199. *Uric Acid Riboside.* Part I. Isolation and Reinvestigation of the Structure.*

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Crystalline uric acid riboside has been isolated from beef blood, and its properties have been compared with those of 3-, 7-, and 9-methyluric acid. It is concluded that the compound is actually uric acid-3 riboside and not uric acid-9 riboside.

Between 1933 and 1939, Gulland and his co-workers compared the ultraviolet absorption spectra of the naturally occurring purine ribosides and deoxyribosides [1] with those of model compounds and concluded that in all cases the ribosyl or deoxyribosyl moiety was attached to the 9-position of the purine ring. For most of these compounds, the position of attachment has been confirmed by synthetic and/or other evidence. One of the compounds examined by Falconer and Gulland [1] was uric acid riboside which was obtained from beef blood and beef liver by a modification of the method of Davis, Newton, and Benedict.[2] The ultraviolet absorption spectrum of this compound, when compared with those of 1-, 3-, 7-, and 9-methyluric acid, closely resembled that of 9-methyluric acid, and it was concluded that the ribosyl group in this compound, as in the other naturally occurring ribosides, was attached to the 9-position. However, there has been no independent confirmation of the structure of uric acid riboside.

In a note appearing in 1952, Carter and Potter [3] reported the isolation from beef blood

* To avoid further confusion, the name uric acid riboside has been retained and used throughout this paper; ribosyluric acid is, however, the preferred name, and uric acid ribonucleoside could also be used. H. S. F.

[1] Falconer and Gulland, *J.*, 1939, 1369.
[2] Davis, Newton, and Benedict, *J. Biol. Chem.*, 1922, **54**, 595.
[3] Carter and Potter, *Fed. Proc.*, 1952, **11**, 195.

of a uric acid riboside by a simpler method. They noted that its ultraviolet absorption spectrum was not identical with that given by Falconer and Gulland. No explanation was offered for this discrepancy. After noting that the absorption spectrum could be reconciled with that to be expected if the ribosyl group was in the 3- or 7-position of the purine ring, they evidently retained the belief that the ribosyl group was in the 9-position and suggested that the anomalous spectrum was possibly due to an interaction between the ribose moiety and the oxygen on position 8 of the purine ring. Leone [4] has also isolated a uric acid riboside from beef blood by a method somewhat similar to that of Falconer and Gulland; the ultraviolet absorption spectrum of his compound is apparently very similar, qualitatively at least, to that of the material isolated by Carter and Potter.

In an attempt to resolve the contradictions in the published data on uric acid riboside, we have isolated this compound from beef blood by a simple method and have reinvestigated some of its properties. The conclusion drawn from this work is that the uric acid riboside isolated by Carter and Potter, by Leone, and by us is not the 9-riboside but is, in fact, the 3-riboside. The evidence for this is based on comparison of the isolated material with 3-, 7-, and 9-methyluric acid by a number of techniques, including ultraviolet spectrophotometry.

The compound was isolated from an aqueous-acetone extract of beef red-blood cells by using a Dowex 1 anion-exchange column and crystallized from a gelatinous aqueous solution as colourless needles. From the elementary analysis, it appears that the compound contains 1·5 mol. of water of crystallization, only one of which is lost at 100° *in vacuo*: the only other published analysis of uric acid riboside [1] is a nitrogen analysis which corresponds to that for an anhydrous compound. A significant difference was also found in the optical rotation of our material, which had $[\alpha]_D^{25}$ −19·8° as compared with Falconer and Gulland's value [1] of $[\alpha]_D^{25}$ −40·8°, the conditions of measurement and solvent being approximately comparable.

When subjected to the action of a nucleosidase preparation [5] from the bacterium *Lactobacillus pentosus*, the compound was degraded to uric acid and a sugar, which was quantitatively estimated by Nelson's method [6] as used by Wang,[7] and shown to be identical with ribose by paper chromatography. As would be expected for a ribofuranoside, the compound consumed one mol. of sodium metaperiodate. All these results indicate that our compound is a uric acid riboside.

