

A STUDY OF THE METABOLISM OF THEOBROMINE, THEOPHYLLINE, AND CAFFEINE IN MAN*

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Previous studies (1, 2) have shown that after the ingestion of caffeine or theophylline by man there was an increased urinary excretion of material which gave a blue color with the alkaline arsenophosphotungstate reagents used in the determination of uric acid. Myers and Wardell (3) and Buchanan, Christman, and Block (1) presented evidence that this extra color was caused by methyluric acids rather than by an increase in true uric acid excretion. When similar amounts of theobromine were taken, there was no increase in the excretion of chromogenic material.

Subsequent work (2) demonstrated the presence of 1-methyl and 1,3-dimethyluric acids in urine after the administration of caffeine and theophylline. The excretion of 7-methylxanthine, 1-methylxanthine, and 1,7-dimethylxanthine after the ingestion of caffeine has recently been reported by Weissmann *et al.* (4).

The present investigation concerns the identification and quantitative determination of the methyluric acids and methylxanthines excreted in the urine of man after the ingestion of theobromine, theophylline, and caffeine. 65 to 75 per cent of 1 gm. oral doses of these compounds were accounted for in the urine as methyluric acids or methylxanthines.

EXPERIMENTAL

Two individuals were placed on a diet free from coffee, tea, and cocoa. 24 hour control urine samples were collected, weakly acidified, and preserved by freezing. A 1 gm. sample of theobromine, theophylline, or caffeine was taken over a 4 hour period as two 500 mg. doses. Subsequent 24 hour urine samples were collected over a 3 day period and kept frozen until analyzed.

Chromatography—5 ml. aliquots of the urine samples were passed through a Dowex 2 anion exchange resin column which had been equilibrated with 0.1 N HCl. Washing the column with water removed the methylxanthines which are not strongly absorbed under these conditions. Uric acid and

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methyluric acids were retained on this column and subsequently eluted with 0.01 N HCl. This fraction will be referred to subsequently as the uric acid fraction.

Both the water washings (methylxanthine fraction) and the 0.01 N HCl eluate (uric acid fraction) were evaporated to dryness, dissolved in a minimal volume of weak alkali, and each was chromatographed on Whatman No. 1 filter paper by the ascending technique (solvent: *n*-butanol, water, acetic acid in a 4:1:1 ratio by volume). Known samples of the various methylxanthines and methyluric acids were chromatographed simultaneously to determine the R_F values for these compounds. After chromatography it was possible to render the methylxanthines and methyluric acids visible under ultraviolet light (5).

The strips containing these compounds were eluted with 10 ml. of 0.1 N HCl in the manner described by Dent (6). The ultraviolet absorption spectrum of each eluate was determined in acid (0.1 N HCl) and alkaline (0.05 N NaOH) solution by means of a Beckman model DU spectrophotometer. Light absorption measurements were made against a blank eluate from paper chromatographed in the same solvent.

Since all the methyluric acids which can be formed from theophylline are chromogenic, 0.2 ml. portions of the urine samples were chromatographed directly on paper with a solvent composed of methanol, benzene, *n*-butanol, and water (2:1:1:1 by volume). After detection of the absorbing areas under ultraviolet light, the chromatograms were cut into appropriate strips, the methyluric acids eluted with urea-cyanide solution (7), and the colorimetric determination made in the usual manner.

Silver Salt Precipitation—Another method for the identification of the methylxanthines was based on the solubility of their silver salts at various pH values. 5 ml. of an aqueous solution containing from 50 to 100 γ of the methylxanthine were adjusted to the desired pH and 5 ml. of 5 per cent silver nitrate were added. The tube was stoppered and allowed to stand for 10 to 15 minutes with occasional shaking. After centrifugation at moderate speed for 5 minutes, the supernatant fluid was decanted and the tube allowed to drain. The methylxanthine was reconstituted by shaking the precipitate vigorously for 5 minutes with 5 or 10 ml. of 10 per cent NaCl in 0.1 N HCl. After centrifugation, its concentration was determined in the supernatant solution by measurement of the optical density at 270 $m\mu$. All ultraviolet absorption measurements were made against a reagent blank carried through the entire silver precipitation procedure.

