absorption from the oral route with gut and hepatic clearance (metabolism) of ASA. If, however, the oral to VC areas under the metabolite curves differed, but their ratio was greater than the ratio of the intact compound then this would indicate both incomplete absorption and organ clearance.

SUMMARY

The influence the route of administration may have on areas under plasma concentration-time curves is discussed.

It is shown that in dogs, acetylsalicylic acid is metabolized in the liver and in the gastrointestinal tract to a considerable extent.

The effect of hepatic and gastrointestinal metabolism of ASA when ASA is administered orally or *via* the hepatic portal vein is to reduce the area under the plasma concentration-time curves compared to the area of an equal intravenous dose.

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Nucleosides in Human Urine I: Isolation and Identification of N_2-Dimethylguanosine, 1-Methylinosine, and N_2-Methylguanosine from Normal Human Urine

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Abstract □ Using partition column chromatography, three methylated nucleosides have been isolated from normal human urine. The lyophilized urine was fractionated on a diatomaceous earth column using a formic acid gradient in an ethyl acetate–2-ethoxyethanol solvent system. Purification of the major fractions, using repeated paper chromatography, led to the isolation of methylated nucleosides, N_2-dimethylguanosine, 1-methylinosine, and N_2-methylguanosine. The identity of these nucleosides was established by comparison of their paper chromatographic and electrophoretic mobilities and UV spectra with those of authentic samples. Acid hydrolysis and the orcinol color reaction were in agreement with their identity as nucleosides.

Keyphrases □ Nucleosides—human urine □ N_2-dimethyl- and methylguanosine, urine—isolation, identification □ 1-Methylinosine, urine—isolation, identification □ Column chromatography—separation □ Paper chromatography—separation, identification □ UV spectrophotometry—analysis

In the last fifteen years, a number of workers (1–10) have contributed to the growing list of urinary purines and pyrimidines that most probably are metabolites of nucleic acids. In particular, Weissmann *et al.* (3–5) have characterized several methylated purines in normal human urine. The elevated levels of the methylated purines excreted by leukemic subjects was observed by Adams *et al.* (6) and by Park *et al.* (11).

It is well established that the minor nucleosides occur mostly in transfer ribonucleic acid (tRNA) (12, 13) and to a small extent in ribosomal RNA (13). The minor bases isolated from urine appear to be the metabolic end products of tRNA (14). Thus, in order to study the metabolism of tRNA in normal and diseased human subjects, it was of importance to determine if any of the nucleosides were excreted in normal human urine in addition to the methylated bases. Reports in the literature indicate that pseudouridine is the most commonly found minor nucleoside of tRNA in urine (7). Fink *et al.* (10) have characterized N_2-dimethylguanosine from one urine sample and the base N_2-dimethylguanine from several normal and leukemic human urine samples.

The methodology developed by Weissmann *et al.* (3) for the isolation of methylated purines cannot be applied to the isolation of nucleosides, since it involves

the use of hot hydrochloric acid, which can hydrolyze the glycosidic bond. In order to determine whether some of these methylated purines were also present as nucleosides, partition chromatography on diatomaceous earth,[1] with aqueous-organic solvents for elution, was used. This paper confirms the presence of N_2-dimethylguanosine (10) and describes the isolation and characterization of two additional methylated purine nucleosides, 1-methylinosine and N_2-methylguanosine, from normal human urine.

MATERIALS AND METHODS

Materials—All solvents used were either reagent grade or freshly distilled materials. The diatomaceous earth was washed successively with 6 *N* hydrochloric acid, water, and ethanol (12). The washed diatomaceous earth was then dried at 100° for 24 hr. Authentic samples of N_2-dimethylguanosine, 1-methylinosine, N_2-methylguanosine,[2] 1-methylguanosine, and 7-methylguanosine, were used.[3]

Column Fractionation—Diatomaceous earth columns have been widely used for the isolation of minor nucleosides from tRNA hydrolysates (12). These columns offer certain advantages over the acidic or basic resins, since neutral and aqueous-organic solvent systems can be used for fractionation. In this procedure, in order to prevent degradation during the process of isolation, solvents containing mineral acids and strong alkalies were avoided.

As a general procedure, urine from normal subjects and patients was collected under toluene for 24 hr. The subjects were kept on low-purine diets for 5 days prior to and during the collection. One-fifth of the 24-hr. urine sample was lyophilized, and the lyophilizate was dissolved in 25 ml. of the lower phase of the solvent system ethyl acetate–2-ethoxyethanol–4% formic acid (4:1:2). This was then triturated with 50 g. of diatomaceous earth and packed on the top of the prepacked column (150 g. diatomaceous earth, 80 × 2.54 cm.) according to the technique of Hall (12). The column was eluted with a gradient of 1,700 ml. each of ethyl acetate–2-ethoxyethanol–4% formic acid (4:1:2), and ethyl acetate–2-ethoxyethanol–16% formic acid (4:1:2), and 20-ml. fractions were collected. The absorbance of each alternate fraction was measured at 265 mμ, and the profile of absorbance *versus* volume was obtained. The fractions were lyophilized and purified by repeated paper chromatography, using mildly acidic and basic solvent systems.

