2 volumes of cold 1.5 m HClO₄, centrifuging, and washing off the extract. The precipitate was washed once with cold 0.6 m HClO₄. The extract and washing were combined and adjusted to pH 8 with 12 m KOH, the KClO₄ precipitate was removed by centrifugation, and the clear extract used for chromatography. These extracts were kept frozen at −20° until the analyses were started.

Chromatography—The extract was placed on an anion exchange column (Dowex 1-X8, 200 to 400 mesh, 25 cm × 0.78 cm², formate form) (9, 10). The column was then washed with distilled water until the absorbancy of the effluent was zero.

The nucleotides were eluted by three linear gradient systems (11), applied in succession, with the eluants suggested by Hurlbert et al. (10). With the first elution mixture, collected in Fractions 1 to 90, the concentration of formic acid was increased from 0 to 0.5 m. With the second elution mixture, collected in Fractions 91 to 170, the concentration of formic acid was increased from 0.5 to 4 m. In the third elution mixture, collected in Fractions 171 to 400, the concentration of ammonium formate 14 m formic acid was increased from 0 to 0.8 m. The flow rate was about 0.3 ml per minute, and fractions of 110 drops were collected with the aid of a fraction collector. The nucleotides in the eluates were located by ultraviolet absorption at 290 mμ, and preliminary identification was made through the order of emergence from the chromatographic column and the ratio of absorbancies measured at 275 and 260 mμ. Appropriate fractions were pooled, formic acid and ammonium formate were removed under reduced pressure by distillation and sublimation in a closed system in which the extracts were at room temperature while a condenser was cooled with liquid nitrogen. The nucleotides from each pooled fraction were further studied by rechromatography on anion exchange resin, by paper chromatography (12, 13), by paper chromatography of the bases obtained after hydrolysis for 1 hour at 100° with 12 m HClO₄ (14), total phosphorus analyses (15, 16), and by tests for the following carbohydrates or their derivatives: deoxypentose (16-19), N-acetyl-amino sugars (20), amino sugar (21), and uronic acid (22). Urease (23) was used in attempts to identify uric acid, and the differentiation between the pyrimidine nucleotides was made with the use of rabbit muscle lactic dehydrogenase (24), which reacted with TPN at less than 1% of the rate observed with DPN (25).

RESULTS

The following carbohydrates and carbohydrate derivatives were not detectable in the extracts: deoxypentose: (indole reaction (19), cysteine-sulfuric acid reaction (19)), and bromination followed by diphenylamine reaction (19)); N-acetyl-amino sugars (aniline acid phthalate reaction (21)); uronic acids (carbazole reaction (22)). These tests, in conjunction with a positive orcinol test give presumptive evidence that the carbohydrate moiety of the nucleotides is a pentose, although the orcinol test is not absolutely specific (26).

Fig. 1 shows a typical pattern of emergence of the nucleotides. In addition to the main fractions which are further described in Table I, bovine thrombocytes contain several minor components in the nucleotide fraction as revealed by increased absorbancies in Tubes 135 to 138, 152 to 156, 223 to 236, and 300 to 303, re-
Nucleotides in Thrombocytes

**Fig. 1.** Emergence pattern of nucleotides from Dowex 1-X8 column. F.A., formic acid; Am.F., ammonium formate.

<table>
<thead>
<tr>
<th>Property</th>
<th>Fraction</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I fast</th>
<th>I slow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rp of nucleotide</td>
<td>A</td>
<td>0.40</td>
<td>0.32</td>
<td>0.40</td>
<td>0.48</td>
<td>0.98</td>
<td>0.25</td>
<td>0.05</td>
<td>0.20</td>
<td>0.05</td>
<td>0.06</td>
</tr>
<tr>
<td>Rp at pH 2, mm</td>
<td></td>
<td>280</td>
<td>280</td>
<td>288</td>
<td>288</td>
<td>257</td>
<td>255</td>
<td>255</td>
<td>255</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>Rp at pH 2, mm</td>
<td></td>
<td>242</td>
<td>240</td>
<td>240</td>
<td>236</td>
<td>240</td>
<td>230</td>
<td>235</td>
<td>235</td>
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<tr>
<td>Rp at pH 12, mm</td>
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<td>270</td>
<td>270</td>
<td>280</td>
<td>280</td>
<td>235</td>
<td>255</td>
<td>255</td>
<td>255</td>
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<td>255</td>
</tr>
<tr>
<td>Rp of base a</td>
<td></td>
<td>245</td>
<td>250</td>
<td>250</td>
<td>282</td>
<td>240</td>
<td>240</td>
<td>240</td>
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<td>240</td>
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<td>Probable identity</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Approximate identity</td>
<td></td>
<td>CDP/E</td>
<td>CMP</td>
<td>DPN</td>
<td>AMP</td>
<td>ADP</td>
<td>e GDP</td>
<td>ATP</td>
<td>GTP</td>
<td>UTP</td>
<td></td>
</tr>
<tr>
<td>Amount, moles per 10^9 thrombocytes</td>
<td></td>
<td>2.5 ± 0.18</td>
<td>1.2 ± 0.03</td>
<td>0.17 ± 0.16</td>
<td>1.4 ± 0.16</td>
<td>22.0 ± 0.16</td>
<td></td>
<td>1.0 ± 0.16</td>
<td>56.9 ± 4.8</td>
<td>4.8 ± 0.76</td>
<td>6.2 ± 0.74</td>
</tr>
</tbody>
</table>

**Notes:**

- Fraction A contained in addition a ninhydrin-positive component.
- Fraction C upon hydrolysis afforded a second base with *Rp* of 0.30 which fluoresced under ultraviolet light.
- Applies to base with *Rp* 0.38.
- Cytidine diphosphoethanolamine.
- Probably an adenosine derivative.
- Mean of 10 specimens ± standard error of the mean.

spectively. The small concentration of these compounds in the extracts did not permit identification.