Evidence for the position of attachment of the ribosyl group was obtained by comparison of the isolated material with uric acid derivatves (3-, 7-, and 9-methyl- and 9-2′-hydroxyethyl-uric acid) in as many different ways as could be devised. All of these compounds and uric acid give colour reactions with *N*,2,6-trichlorobenzoquinone imine [8] which appear to be characteristic of the unsubstituted compound and of the position of nitrogen-substitution. The colour can be developed by spraying the reagent on paper chromatograms, and the test is made more sensitive by subsequent spraying with a slightly alkaline buffer. The colour reactions given by the various compounds are recorded in Table 1. Uric acid riboside gives a yellow colour with this reagent which is similar to that given by 3-methyluric acid and is distinguishable from those of all the other compounds.

Comparison of the ultraviolet spectrum of uric acid riboside with those of the model compounds at different pH values (Fig. 1 and Table 2) confirmed the close structural resemblance between this compound and 3-methyluric acid and revealed obvious differences from 9-methyluric acid, though the spectra of all the compounds are rather similar. From the data available,[4,9] it appears that Carter and Potter's compound and Leone's compound

[4] Leone, *Boll. Soc. ital. Biol. sper.*, 1955, **31**, 622.
[5] Lampen and Wang, *J. Biol. Chem.*, 1952, **198**, 385.
[6] Nelson, *J. Biol. Chem.*, 1944, **153**, 375.
[7] Wang in "Methods in Enzymology," Vol. II, ed. Colowick and Kaplan, Academic Press, Inc., New York, 1955, p. 456.
[8] Berry, Sutton, Cain, and Berry, *Univ. Texas Publ. No.* 5109, 1951, 22.
[9] Unpublished spectrum supplied by Dr. C. E. Carter.

have the same ultraviolet absorption spectrum as the uric acid riboside described in this paper.

Uric acid is known to decompose rapidly in alkaline solution,[10] and 3-, 7-, and 9-methyluric acid behave similarly. This provided an additional method of comparison, since the rates of decomposition could be conveniently followed by measuring the decrease in ultraviolet absorption at the maxima of the various compounds with time. Thus,

TABLE 1. *Summary of chromatographic data on uric acid derivatives* [a] *in various solvents.*

Compound	Propan-1-ol–1% aq. NH_3 (3 : 2)	$NMe_2 \cdot CHO$–Bu^nOH–H_2O (1 : 1 : 1)	Bu^nOH–AcOH–H_2O (4 : 1 : 1)	Bu^sOH–$H \cdot CO_2H$–H_2O (8 : 2 : 5)
Uric acid [b]	0·37, Y–O, O	0·51, Y–O, O	0·17, Y–O, O	0·42, NC, O
Uric acid riboside	0·48, R–P, Y	0·63, R–P, Y	0·16, NC, Y	0·53, NC, Y
3-Methyluric acid	0·47, NC, Y	0·60, NC, Y	0·28, NC, Y	0·54, NC, Y
7-Methyluric acid	0·48, R–B, R–B	0·60, Y–B, R–B	0·32, Y–B, R–B	0·64, NC, R–B
9-Methyluric acid	0·48, Y, Y–O	0·59, Y, Y–O	0·26, NC, Y–O	0·41, NC, NC
9-2′-Hydroxyethyluric acid	0·48, Y–B, Y–O	0·57, Y, Y–O	0·21, Y, Y	0·51, NC, Y

[a] R_F values (1st column) and colour reactions with *N*,2,6-trichloroquinone imine before (2nd column) and after (3rd column) spraying with buffer. NC, no colour; Y, yellow; O, orange; R, red; B, brown; P, purple. [b] Leone and Guerritorie (*Boll. Soc. ital. Biol. sper.*, 1950, **26**, 609; *Chem. Abs.*, 1951, **45**, 7621) used different solvent systems to separate uric acid and uric acid riboside.

