It is therefore important to consider the evidence relating to the molecular weight of the three types of dextran sulphate preparation used in the biological investigation. The intrinsic viscosity of the dextrans and of the dextran sulphates derived from them indicate corresponding differences in molecular weight. The critical distinction in toxicity is between the *E* type and the *I* type and here the osmotic pressures recorded support the contention that the *I* type is of considerably lower molecular weight than the *E* type, being of the order of 7000.

The anticoagulant activity expressed in units/mg. appears to be independent of the molecular weight but depends on a certain minimum number of sulphate groups per glucose unit. At 1.0–1.3 sulphate groups per glucose unit the sharp increase of activity to its maximum value is indicative of the appearance of some critical pattern of groups in the molecular structure. Heparin has greater activity with considerably fewer sulphate groups in the molecule. From the biological investigations evidence was obtained that the activity of heparin and dextran

sulphate was qualitatively identical, in spite of the sevenfold difference in the weight corresponding with a given amount of anticoagulant activity.

SUMMARY

1. Molecular weight determines the toxicity of dextran sulphate. Molecules smaller than a critical size are not toxic.

2. Maximum blood anticoagulant activity is attained when the number of sulphate groups exceeds an average of 1.3 per glucose unit.

3. Preparation of a potentially useful anticoagulant drug is described.

It is a pleasure to acknowledge here the close and fruitful collaboration of my colleague, Dr Kenneth Walton, whose account of the biological aspects of our investigation will appear elsewhere, and to thank Prof. J. R. Squire for his help and encouragement. Thanks are also due to Prof. M. Stacey, F.R.S., for placing at my disposal the facilities of his Department for the larger scale preparations of dextran sulphate.

I am grateful to Mr M. Hall, for his invaluable help in the laboratory, and to Messrs Dextran Ltd. for gifts of dextran.

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The Occurrence of Substituted Uric Acids in Human Urine

By E. A. JOHNSON

Medical Research Council Spectrographic Unit, London Hospital, London, E. 1

(Received 12 September 1951)

In the course of work on the separation of purines and pyrimidines from biological materials, purine fractions obtained from human urine have been examined. This work is not complete, but as it is being interrupted the results so far obtained are given here.

The purine fractions employed were obtained by ammoniacal silver precipitation of the urine after

phosphate removal using the reagents described by Snell & Snell (1937), and separation was effected on columns of the ion-exchange resin Zeo-Karb 215 by methods similar to those used by Cohn (1949) for the separation of bases from nucleic acids. Uric acid was readily separated from the xanthines, but between these, other well-defined fractions were obtained having ultraviolet absorption spectra scarcely

distinguishable from those of uric acid. These materials were isolated, though in exceedingly small quantities, and were evidently methyluric acids. Confirmation of this was obtained by the complete cessation of the excretion of these materials when the subject abstained from tea, coffee and cocoa, products all containing methylxanthines, and by their appearance in the urine again after ingestion of caffeine and theophylline.

This work therefore extends that of Myers & Hanzal (1946), Buchanan, Block & Christman (1945), Buchanan, Christman & Block (1945) and other workers, well summarized in the papers of these authors, on the metabolism of the methylxanthines.

EXPERIMENTAL

Separation and chromatography

Several methods of separating purines from urine were briefly examined; of precipitation methods none was found to have any marked advantage, at least qualitatively, over the ammoniacal silver method. The CHCl_3 extraction method of Fisher, Algeri & Walker (1949) was found to yield three methylxanthines, probably theobromine, theophylline or paraxanthine, and caffeine, and at least three other unidentified compounds. The products from these separation methods were examined by paper chromatography employing as solvent *n*-butanol- NH_3 (Hotchkiss, 1948) by which the methylated xanthines are well separated (see also Markham & Smith, 1949). For separation of uric acids a single-phase solvent mixture containing *n*-butanol, ethylene glycol monomethyl ether, acetic acid and water in the proportions 7:7:2:4 (v/v) was found satisfactory. Chromatograms were usually run on paper strips 1 in. (2.5 cm.) wide by upward displacement in boiling tubes 12 in. long and 1.5 in. internal diameter (30 $\times$ 3.7 cm.). The tubes were held vertically with the solvent mixture in the bottom. The mouth of each tube was closed with a rubber stopper through which passed a glass rod ending in a hook on which the paper was carried. Two lanes could be run quite satisfactorily on such strips when direct comparison was required. R_F values varied appreciably with the ambient temperature, and values quoted here for the substituted uric acids are given as relative R_F 's, i.e. as ratios to that of uric acid itself. In the above solvent at about 20°, uric acid has an R_F value of 0.25. Spots were located in ultraviolet light (Holiday & Johnson, 1949).