The main criteria that served for tentative identification of the major components and the conclusions drawn therefrom are summarized in Table I. The following additional observations are pertinent. Fraction A gave a positive ninhydrin test; hydrolysis with 1 N HCl at 100° for 30 minutes produced a ninhydrin-positive compound which, on paper chromatography with phenol-water (25:25, volume for volume), had *Rp* of 0.34 similar to phosphoethanolamine.

Fraction C contained a compound with an absorption spectrum of the pyridine nucleotides; it formed a complex with KCN as evidenced by appearance of maximal absorbancy at 327 mm. When Fraction C was treated with lactate and lactate dehydrogenase from rabbit muscle, the absorbancy at 340 mm increased indicating the formation of DPNH. The presence of a pyridine nucleotide was also suggested by the presence of a substance in the acid-hydrolyzed fraction that fluoresced in ultraviolet light and had *Rp* of 0.30 in paper chromatography (14).

Fraction F had an absorption spectrum similar to adenosine and yielded upon acid hydrolysis a compound with *Rp* 0.36, similar to adenine. The small amounts of substance available precluded further identification.

Fraction I was separated by rechromatography on Dowex 1 resin into a faster moving component with properties of GTP and a slower moving component with properties of UTP.

The enzymatic procedure provided no evidence for the presence of uric acid in the thrombocyte concentrates.

The amounts of the individual nucleotides listed in Table I were calculated from their extinction coefficients at 260 mm (27) and on the basis of the cell counts of the thrombocyte concentrates. If the "normal" thrombocyte content of blood from calves is considered to be about 800,000 per cu mm (2), the
quantities of the nucleotides shown in Table I represent those present in the thrombocytes from about 125 ml of normal blood. For the triplicates this estimate may be low inasmuch as some hydrolysis may have occurred during preparation of the thrombocyte concentrates, in spite of constant refrigeration.

The extremely low thrombocyte counts observed in blood from calves with aplastic anemia (2) precluded the preparation of satisfactory concentrates from this source.

DISCUSSION

Our results provide evidence for the presence of at least 10 nucleotides in thrombocyte concentrates prepared from normal calf blood. Nucleotide analyses were made on acid-soluble extracts from erythrocytes from the same blood specimens and it was calculated that the erythrocyte contribution to the nucleotide content of the thrombocyte concentrates is less than 0.1%.

No deoxypentoses were detected in these thrombocytes by the procedures used which is consistent with the earlier observation that DNA cannot be found in the bovine thrombocytes (3).

The relatively high concentrations of ADP and ATP in comparison to the other nucleotides is in accord with the observations of Bestetti and Crosti (5) who reported that adenosine is the main purine or pyrimidine base in acid-hydrolyzed human thrombocytes. In contrast to Fantl and Ward (7) who observed a mole ratio of 5:2 between ADP and ATP in the acid-soluble nucleotides of human blood, this ratio in the extracts of our specimens was about 2:5; moreover, these contained, per unit number of cells from 4 to 8 times the amounts of acid-soluble derivatives of adenosine, guanosine, and pyrimidines which were found in human thrombocytes (7). In his assays for ATP, Born (6) found a higher concentration in the thrombocytes of pigs than in those of humans; his values for the latter are also greater than those reported by Fantl and Ward (7).

Cytidine diphosphoethanolamine has been reported in acid-soluble extracts of rat and hen liver (28) and in calf thymus gland (29). The occurrence of this compound in bovine thrombocytes is of interest in view of its metabolic role in the biosynthesis of phosphatides (28, 30) of which thrombocytes contain relatively high concentrations (29, 31). Wallach et al. (32), through paper chromatography of phospholipids from human thrombocytes, found evidence for the presence of phosphatidylethanolamine. This compound has been implicated (33) as one of the platelet factors involved in blood clotting (34).

Analyses for acid-soluble nucleotides have been reported for a wide variety of tissues, including liver, thymus, and intestinal mucosa. Recent evidence has shown that thrombocytes originate from the fragmentation of the cytoplasm of the megakaryocytes (35), and it is of interest that these formed blood elements of cytoplasmic origin contain most of the nucleotides reported in extracts of whole tissues.

SUMMARY

Compounds with properties similar to the following nucleotides are present in thrombocyte concentrates prepared from normal calf blood: cytosine diphosphoethanolamine, cytidine monophosphate, adenosine monophosphate, adenosine diphosphate, guanosine diphosphate, adenosine triphosphate, uridine triphosphate, guanosine triphosphate, and diphosphopyridine nucleotide. Of these compounds, adenosine triphosphate is present in the largest amount.

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REFERENCES

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