Table I records the percentages of the methylxanthines which were precipitated by silver nitrate at various pH values and then reconstituted into the acid-NaCl supernatant fluid. It can be seen that mixtures of

some methylxanthines can be separated by this procedure. For example, from a mixture containing theobromine (3,7-dimethylxanthine), 3-methylxanthine, and 7-methylxanthine, only the latter compound is precipitated by silver nitrate at a pH of 1 to 2. Adjustment of the pH of the supernatant fluid with alkali and acetate buffer (8) to a pH of 5.5 and addition of more silver nitrate will precipitate 95 per cent of the 3-methylxanthine, leaving the theobromine in solution. This type of analysis has been applied to the xanthine fractions from resin columns and to eluates from paper chromatograms. When known amounts of 3-methylxanthine, 7-methylxanthine, and 3,7-dimethylxanthine (theobromine) were added to control urine samples, each could be determined by this procedure with an accuracy of 90 to 95 per cent.

Results

Methylxanthine and Methyluric Acid Excretion in Urine of Man after Ingestion of Theobromine—After the ingestion of 1 gm. of theobromine by each of two individuals, there was no increase in the excretion of either true uric acid or material giving residual color as determined by the uricase procedure (7). Paper chromatography of the uric acid and methylxanthine fractions obtained from the anion exchange column indicated the presence of a number of theobromine metabolites in the fractions from both the 0 to 24 hour and 24 to 48 hour samples.

In the chromatogram of the uric acid fraction, ultraviolet light-absorbing areas corresponding to uric acid (R_F 0.18) and 7-methyluric acid (R_F 0.35) were found. 3-Methyluric acid, another possible metabolite of theobromine, was not detected on this chromatogram. After elution from the paper, further identification of 7-methyluric acid was made by its characteristic absorption spectra in acid and alkaline solution and its failure to give a color reaction with alkaline phosphotungstate solutions. As shown in Table II, relatively small amounts of 7-methyluric acid are excreted during the 48 hours following theobromine ingestion.

The paper chromatograms of the methylxanthine fraction had ultraviolet-absorbing areas at R_F values of 0.41, 0.48, and 0.60 corresponding to those of 7-methylxanthine (0.41), 3-methylxanthine (0.48), and theobromine (0.60), respectively. The eluted material with an R_F of 0.60 had absorption maxima at 271 $m\mu$ in acid (0.1 N HCl) and 273 $m\mu$ in alkaline solution (0.05 N NaOH). These values agree well with those for theobromine in acid (272 $m\mu$) and in alkali (274 $m\mu$). The theobromine excreted per 48 hours (Table II) was calculated from the optical density measurements of the acidified eluates.

Since the areas containing 7-methylxanthine and 3-methylxanthine overlapped to some extent, they were eluted from the paper as a single fraction

and separated by fractional precipitation as silver salts at pH 1.0 and 6.0 (Table I). Further identification was made by the characteristic shift in absorption maxima when the medium was changed from acid (0.1 N HCl) to alkali (0.05 N NaOH), 3-methylxanthine shifting from a peak of 270 in acid to 275 $m\mu$ in alkaline solution, 7-methylxanthine from 267 to 290 $m\mu$. The values reported in Table II for the 48 hour excretion of 3- and 7-methylxanthine have been calculated from the optical density values in acid solution.

Methylxanthine and Methyluric Acid Excretion in Urine of Man after Ingestion of Theophylline—The procedure for the determination of true uric acid (7) indicated that, after the ingestion of theophylline, there was a sharp increase in the excretion of chromogenic substances that were not

TABLE I
*Silver Salt Precipitation of Methylxanthines from Water
Solutions at Various pH Values*

Concentration range, 1.0 to 2.0 mg. per 100 ml.

| Compound | Per cent recovery | | |
|------------------------------|-------------------|---------------|-----------------|
| | pH 1.0 to 2.0 | pH 5.0 to 6.0 | pH 10.0 to 12.0 |
| 1-Methylxanthine..... | 30 | 91 | 94 |
| 3-Methylxanthine..... | 0 | 95 | 95 |
| 7-Methylxanthine..... | 92 | 93 | 97 |
| 1,3-Dimethylxanthine..... | 0 | 93 | 84 |
| 1,7-Dimethylxanthine..... | 74 | 92 | 97 |
| 3,7-Dimethylxanthine..... | 0 | 5 | 50-80 |
| 1,3,7-Trimethylxanthine..... | 0 | 0 | 0 |

destroyed by uricase. This increase could be attributed to the presence of one or all of the chromogenic methyluric acids (1-methyl, 3-methyl, and 1,3-dimethyl). After separation by direct paper chromatography, as previously described, the colorimetric analyses indicated that 1-methyluric acid and 1,3-dimethyluric acid were present in considerable quantity (Table II).