The largest column of Zeo-Karb 215 resin employed was 21 cm. long and 1.8 cm. in diameter, and contained 12 g. finely ground resin (settling time 1–10 min.) mixed with an equal weight of Hyflo-Supercel. This was recycled a number of times with KOH or NH_3 and HCl, and finally equilibrated with 0.5N-HCl. Quantities of up to 2 l. of urine were treated with magnesia mixture (Snell & Snell, 1937) and NH_3 to precipitate phosphate, centrifuged, and the supernatant treated with a slight excess of ammoniacal silver reagent. After standing in the dark for at least 1 hr., the precipitate then formed was spun down, washed first with very dilute ammonia and then with water, suspended in a little water and N-HCl added until the pH was about 2. The volume was made up to about 20–30 ml., the mixture heated to boiling and filtered hot. The solution was allowed to crystallize

overnight, depositing principally uric acid, and the supernatant poured on to the column. Even had it been practicable to charge the column with a volume sufficient to retain all the uric acid in solution, it seemed very probable that this would have tended to crystallize out in the column during the separation, especially in view of the solubilizing effects referred to later. 0.5N-HCl was used as eluent, and fractions of 4.04 ± 0.02 ml. were collected by a siphon-operated automatic collector. The optical densities of the fractions were measured at a wavelength of 285 m μ . with a Unicam SP 500 spectrophotometer. Purine from 2 l. of urine obtained as described above tended to overload the column, in that considerable overlap between fractions occurred, but from previous experience it was readily possible to separate the groups containing the individual acids. Smaller quantities gave excellent discrimination, as shown in Fig. 3. Xanthines were retained on the column until all uric acids had been eluted. They could be displaced by increasing the acid strength to 2N, but there was little discrimination within the group. At least four xanthines were shown to be present in this fraction by paper chromatography. The uric acids were isolated from their solutions in 0.5N-HCl by running these down a column of the anion-exchange resin Duolite A. 2 in the free base form. The capacity of the column for HCl was chosen to be slightly greater than that present in the solution, and the uric acid was then displaced by 0.01N-HCl. The resulting solution was evaporated to dryness under reduced pressure. Earlier experiments in which the 0.5N acid solution was evaporated directly indicated that some loss was incurred, and although the column method also involved some losses it was thought that it involved less chance of decomposing the uric acids.

Identification

Absorption spectroscopy. Probably the most straightforward and generally useful method of identification for purines in very small quantities is that of ultraviolet absorption spectroscopy. The absorption spectra of the three uric acid fractions (referred to hereafter as I, II and III) obtained from urine differed, however, from one another very little (Table 1). Examination of the spectra of a number of methyl-substituted uric acids originally made by Prof. H. Biltz and kindly provided by Prof. D. Keilin, F.R.S., showed that those substituted in the 3- and in the 9-positions differed significantly and characteristically from uric acid itself, while 1- and 7-substituted uric acids showed very much smaller differences. The absorption spectra of uric acid itself at various pH values have been published by a number of authors, including Holiday (1930), Fromhertz & Hartmann (1936) and Stimson & Reuter (1943), but it is felt that improved techniques justify republication (Fig. 1, Table 1). These curves were obtained on an automatic recording spectrophotometer (Holiday & Sutton, to be published) with point density checks on a Unicam SP 500 spectrophotometer. Two commercial samples of uric acid were employed, purified by combinations of three methods: solution in conc. H_2SO_4 and fractional precipitation by dilution, precipitation from the lithium salt, and recrystallization from water or dilute hydrochloric acid. The products did not differ significantly.

