166. 3-*Ribosyluric Acid.** *Part II.*[1] *Isolation of the Corresponding Nucleotide from Beef Blood.*

By Dolph Hatfield, Robert R. Rinehart, and H. S. Forrest.

The isolation from beef blood, in very small quantity, of a new uric acid-containing compound is described. Evidence is presented that it is (3-ribosyluric acid)-5′ phosphate.

In Part I of this series,[1] a simple method was described for the isolation of 3-ribosyluric acid from beef blood. During subsequent isolations of this material, it became apparent that a second uric acid-containing compound (as judged on the basis of the colour reaction with *N*,2,6-trichlorobenzoquinone imine, the reagent used for the detection of uric acid and its derivatives) was present in the blood in very much smaller quantity. From its R_F values, it appeared that this compound might be the phosphate ester of 3-ribosyluric acid. Accordingly, since such a compound might be expected to be readily hydrolyzed enzymically in whole blood, a modification of the earlier isolation procedure for 3-ribosyluric acid was devised to yield the maximum amount of the suspected nucleotide and to minimize its loss by enzymic degradation. By this modified procedure, the desired compound was obtained, although in very small amount. (It occurs in beef blood to the extent of about 0·5% of the amount of 3-ribosyluric acid.) Structural determinations described below revealed that it is indeed (3-ribosyluric acid)-5′ phosphate.

Basically, the differences in the new procedure from that used previously involved (1) the immediate addition of sodium fluoride (a potent inhibitor of phosphate-cleaving enzymes) to the fresh beef blood, and (2) preliminary concentration of acids by treatment of an aqueous-acetone extract of red blood cells with Dowex 1 in the hydroxide form. Elution of the adsorbed acids from the resin was accomplished with 98% formic acid, and addition of the eluate to an excess of ether gave a flocculent precipitate which could then be resolved into its components, after dissolution and neutralization, on a preparative Dowex 1 column. From this column, in addition to a relatively large amount of 3-ribosyluric acid, there was obtained a fraction containing a compound with an absorption spectrum very similar to that of 3-ribosyluric acid, but with different R_F values in a number of solvents. This material was further purified by paper chromatography and was freed from impurities from the paper by absorbing it on, and eluting it from, a Dowex 1 column in the way described above for its concentration from the beef-blood extracts. The white solid obtained by ether-precipitation from the formic acid eluate of the column was chromatographically homogeneous and was used for most of the structural determinations.

Treatment of a buffered solution of the unknown compound with an alkaline phosphatase preparation caused its breakdown into a single compound which was indistinguishable, chromatographically or spectrophotometrically, from 3-ribosyluric acid. This observation, together with the absorption spectrum of the compound (see

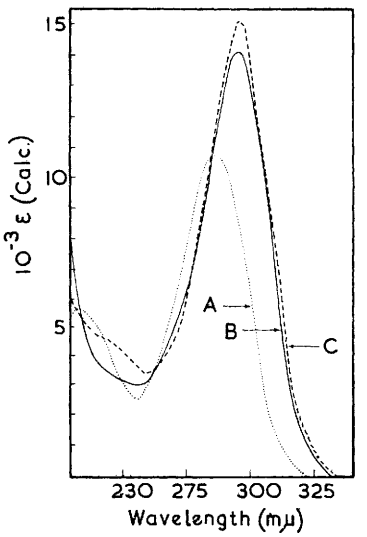
* In Part I, the old name, uric acid riboside, was used, but in this and future publications, the more correct name, 3-ribosyluric acid, will be used.

[1] Part I, Forrest, Hatfield, and Lagowski, *J.*, 1961, 963.

Figure), clearly indicated that the new compound was a derivative of 3-ribosyluric acid. Further evidence on its structure was obtained by estimation of the proportions of phosphate and sugar residues per mole of uric acid calculated from the absorption spectrum. The three residues were found to be present in the molecule in a 1 : 1 : 1 ratio. Finally, the position of the phosphate group on the ribosyl portion was unequivocally demonstrated by the fact that the compound reacted with an equimolar amount of sodium metaperiodate. The only possible place of attachment of the phosphate group is, therefore, the 5′-position.