A preliminary separation of the methylxanthines from the uric acids was made on Dowex 2 anion exchange resin, as previously described. 3-Methylxanthine (R_F 0.48) has absorption maxima in acid and alkaline solutions at 270 and 275 $m\mu$, respectively. The fraction separated from urine at an R_F value of 0.45 to 0.59 had absorption maxima at 267 and 275 $m\mu$ in acid and alkaline solutions. The presence of a small amount of 1-methylxanthine (R_F 0.55) in this fraction cannot be ruled out, although the two maxima at 242 and 277 $m\mu$, characteristic of 1-methylxanthine in

alkaline solution, were not observed. The fraction eluted from the paper with an R_F of 0.70 (0.65 to 0.75) had absorption maxima in acid and alkaline solutions at 267 and 273 $m\mu$, respectively, as compared to 271 and 275 $m\mu$ for a known sample of theophylline (R_F 0.70). The values for the 3-methylxanthine and theophylline (Table II) were calculated from the optical density measurements of the corresponding acid eluates.

Methylxanthine and Methyluric Acid Excretion in Urine of Man after Ingestion of Caffeine—After the ingestion of 1 gm. of caffeine, there was a

TABLE II

Methylxanthine and Methyluric Acid Excretion during 48 Hour Period after Ingestion of 1 Gm. of Designated Methylxanthine

All the values are expressed as mg. of the administered compound required to give the excretory product.

| Compound excreted | Compound ingested | | | | | |
|--|-------------------|------------------|------------------|------------------|------------------|------------------|
| | Theobromine | | Theophylline | | Caffeine | |
| | Subject H. C. | Subject F. M. | Subject H. C. | Subject F. M. | Subject H. C. | Subject F. M. |
| Theobromine (3,7-dimethylxanthine)..... | 124 | 117 | | | | |
| 3-Methylxanthine..... | 182 | 215 | 155 | 111 | | |
| 7-Methylxanthine..... | 280 | 281 | | | 90 | 40 |
| 7-Methyluric acid..... | 42 | 36 | | | | |
| Theophylline (1,3-dimethylxanthine)..... | | | 87 | 111 | | |
| 1-Methyluric acid..... | | | 186 | 186 | 254 | 304 |
| 1,3-Dimethyluric acid..... | | | 382 | 318 | 142 | 46 |
| Caffeine (1,3,7-trimethylxanthine)..... | | | | | 11 | 12 |
| 1-Methylxanthine..... | | | | | 182 | 199 |
| 1,7-Dimethylxanthine..... | | | | | 74 | 19 |
| Total..... | 628 | 649 | 810 | 726 | 753 | 620 |

marked increase in the excretion of non-uric acid chromogens by both experimental subjects. In confirmation of the results obtained by Weinfeld and Christman (2), the present work demonstrates that 60 to 80 per cent of this chromogenic material is 1-methyluric acid and the remainder 1,3-dimethyluric acid (Table II).

The analytical procedures were similar to those previously described. Eluates from paper chromatograms corresponding to those of uric acid (R_F 0.20), 1-methyluric acid (R_F 0.42), and 1,3-dimethyluric acid (R_F 0.54) gave absorption maxima at 285 $m\mu$. The values given in Table II were calculated from the optical densities observed at this wave length. A small amount of material (10 to 15 mg. per 24 hours) with a spectra characteristic of the uric acids was found in the eluate from the chromatogram

at R_F 0.63 (0.58 to 0.68). The only methyluric acid found at this R_F range is 1,7-dimethyluric acid (R_F 0.60). Johnson (9) has reported the presence of 1,7-dimethyluric acid in the urine of individuals on a regular diet, which included coffee and tea. No evidence was found for the presence of 3-methyluric acid, which has an R_F value of 0.32.

If demethylation of caffeine without oxidation occurred, six mono- and dimethylxanthines, as well as unchanged caffeine, might be present in the xanthine eluate. Unchanged caffeine (R_F 0.78) was well separated from the other xanthines by paper chromatography. The material eluted from this area of the chromatogram (R_F 0.73 to 0.84) was characteristic of caffeine since it was not precipitated as a silver salt and showed no shift in absorption peak in changing from acid to alkaline solution. After the ingestion of 1 gm. of caffeine, 11 and 12 mg. were excreted unchanged by subjects H. C. and F. M., respectively.