Arsenophosphotungstic acid colour reaction. The Benedict arsenophosphotungstic acid colour reaction also offered a valuable method of distinguishing between the various substituted uric acids. Unfortunately, the original method of

Table 1. *Ultraviolet absorption of uric acid and of some methyluric acids*

(Wavelengths given in $m\mu$. Values given for uric acid and for 7-methyluric acid are believed to be accurate in wavelength to 0.5 $m\mu$. and in molecular extinction coefficient ϵ to 0.01×10^4 ; for the other compounds values are given for purposes of comparison and are subject to revision, particularly those in alkaline solutions.)

Compound	pH 3			pH 8-8.5			pH > 12		
	$\lambda_{\max.}$	$\lambda_{\min.}$	$\epsilon \times 10^{-4}$	$\lambda_{\max.}$	$\lambda_{\min.}$	$\epsilon \times 10^{-4}$	$\lambda_{\max.}$	$\lambda_{\min.}$	$\epsilon \times 10^{-4}$
Uric acid	284	—	1.21	291.5	—	1.24	294.5	—	1.35
	—	255	0.365	—	260.5	0.26	—	262	0.25
	230.5	—	0.87	235	—	0.985	219.5	—	2.54
7-Methyluric acid	—	212	0.52	—	218.5	0.59	—	—	—
	286	—	1.14	293	—	1.22	296.5	—	1.31
	—	258	0.39	—	263	0.30	—	262	0.255
1:7-Dimethyluric acid (fraction III)	234	—	0.87	237	—	0.97	222	—	2.37
	—	213	0.50	—	219	0.595	—	—	—
	285.5	—	1.10	—	—	—	295.5	—	1.27
1-Methyluric acid	—	258	0.435	—	—	—	—	263.5	0.33
	234	—	0.84	—	—	—	222	—	2.60
	—	220	0.64	—	—	—	—	—	—
3-Methyluric acid	283.5	—	1.14	—	—	—	292.5	—	1.23
	—	253.5	0.39	—	—	—	—	260.5	0.30
	231	—	0.79	—	—	—	217.5	—	2.14
1:3-Dimethyluric acid	—	218	0.66	—	—	—	—	—	—
	287	—	1.10	—	—	—	292.5	—	1.49
	—	257	0.31	—	—	—	—	257.5	0.24
1:3-Dimethyluric acid	232	—	0.79	—	—	—	214	—	1.84
	—	215	0.49	—	—	—	—	—	—
	285.5	—	1.16	—	—	—	294	—	1.69
1:3-Dimethyluric acid	—	258	0.41	—	—	—	—	260	0.335
	234	—	0.88	—	—	—	214.5	—	1.90
	—	221	0.77	—	—	—	—	—	—

Benedict & Franke (1922) and the adaptation used by Buchanan, Block & Christman (1945) are not suitable for application to microgram quantities such as are eluted from paper chromatograms, and yet another modification had to

Fig. 1. Ultraviolet absorption spectra of uric acid. —, pH 3; — —, pH 8; - - - -, pH > 12. For short wavelength peak at pH > 12 (curve A), values of ϵ should be multiplied by 2.

be worked out. It was most convenient to measure the ratio between the colour developed and the peak optical density in alkaline solution, giving a value characteristic for each compound without requiring any determination of absolute quantity present. The colour development was carried out as follows. To 2.5 ml. uric acid solution was added 0.5 ml. of an