Thus, although insufficient material has been isolated to perform elementary analyses, the above observations provide conclusive evidence that this component of beef blood is (3-ribosyluric acid)-5′ phosphoric acid.

Other experiments [2] have shown that this phosphate ester is probably the first product in the biosynthesis of 3-ribosyluric acid from uric acid, the phosphate group being then



Ultraviolet absorption spectra of (3-ribosyluric acid)-5′ phosphate at pH 1 (A), pH 14 (B), and pH 7 (C). The extinction coefficients are calculated from the known values for 3-ribosyluric acid.[1]

lost by enzymic hydrolysis to yield 3-ribosyluric acid. The hypothesis that 3-ribosyluric acid is a more soluble " carrier " for uric acid still seems to be the best explanation for the occurrence of these compounds in beef blood. Blood from a number of other animals has been examined for the presence of 3-ribosyluric acid,[2] but it has been found only in the buffalo's blood which also contains (3-ribosyluric acid)-5′ phosphate. The metabolic peculiarity which gives rise to these compounds, apparently only in cattle and buffalo, still awaits elucidation.

Experimental

Isolation of 3-Ribosyluric Acid and (3-Ribosyluric Acid)-5′ Phosphate.—Fresh defibrinated beef blood (2·7 l.) was treated with sodium fluoride (*ca.* 35 mg.) and chilled as quickly as possible. The red blood cells were collected by centrifugation, then water (800 ml.) and acetone (2 l.) were added consecutively with stirring to the packed cells. The bulk of the precipitated material was collected by filtration through cloth and the precipitate was washed with water (700 ml.) and again collected on cheese cloth. The combined filtrates were then passed through a pad of Celite supported on filter paper. The clear filtrate was stirred for 20 min. with a thick slurry in water of Dowex 1 anion-exchange resin (100—200 mesh; 20 ml.) in the hydroxide form. The resin was collected on a chromatographic column and freed from the excess of solvent by suction. 98% Formic acid (40 ml.) was added to the column in increments of about 5 ml. each, and the effluent was allowed to drop into anhydrous ether (250—300 ml.). After storage for

[2] Hatfield and Forrest, *Biochim. Biophys. Acta*, 1962, **62**, 185.

16 hr. at 4°, the flocculent precipitate was collected and combined with precipitates obtained from two similar preparations, and with one obtained from a final treatment with Dowex 1 (25 ml.), in the hydroxide form, of the combined aqueous-acetone extracts from all three.

The ether-insoluble precipitates (representing an extract from 8 l. of blood) were dissolved in water (2 l.), the pH was adjusted to 8·5 with 1% aqueous ammonia, and the solution passed through a column (2·7 × 13 cm.) of Dowex 1 anion-exchange resin (100—200 mesh) in the formate form. After the column had been washed with water and 0·01M-formic acid (500 ml.), material absorbing at 310 mμ and shown to be identical with 3-ribosyluric acid by paper chromatography was mostly eluted with 0·02M-formic acid (1500 ml.). Its removal from the column was completed with 0·05M-formic acid (500 ml.). Addition of more concentrated formic acid (0·1M, 500 ml.; 0·5M, 500 ml.) caused no further elution of ultraviolet-absorbing materials, nor did successive elutions with mixtures of 0·05M-ammonium formate and 0·01M-formic acid (500 ml.), 0·05M-ammonium formate and 0·1M-formic acid (500 ml.), and 0·1M-ammonium formate and 0·1M-formic acid (1 l.). Finally, additional absorbing material (as measured at 290 mμ) was eluted with a mixture of 0·15M-ammonium formate and 0·15M-formic acid (1500 ml.).

The pooled fractions containing 3-ribosyluric acid were adjusted to pH 8 with 1% aqueous ammonia and passed through a column (1 : 2 × 5 cm.) of Dowex 1 in the acetate form. 3-Ribosyluric acid was retained on this column and was subsequently eluted from it with 0·1N-acetic acid (500 ml.). Concentration of the eluate *in vacuo* to a volume of 40 ml. caused incipient crystallization of 3-ribosyluric acid. After several days at 4°, the solution was filtered to yield *ca.* 400 mg. of the product as white needles.