The material which was eluted from the chromatogram with an R_F of 0.66 (0.59 to 0.73) had absorption peaks in acid at 262 $m\mu$ and in alkaline solution at 283 $m\mu$. This shift is typical of both 7-methyl- and 1,7-dimethylxanthine but the R_F value is that of 1,7-dimethylxanthine (0.66) rather than of 7-methylxanthine (0.43). After silver precipitation at pH 1.0, the absorption curve of the reconstituted xanthine was still typical of 1,7-dimethylxanthine with some evidence, however, for the presence of a small amount of 1-methylxanthine (R_F 0.55). Upon adjustment of the supernatant fluid to a pH of 5.0 to 6.0, a second silver precipitate was obtained. The reconstituted xanthine from this precipitate had an absorption spectrum in alkaline solution with maxima at 240 and 272 $m\mu$, typical of 1-methylxanthine (242 and 275 $m\mu$). The small amount of 1,7-dimethylxanthine that was not precipitated at a pH of 1.0 to 2.0 would be included in this fraction.

Approximately one-third of the ultraviolet-absorbing material of the eluate with an R_F of 0.53 (0.47 to 0.59) was precipitated as the silver salt at pH 1.0 and 90 per cent of the remainder at pH 5.0 to 6.0. Similar percentages of 1-methylxanthine added to a control urine were precipitated as silver salts at these pH values. The spectra of the xanthine released from these silver precipitates had peaks in alkaline solution at 240 and 277 $m\mu$ characteristic of 1-methylxanthine.

The material eluted from the chromatogram with an R_F of 0.42 (0.37 to 0.47) appeared to be 7-methylxanthine for several reasons. (1) The R_F value of 7-methylxanthine in this solvent is 0.43. (2) The absorption peak of the material in acid solution is at 269 $m\mu$, which is similar to that of 7-methylxanthine. Moreover, the 18 $m\mu$ shift in peak in alkaline solution to 287 $m\mu$ is characteristic of 7-methylxanthine. (3) Data in Table I indicate that, at pH 1.0, 92 per cent of 7-methylxanthine is precipitated as the

silver salt. 90 per cent of the ultraviolet-absorbing material in the eluate with an R_F of 0.42 was also precipitated at pH 1.0.

The values given in Table II for the amounts of the various catabolites of caffeine excreted per 48 hours were calculated from the optical densities of the reconstituted xanthines after silver precipitation. Since caffeine is not precipitated as a silver salt, the values for caffeine were obtained from the optical densities of the eluates from the paper chromatograms.

DISCUSSION

Since the development of the enzymatic uricase method for the determination of uric acid, all studies have indicated that there is no appreciable

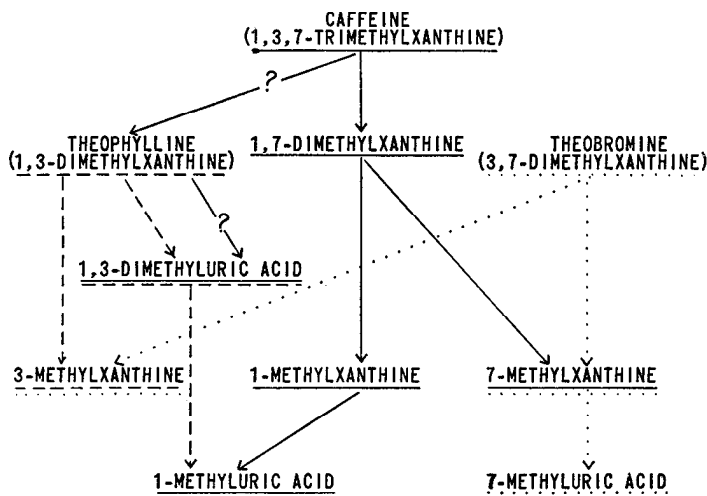


FIG. 1. Compounds excreted by man after the ingestion of caffeine (solid line) theophylline (dashed line), and theobromine (dotted line).

increase in true uric acid excretion upon the ingestion of caffeine, theophylline, or theobromine. Increases in the excretion of material which gives color with uric acid reagents after the ingestion of caffeine and theophylline can be attributed to the presence of the chromogenic methyluric acids.