aqueous solution containing 15% Na_2CO_3 and 15% urea, 0.5 ml. 10% aqueous NaCN, and 0.5 ml. arsenophosphotungstic acid reagent (Benedict & Franke, 1922). The solution was then 'seeded' with a little of the precipitate which always forms after a variable delay, and left to stand for 30 min. It was then centrifuged and the optical density of the supernatant measured against that of a reagent blank at the absorption peak 700 $m\mu$. in the Unicam SP 500 spectrophotometer. This value was divided by the optical density at 295 $m\mu$. given by the same amount of the uric acid in 40 ml. aqueous solution at a pH > 11.5. 'Colour ratios' obtained in this manner have proved quite reproducible, and not significantly affected by the age of the reagents. The order of the colour ratios obtained in this manner agrees, for those acids used, with that given by Buchanan, Block & Christman (1945). All the acids substituted in position 7 give very low values. The reaction is also applicable in simplified form to paper chromatograms. These are lightly sprayed with saturated Na_2CO_3 solution, and then with the arsenophosphotungstic acid solution diluted 1 in 3 with water.

Acid strength determinations. It would be expected that substitution of the possibly ionizable hydrogen atoms in the uric acid molecule by methyl groups would affect the acid strength. Biltz (1936) gives the order of acid strength of the four hydrogen atom positions as $9 > 3 > 1 > 7$, and states that uric acids not substituted in either the 9- or the 3-positions have very similar acid strengths. As a further check therefore on the substitution positions of the acids isolated from urine, spectrophotometric titrations were carried out on them and also on uric acid, 7-methyluric acid and 3-methyluric acid. The change with pH of optical density at a suitable wavelength was determined; buffers were not used, but, using a glass electrode system, solutions in very dilute acid or

alkali were titrated with very small volumes of strong alkali or acid, so that changes in volume were negligible. The wavelengths chosen were 244 and 302 $m\mu$, of which the second proved the more suitable. Curves of the type shown in Fig. 2 were obtained, from which pK values could be estimated with an accuracy of about ± 0.1 pH unit.

Fig. 2. Relation between optical density and pH at 302 $m\mu$ for fraction II of urinary uric acids.

Crystallization. The isolated uric acids were recrystallized from water and possessed distinguishable crystalline forms. Myers & Hanzal (1946) give some data on the solubilities of methyluric acids which, as with the xanthines, increase with an increasing degree of methylation. It was observed that the substituted acids from urine were very much more soluble in hot water than uric acid itself.

RESULTS

The purines from normal urine samples with no dietary precautions yielded by chromatography on a Zeo-Karb 215 resin column three fractions, as shown in Fig. 3 *b*, all showing very similar ultra-violet absorption spectra. The first of these was usually present in the greatest quantity, and was shown by paper chromatography, colour ratio and crystalline form to be uric acid itself.

Fraction II invariably proved on isolation to be heterogeneous. The first crystallization of the evaporation residue gave an aggregate of colourless crystals mixed with yellowish spherical granules, with very variable colour ratio, 0.9–2.2, compared with 2.9 for uric acid itself. On paper chromatography the material streaked badly, but gave a principal spot exactly corresponding in position to that of 7-methyluric acid, with a relative R_f of 1.4. Colour reaction on the paper was largely confined to the streak behind this spot and to material left at the

starting point. On recrystallization from water the yellowish material remained largely undissolved, and the product which was visually free from it formed very characteristic irregular stellate clusters

Fig. 3. Elution of urinary uric acids from a Zeo-Karb 215 resin column. (a) Full line: after 2 days' abstinence from foods and beverages containing methylated xanthines; broken line: after 4 days' abstinence. (b) After administration of 400 mg. caffeine. (c) After administration of 250 mg. theophylline.

of blades. On paper chromatography after recrystallization the colour-forming streak was much reduced, and the colour ratio was also reduced in solution to 0.47. Absorption spectra of this material were determined at pH values of 3 and > 12 , at a concentration of 0.245 mg. in 20 ml. They were not distinguishable from those of 7-methyluric acid (Table 1) in shape and band positions, and values were obtained for $E_{1\%}^{1\text{cm}}$ of 553 at 286.5 $m\mu$, pH 3, and 627 at 296.5 $m\mu$, pH > 12 , compared with corresponding values of 625 and 720 for 7-methyluric acid. Further purification was not attempted, since too little of the material was available.