TABLE 2. *Ultraviolet absorption spectra.*

Compound	pH of solvent	$\lambda_{max.}$ (mμ)	$10^{-3}\varepsilon$	$\lambda_{min.}$ (mμ)	$10^{-3}\varepsilon$
7-Methyluric acid	1	286, 234	11·1, 8·6	258, 214	3·7, 5·0
	7	294, 238	12·1, 10·2	263, 219	3·3, 6·4
	14	297	13·6	263	2·8
3-Methyluric acid	1	288, 233	11·3, 8·3	258, 215	3·2, 5·2
	7	296, 241 (sh.)	16·0, 4·4	262	2·7
	14	292, 245 (sh.)	15·3, 3·2	256	2·6
Uric acid riboside	1	288, 233	12·4, 6·8	256, 220	2·9, 5·7
	7	298, 243 (infl.)	17·7, 2·9	257	2·3
	14	297	16·5	254	1·6
9-Methyluric acid	1	286, 233	13·0, 7·9	256, 215	3·7, 4·7
	7	292, 238	11·9, 9·2	262, 224	2·7, 5·6
	14	299, 250	10·3, 9·9	272, 228	4·5, 4·4
9-2′-Hydroxyethyluric acid	1	287, 233	12·6, 7·5	256, 215	3·3, 4·2
	7	292, 238	11·7, 9·1	263, 224	2·5, 5·6
	14	301, 249	9·9, 9·7	273, 228	3·9, 4·0

TABLE 3. *Calculated* [a] pK_a *values of uric acid derivatives.*

Compound	pK_a values	
Uric acid riboside	6·0	10·9
3-Methyluric acid	6·1	11·3
9-Methyluric acid [b]	5·6	11·3
7-Methyluric acid	5·4	10·6
Uric acid [b]	5·4	11·3

[a] Cf. Fox and Wempen, *Adv. Carbohydrate Chem.*, 1959, **14**, 304. [b] Slightly different values are given by Bergmann and Dikstein (*J. Biol. Chem.*, 1922, **54**, 601).

9-methyluric acid (and 9-2′-hydroxyethyluric acid) decomposed the fastest and 7-methyluric acid the slowest. 3-Methyluric acid and uric acid riboside decomposed in alkali at intermediate, and almost identical, rates (Fig. 2).

Finally, p*K* values for the model compounds and uric acid riboside were calculated by the spectrophotometric method described by Shugar and Fox.[11] Although the evidence (Table 3) is not so clear cut in this case, it is again apparent that the values for uric acid riboside most closely resemble those for 3-methyluric acid.

[10] Griffiths, *J. Biol. Chem.*, 1952, **197**, 399; Albert and D. J. Brown, *J.*, 1954, 2060.
[11] Shugar and Fox, *Biochim. Biophys. Acta*, 1952, **9**, 199.

By these four criteria, the uric acid riboside which we have isolated from beef blood resembles 3-methyluric acid most closely and is quite clearly different from the 7- and the 9-methyl compound. It seems therefore that it should be described as the 3-isomer. We are attempting to provide final confirmation of this by synthetic means.

FIG. 1. *Ultraviolet absorption spectra of* 9-*methyluric acid* (A), *uric acid riboside* (B), 3-*methyluric acid* (C), *and* 7-*methyluric acid* (D). *The spectra are arranged without regard to their specific absorbancies to show clearly the relative positions of the maxima and minima.*