In Fig. 1, the probable metabolic pathways of caffeine, theophylline, and theobromine in man are presented. After the ingestion of 1 gm. of theobromine, the major excretory products found in the urine of two subjects expressed as per cent of dose were 7-methylxanthine (28 to 30), 3-methylxanthine (14 to 21), and unchanged theobromine (11 to 12). A small amount of 7-methyluric acid (3 to 4 per cent) was also excreted. Since more 7- than 3-methylxanthine was excreted, it would appear that demethylation occurs more readily at the 3 than at the 7 position. The

7-methyluric acid may be formed by the oxidation of 7-methylxanthine or by demethylation of 3,7-dimethyluric acid formed by the direct oxidation of theobromine.

Theophylline (1,3-dimethylxanthine) differs from theobromine in that both of the methyl groups are on the pyrimidine ring, leaving the imidazole ring unsubstituted. The main excretory products after theophylline ingestion were 1,3-dimethyluric acid (35 per cent), 1-methyluric acid (19 per cent), 3-methylxanthine (13 per cent), and unchanged theophylline (10 per cent). The oxidation of theophylline without demethylation appears to be the major metabolic pathway. In addition a portion of the theophylline was demethylated at position 1 to give 3-methylxanthine. Two possible pathways for the formation of 1-methyluric acid are as follows: (1) demethylation of 1,3-dimethyluric acid or (2) direct oxidation of 1-methylxanthine. Although this latter compound has not been detected as one of the excretory products of theophylline metabolism, it may be an intermediate.

It would appear that theobromine and theophylline follow somewhat different pathways of metabolism. The major products of theophylline metabolism in man are methyluric acids, and the major products of theobromine metabolism are methylxanthines. Direct oxidation of theophylline must occur with relative ease to produce large amounts of 1,3-dimethyluric acid. On the other hand, no 3,7-dimethyluric acid was found upon the ingestion of theobromine, although small amounts may have been formed and further metabolized. Apparently a methyl substitution at position 7 hinders oxidation at the adjacent C atom (position 8). That oxidation is not completely prevented is shown by the excretion of small amounts of 7-methyluric acid after the ingestion of theobromine.

Excretion of 1-methyluric acid and 1-methylxanthine accounted for approximately 46 per cent of the ingested caffeine. In addition to these two products, 1,7-dimethylxanthine, 7-methylxanthine, 1,3-dimethyluric acid, and unchanged caffeine were present. All of these products, except the 1,3-dimethyluric acid, may be accounted for by a direct pathway of demethylation and oxidation via 1,7-dimethylxanthine. Weinfeld (10) has pointed out that, if the primary step is a demethylation in the 7 position, caffeine would be converted to theophylline which would then be metabolized to yield the series of compounds characteristic of this dimethylxanthine. On the other hand an initial demethylation at position 1 would yield theobromine, which would be further metabolized to give its characteristic products.

Since the pattern of the excretory products after caffeine ingestion is different from that of theophylline or theobromine, other metabolic pathways must be considered. 1,7-Dimethylxanthine, which is excreted in small amounts, may be the intermediate in the formation of both the 1-

methyl and 7-methylxanthine. Oxidation of 1-methylxanthine would give 1-methyluric acid. The 1,3-dimethyluric acid could arise from the demethylation of 1,3,7-trimethyluric acid formed by direct oxidation, even though none of this compound is excreted. It is more probable that this dimethyluric acid comes from the oxidation of 1,3-dimethylxanthine. Although this latter compound is not excreted after caffeine ingestion, it may be formed at a rate which permits its complete oxidation.

The present work indicates that demethylation may occur at either the 1, 3, or 7 position. In man, the order of increasing stability of these methyl groups is 3, 7, and 1. This is consistent with the chemical lability of these positions as determined by Cavalieri *et al.* (11), who found that the most readily dissociated hydrogen of xanthine is at position 3 and the least acid hydrogen is at position 1. That demethylation of caffeine, theobromine, and theophylline in man does not go beyond the monomethylxanthines is supported by two lines of evidence: (1) no accumulation of xanthine in the urine was observed in the chromatographic studies; and (2) insignificant, if any, increases in uric acid excretion.

SUMMARY

After suitable control periods on diets low in methylated purines, two human subjects received 1 gm. doses of theobromine, theophylline, and caffeine. 62 per cent of the theobromine, 77 per cent of the theophylline, and 66 per cent of the caffeine were excreted in the form of methylxanthines and methyluric acids during the subsequent 48 hours. The major part of the theobromine was excreted as methylxanthines. In contrast, the methyluric acids were the predominant excretory products of theophylline. After caffeine administration, approximately equal amounts of the methylxanthines and methyluric acids were present in the urine.

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