Fraction III appeared to be quite homogeneous. It was quite soluble in hot water, from which it crystallized in plates, though sometimes small needles appeared singly or in radiating clusters. The colour ratio was 0.175, comparing closely with the value of 0.15 obtained for 7-methyluric acid, but the relative R_f was 1.8. Absorption spectra were determined at pH 3 and > 12 on solutions of concentration 0.303 mg. in 30 ml. The results are given in Table 1; the reasons for considering this compound to be 1:7-dimethyluric acid are given in the discussion.

Table 2. *Acid strengths, colour ratios and relative R_F values of uric acid and some methyl derivatives*

(Colour ratios and acid strengths are determined by the methods described in the text. The R_F values are relative to that of uric acid, using the butanol-methyl cellosolve-acetic acid solvent.)

Compound	Colour ratio	Relative R_F	pK_1	pK_2
Uric acid	2.9	1.0	5.4	10.6
1-Methyluric acid	3.4	1.5	—	—
		(streaks)		
3-Methyluric acid	1.6	0.9	6.2	>11.4 (?)
		(streaks)		
7-Methyluric acid	0.15	1.4	5.5	10.6
1:3-Dimethyluric acid	1.7	2.0	—	—
1:7-Dimethyluric acid (fraction III)	0.175	1.8	5.7	10.9

Values for the pK 's of these compounds were determined spectrophotometrically, and it was found that the differences between them were very small, scarcely exceeding the estimated error of ± 0.1 pH unit. Fig. 2 shows the curve obtained for fraction II, from which $pK_1 = 5.6$, $pK_2 = 10.6$. This particular curve was chosen since it is continuous; in other cases, the concentrations for pK_1 differed from those used for the pK_2 determinations, but results were very similar. The other values are given in Table 2; those obtained for uric acid differ from the results of His & Paul (1900) quoted by Holiday (1930), which correspond to values of 5.7 and 8.7.

Since it appeared probable that methyl groups constituted the substituents in these uric acids and that they were therefore metabolic products from methylated xanthines, some runs were carried out on purines from urine obtained after 2 and 4 days' abstinence from foods or beverages containing such compounds (Fig. 3a) and also after oral administration, with similar diet, of 400 mg. caffeine (Fig. 3b) and 250 mg. theophylline (Fig. 3c). In each case the urine samples of 25 ml. were treated similarly, and the purine solutions in 4.3 ml. of very dilute hydrochloric acid were run on a column 14 by 1.3 cm. containing 5 g. resin and a similar weight of Hyflo-Supercel, which had been in use for some time and gave quite reproducible results. It can be seen that if consumption of methylxanthines is avoided, only one uric acid fraction is obtained, that of uric acid itself. After the administration of caffeine the pattern found corresponds exactly to that of the normal urine samples previously investigated, with two methyluric acid fractions in addition to that of uric acid, whereas the metabolism of theophylline yields only one methyluric acid fraction under the conditions described.

A phenomenon which may be observed in Fig. 3, and which was conspicuous throughout this work, is the increased amount of uric acid which may remain dissolved in a given volume of water or dilute hydrochloric acid in the presence of methyluric acids, or possibly of methylxanthines. This solubilizing effect was particularly conspicuous when larger quantities of purine materials were dealt with; uric

acid fractions from the column proved to be supersaturated and crystallized out on standing from volumes of solution greater than those originally charged to the column, which had themselves been allowed to crystallize to equilibrium. Examination by paper chromatography of the material crystallizing out of solutions from the decomposition of the silver purines showed that although this was principally uric acid, substituted uric acids and xanthines were also present.

A synthetic mixture of uric acid, 3-methyluric acid and 3:7-dimethyluric acid was also run on the smaller column. 3-Methyluric acid was eluted immediately after uric acid, the peaks being at 28 and 45 ml., and the peak of the 3:7-dimethyluric acid fraction came at 115 ml. This experiment was carried out in the earlier stages of the work to determine the behaviour of known methyluric acids on the column. These particular acids were chosen since they could readily be distinguished by absorption spectra or colour ratios.