The pooled fractions from the Dowex column containing the suspected nucleotide were adjusted to pH 8 with 1% aqueous ammonia and treated with a slurry in water of Dowex 1 (20 ml.) in the hydroxide form which completely adsorbed all the ultraviolet-absorbing material. Again, this material was recovered by eluting it from the resin with 98% formic acid and allowing the eluate to drop into anhydrous ether. The resulting white precipitate was collected by centrifugation and redissolved in water (4 ml.), and the solution adjusted to pH 8 with aqueous ammonia (*d* 0·88). This solution was then chromatographed on filter paper (Whatman, No. 3MM, washed previously with 5% acetic acid; 46 × 57 cm.; 2 sheets; descending) with propan-1-ol–1% ammonia (7 : 3). The desired compound was located on the developed chromatogram by its absorption of ultraviolet light (Mineralight lamp) and by its colour reaction on a representative strip with *N*,2,6-trichlorobenzoquinone imine. The material was eluted from the paper with water and rechromatographed twice with propan-1-ol–water (3 : 2, ascending; and 7 : 3 descending; consecutively). The product obtained from the final chromatogram was used for some of the degradative tests described below. However, for others, it was necessary to remove the inevitable carbohydrate impurities which arise in paper-chromatographic purification, and for this purpose, the combined Dowex column–ether precipitation procedure was used to obtain a solution of the pure compound.

The yields of the two products calculated from the optical densities of the preparative column effluents were: 3-ribosyluric acid, 460 mg.; new compound, 6 mg. (yield after final purification, *ca.* 2 mg.).

Paper Chromatography.—R_F values in various solvents for the new compound, isolated as described above, are as follows: Pr^nOH–1% aq. NH_3 (3 : 2; ascending), 0·20; NMe_2·CHO–Bu^nOH–H_2O (1 : 1 : 1; ascending), 0·41; Bu^nOH–AcOH–H_2O (4 : 1 : 1; ascending, 0·04; Pr^nOH–H_2O (7 : 3; descending), 0·19. R_F values for 3-ribosyluric acid and uric acid in most of these solvents have been reported previously.[1] The compound was detected either by its absorption of ultraviolet light (lamp with maximum emission at 260 mμ), or by its colour reaction with *N*,2,6-trichlorobenzoquinone imine, which was essentially identical with that exhibited by 3-ribosyluric acid.

Action of Alkaline Phosphatase.—A small amount of the solution containing the new compound was adjusted to pH 9 and treated with alkaline phosphatase (Nutritional Biochemicals Corporation). Test samples submitted to paper chromatography showed that the compound was degraded completely, with time, into a uric acid-containing compound that was indistinguishable from 3-ribosyluric acid paper chromatographically or spectrophotometrically. This was the only ultraviolet-absorbing material formed by the catalytic action of the enzyme.

Estimation of Phosphate.—The quantity of the presumed nucleotide in an aliquot part of the purified aqueous solution was calculated from the extinction coefficient [1] of 3-ribosyluric acid.

Phosphate was then determined by the method of Fiske and SubbaRow [3] as described by Leloir and Cardini.[4] From 125 μg. of the compound 34·4 μg. of phosphate were obtained (theory, 31·6 μg.).

Estimation of Ribose.—The ribose present in another aliquot part of the most highly purified material was determined by using the orcinol reagent.[5] From 28 μg. of the compound, 12 μg. of sugar calculated as ribose were obtained (theory, 11 μg.).

Periodate Oxidation.—The spectrophotometric micro-technique of Dixon and Lipkin [6] was used to determine the uptake of sodium metaperiodate in an aliquot part of the highly purified sample; 0·9 mol. of oxidant was consumed in 10 min., after which there was no further uptake.

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GENETICS FOUNDATION, THE UNIVERSITY OF TEXAS,
AUSTIN 12, TEXAS, U.S.A. [*Received, August 8th,* 1962.]

[3] Fiske and SubbaRow, *J. Biol. Chem.*, 1925, **66**, 375.
[4] Leloir and Cardini in "Methods in Enzymology," Vol. III, eds. Colowick and Kaplan, Academic Press, Inc., New York, 1955, p. 843.
[5] Ashwell, ref. 4, p. 87.
[6] Dixon and Lipkin, *Analyt. Chem.*, 1954, **26**, 1092.