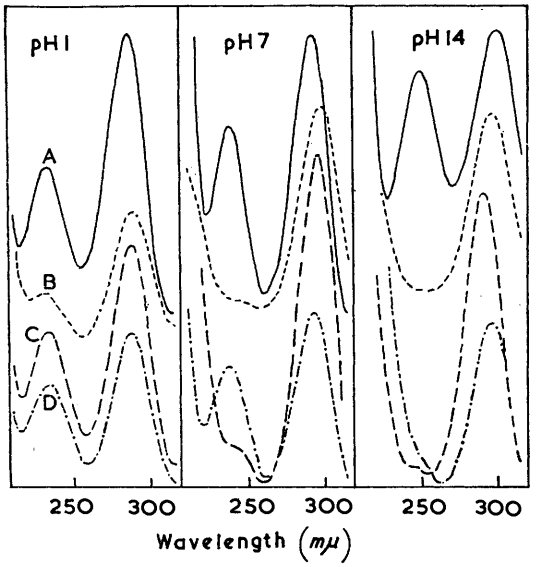
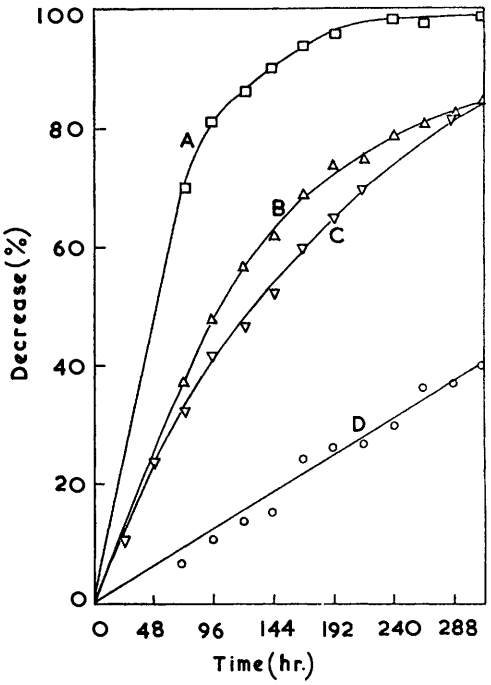


FIG. 2. *Decomposition of* 9-*methyluric acid* (A), *uric acid riboside* (C), 3-*methyluric acid* (B), *and* 7-*methyluric acid* (D), *in* 0·1N-*sodium hydroxide expressed as percentage decrease in heights of the longer-wavelength maxima.*



EXPERIMENTAL

Isolation of Uric Acid Riboside.—Fresh, defibrinated beef blood (2 l.) was centrifuged at 6000 r.p.m. for 15—20 min. The packed red-blood cells were resuspended in physiological saline solution and again collected by centrifugation. Acetone (1·2 l.) and water (120 ml.) were then added to the packed mass, and the whole was heated gently on the steam-bath with stirring until the precipitated solids became dark brown (5—10 min.). An additional quantity of water (500 ml.) was added and most of the solid was removed by filtration through cheese-cloth. The solution was finally clarified by filtration through Celite.

This extract was concentrated to about 250 ml. and again filtered through Celite. The clear filtrate was added to a column (20 × 2 cm.) of Dowex 1 anion-exchange resin in the acetate form. The column was washed with ammonium acetate solution (0·6N-ammonia adjusted to pH 8 with acetic acid; 200 ml.), then with water (500 ml.), and finally with 0·01M-acetic acid (500 ml.). The eluate from the last two washings contained no material absorbing at 310 mμ. Elution was then commenced with 0·02M-acetic acid; after about 350 ml. of this had passed through the column, the absorption of the eluates at 310 mμ began to rise, reached a maximum, and then declined. The fractions containing the absorbing material were combined (800 ml.) and evaporated *in vacuo*. The residue was dissolved in hot water (10 ml.) and the solution set aside, whereupon it became gelatinous. Over a period of days masses of colourless needles separated; these were collected by filtration (50—60 mg.). A further quantity of material (20—30 mg.) was obtained by combining the mother-liquors and end-fractions from the column and repurifying them through a second column.