DISCUSSION

The results obtained are essentially qualitative. Myers & Hanzal (1946) state that the methyluric acids are all precipitated on standing by both ammoniacal silver magnesium mixture and by Folin's silver lactate-lactic acid solution. Buchanan, Christman & Block (1945), however, state that under the conditions used in their work 1:3-dimethyluric acid and 3-methyluric acid were not precipitated by ammoniacal silver. In the present work no evidence of the presence of any 3-substituted acids was found. Further possible segregation was caused by solubility limitations, which made it impracticable to employ total purine fractions for analysis on resin columns. The effect here, however, was one of relative enrichment of the more soluble methylated acids at the expense of uric acid, though it was found that some methyluric acids were left in the crystallized material.

Of the separated fractions, fraction II may reasonably be inferred to consist of a mixture of 7-methyluric acid and 1-methyluric acid with the

yellowish granules as a possible third component. Both 7- and 1-methyluric acid have absorption spectra closely resembling that of uric acid (Table 1), and mixtures of the two of varying composition could yield the colour ratios reported, corresponding to an increase in the proportion of 7-methyluric acid in the product from recrystallization. 1-methyluric acid tends to streak during paper chromatography, but the principal spot is found at almost the same position as 7-methyluric acid (Table 2). After ingestion of theophylline (Fig. 3c) no trace is observed of 3-methyluric acid, which would be eluted immediately after uric acid, and the single methyluric acid fraction must be attributed, therefore, to the 1-methyl or 1:3-dimethyl compound; absorption band positions and relative intensities indicated that it was 1-methyluric acid. The elution peak of the corresponding fraction obtained after ingestion of caffeine lies closer to that of uric acid. This fraction presumably contains the two overlapping elution bands of 7- and 1-methyluric acids.

Fraction III, if derived from the oxidation, with or without demethylation, of methylxanthines, might possibly then be 1:3-, 1:7- or 3:7-dimethyluric acid, or 1:3:7-trimethyluric acid. Since this fraction is not obtained from theophylline, the first is improbable and is in any case excluded, since fraction III gives a very low colour. The last two may also be eliminated since their absorption spectra are quite distinct from that of fraction III. Unfortunately, no sample of 1:7-dimethyluric acid was available for direct comparison. The absorption spectrum, however, was scarcely distinguishable from that of 7-methyluric acid, and it had been observed that the spectra of the corresponding 7-methyl and 1:7-dimethylxanthines are, in fact, practically indistinguishable, and give almost identical molar extinction coefficients. Allowing for the very small amount, 0.303 mg., on which the determination was carried out, the values obtained for fraction III show good agreement with those for 7-methyluric acid (Table 1).

The almost identical acid strengths of uric acid, 7-methyl and the presumed 1:7-dimethyluric acids (Table 2) are in agreement with the statement by Biltz (1936) that all such acids not substituted in the 3- or 9-positions will have similar strengths, and it was found that 3-methyluric acid is significantly weaker. It appears from these results that the separation obtained on resin columns does not depend on acid strength. It does, however, bear some relation to the position and degree of methylation; so far as has been determined the order of elution, after uric acid, is 3, 7, 1, 3:7, 1:7.

Little can be added on the basis of this work to the deductions summarized by Myers & Hanzal (1946) concerning resistance to demethylation in various positions. The results give no reason to dispute the general conclusion that methyl groups in the 3-position are the least stable, and provide definite evidence that demethylation also occurs in both the 1- and 7-positions.

SUMMARY

1. Substituted uric acids have been isolated from the urine of a human subject on a normal diet.
2. The positions of substituent groups have been determined by comparison with known synthetic methyluric acids, using spectroscopic and other methods. Results indicate that these isolated compounds are also methyluric acids substituted in the 7-, 1-, and 1:7-positions.
3. These compounds have been shown to be metabolic products of exogenous methylxanthines, i.e. caffeine, theobromine or theophylline.
4. Demethylation during metabolism of these compounds can occur in the 3-, 1- and 7-positions, but no definite order of stability has been demonstrated.

The author wishes to express his appreciation to Dr E. R. Holiday and Dr G. H. Beaven for their interest and encouragement during this work.

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