Small amounts of material (5—10 mg.) were isolated by paper chromatography of the concentrated aqueous acetone extracts on heavy filter paper (Whatman No. 17; 1 rectangular sheet/*ca.* 12 ml. of extract). After development of the chromatogram with butan-1-ol–dimethylformamide–water (1 : 1 : 1), the appropriate band was detected by its ultraviolet absorption or by the use of a *N*,2,6-trichlorobenzoquinone imine spray on a representative strip of the chromatogram, cut out, and eluted with water, and the material was rechromatographed with propan-1-ol–1% aqueous ammonia (3 : 2) as solvent. This gave an amorphous solid which was shown to be about 70% pure by its specific ultraviolet absorption.

The chromatographic technique was used to show that uric acid riboside was the sole uric acid-containing compound present in beef red-blood cells (cf. Benedict [12]); it did not occur in blood serum, which however contained small amounts of uric acid.

A portion of the crystalline material, recrystallized from water for analysis and dried at 25° *in vacuo* over phosphorus pentoxide, had m. p. >350°, $[\alpha]_D^{25}$ −19·8° (*c* 2·13 in 0·1N-NaOH) (Found: C, 36·8; H, 4·6; N, 16·7. $C_{10}H_{12}N_4O_7,1{\cdot}5H_2O$ requires C, 36·8; H, 4·6; N, 17·1. Found, after drying at 120°/2 mm.: C, 39·4; H, 3·9; N, 18·1. $C_{10}H_{12}N_4O_7,0{\cdot}5H_2O$ requires C, 39·0; H, 4·2; N, 18·1%).

Paper Chromatography.—The R_F values of the above material in a variety of solvents are listed in Table 1, with data for uric and methyluric acids.

Periodate Oxidation of Uric Acid Riboside.—Crystalline material was oxidized with sodium metaperiodate, the spectrophotometric microtechnique of Dixon and Lipkin [13] being used to determine the uptake of oxidant. One mol. of oxidant was consumed in 5 min., after which oxidation ceased.

Identification of Cleavage Products from Uric Acid Riboside.—Uric acid riboside (1·44 mg.) was incubated at 37° with a crude enzyme preparation from *L. pentosus* [*Lactobacillus planatarum* (*pentosus*), strain 124-2, ATCC 8041]. After 4 hr. the reducing sugar was quantitatively estimated in aliquot parts of the solution (which were calculated to contain initially 0·24 mg. of uric acid riboside) by the method of Nelson [6] as described by Wang.[7] The yield was 0·100 mg.; theor., 0·106 mg.

In another experiment, after incubation the protein was precipitated by heating its solution at 100° for 3 min. and the clear supernatant liquid was subjected to paper chromatography, with appropriate controls. The sole ultraviolet-absorbing material present was shown to be uric acid (appropriate solvents listed in Table 1), and the reducing sugar was identical with ribose (butan-1-ol–acetic acid–water; 4 : 1 : 1).

The compound was readily hydrolyzed by N-hydrochloric acid (66% after 1 hr. at 100° as measured by the production of uric acid). Falconer and Gulland's results [1] seem to indicate that their compound was much more stable to hydrolysis (6 hr. in 16% sulphuric acid for complete hydrolysis).

Ultraviolet Absorption Spectra.—The ultraviolet absorption spectra of uric acid riboside and the model compounds are shown in Fig. 1. These are arranged arbitrarily to make the similarities and differences among the maxima and minima more easily discernible. The extinction coefficients for these compounds and 9-2′-hydroxyethyluric acid are given in Table 2.

Alkaline Decomposition of Uric Acid Derivatives.—The rates of decomposition of uric acid riboside and 3-, 7-, and 9-methyluric acid in 0·1N-sodium hydroxide at 25°, as measured by the percentage decrease in heights of the longer wavelength maxima, are recorded in Fig. 2. 9-2′-Hydroxyethyluric acid decomposed at the same rate as 9-methyluric acid. No attempt was made to elucidate the mechanism of decomposition of these compounds.

p*K Values.*—The p*K* values of the compounds were determined by the spectrophotometric method,[11] and the results are given in Table 3.

To provide a second example of a 9-substituted derivative, 9-2′-hydroxyethyluric acid was synthesized in an unambiguous manner from 2,4-dihydroxy-6-2′-hydroxyethylaminopyrimidine. In the comparisons described above, it behaved exactly as the 9-methyl compound; therefore, to avoid confusion it was not included in the Figures.

9-2′-Hydroxyethyluric Acid.—5-Amino-2,4-dihydroxy-6-2′-hydroxyethylaminopyrimidine bisulphite compound was prepared from 2,4-dihydroxy-6-2′-hydroxyethylaminopyrimidine (500 mg.) according to Masuda's procedure.[14] Without isolation of the product, the mixture

[12] Benedict, *J. Biol. Chem.*, 1915, **20**, 633.
[13] Dixon and Lipkin, *Analyt. Chem.*, 1954, **26**, 1092.
[14] Masuda, *Pharm. Bull.* (*Japan*), 1957, **5**, 28; McNutt, *J. Amer. Chem. Soc.*, 1960, **82**, 217.

was concentrated *in vacuo* at 25°, 10% aqueous sodium hydroxide (9 ml.) was added, and carbonyl chloride was bubbled through the solution, which was kept in an ice bath, for 0·75 hr.[15] The precipitate, which began to separate immediately on addition of the carbonyl chloride, was isolated by filtration and washed with cold water. The filter cake was dissolved in a minimum of warm 0·1N-sodium hydroxide; on acidification of the warm solution with glacial acetic acid, clusters of white needles separated [146 mg., overall yield 23·6%; m. p. >350° (decomp.)] (Found: N, 26·3. $C_7H_8N_4O_4$ requires N, 26·4%).

Discussion

There is no doubt that we have isolated the major uric acid-containing compound in beef red-blood cells. Two other investigators also appear to have isolated this compound. The question still remains—how did Falconer and Gulland obtain a compound from beef blood (and beef liver) whose ultraviolet absorption spectrum closely resembled that of 9-methyluric acid? We have repeated Falconer and Gulland's experimental procedure as closely as possible, reasoning that possibly some step in this method was causing a rearrangement of the uric acid riboside. We could find no evidence of the appearance of a riboside different from the one we have isolated. There is the possibility that under unusual dietary conditions (*e.g.*, a high-protein diet) uric acid-9 riboside could occur in beef blood, and the most reasonable conclusion, then, would be that Falconer and Gulland, by chance, obtained their blood from cattle under such dietary conditions. An alternative possibility that a different uric acid riboside occurs in different breeds of cattle seems to be scarcely feasible. A fortuitous contamination of uric acid with ribose cannot be ruled out, although the ultraviolet absorption spectral evidence would appear to eliminate this possibility. Further, and contrary to a previous report,[16] we could find no paper-chromatographic evidence that a uric acid riboside occurs in human, chicken, or pig blood.

In beef blood, uric acid riboside occurs only in the red blood cells. Presumably it is used metabolically as a more soluble " carrier " for uric acid from the tissues to the kidney. There is no evidence that it is concerned with or derived from the other purine nucleosides in the biochemical processes in which they are involved. Therefore, it is not unreasonable that its structure should differ from those of the other naturally occurring purine nucleosides and it might even be predicted that in mammalian blood containing this riboside, specific enzymes would be involved in its synthesis and/or degradation. An enzyme preparation obtained from various human tissues and from a number of other mammalian tissues [17,18] is capable of splitting uric acid riboside, but its specificity has not been determined. Obviously this problem warrants further study, especially in view of the structural evidence for uric acid riboside presented in this paper.

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[15] Cavalieri and Bendich, *J. Amer. Chem. Soc.*, 1950, **72**, 2587.
[16] Newton and Davis, *J. Biol. Chem.*, 1922, **54**, 601.
[17] Laster and Blair, *Fed. Proc.*, 1958, **17**, 261.
[18] Laster and Blair, *J. Clin. Invest.*, 1958, **37**, 909.