Chromatography and Electrophoresis—The following solvent systems were used for paper chromatography of the column eluates:

A—Isopropanol–concentrated ammonium hydroxide–water (7:1:2)
B—Isopropanol–concentrated hydrochloric acid–water (680:170:144)
C—Isopropanol–1% aqueous ammonium sulfate (2:1)
D—Dimethylformamide–chloroform–water (50:40:10)
E—Ethyl acetate–2-ethoxyethanol–16% formic acid (4:1:2)
F—1-Butanol–concentrated ammonium hydroxide–water (86:14:5)
G—Ethyl acetate–*n*-propanol–water (4:1:2)

Electrophoresis was carried out in a mixture of 1.0 *M* acetic acid and 0.75 *M* formic acid at pH 2 on Savant flat-bed apparatus or in 0.05 *M* ammonium formate buffer at pH 3.5 in a electrophorator tank.[4]

Whatman No. 3 MM paper was used for the initial preparative paper chromatography of column eluates. Whatman No. 1 was used for comparative identification. All chromatograms were developed by the descending method. In all instances, appropriate authentic samples of nucleosides were cochromatographed with the purified urinary nucleosides.

Chromatograms were viewed under a short-wave UV lamp. All UV-absorbing areas were eluted and analyzed in a spectrophotometer.[5]

[1] Celite, Johns-Manville, New York, N. Y.
[2] Obtained from Prof. R. H. Hall.
[3] Cyclo Chemical Corp.
[4] Gilson.
[5] Cary.

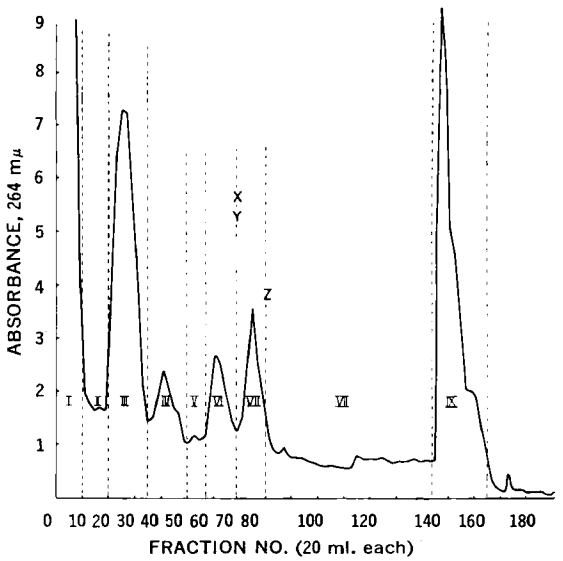

Figure 1—*Fractionation of urine lyophylizate on a diatomaceous earth partition column. The column was eluted with a gradient of 1,700 ml. each of ethyl acetate–2-ethoxyethanol–4% formic acid (4 : 1 : 2) → ethyl acetate–2-ethoxyethanol–16% formic acid (4 : 1 : 2). Fractions 145–190 were collected when the column was washed with 10% formic acid. Key: X, Y, and Z = nucleosides.*

Suspected nucleosides were subjected to quantitative (15) and qualitative (16) sugar analysis and acid hydrolysis. The unknowns were then compared with the several known methylated nucleosides with regard to their UV spectra and mobility upon paper chromatography and electrophoresis.

RESULTS

Figure 1 represents an elution profile of a urine sample when the lyophilizate from one-fifth of a 24-hr. collection was fractionated on a diatomaceous earth column. The elution pattern was similar for all urine samples examined. On the basis of model experiments, Fractions VI and VII were selected for further examination.

Isolation and Identification of Compound X (N_2-Dimethylguanosine) and Compound Y (1-Methylinosine)—Fraction VI (Fig. 1) was concentrated and lyophilized to a white powder. This material was dissolved in a small amount of distilled water and was applied to several Whatman No. 3 MM papers. These were developed in solvent System *A* for 18 hr. All UV-absorbing bands were cut out and eluted with water. A positive orcinol reaction (15) was obtained from aliquots of the third and fourth bands from the solvent front. These two suspected nucleosides were designated Nucleosides X and Y. The eluates for X and Y were separately pooled.

Preliminary UV spectrophotometry suggested that Compound X was a guanosine-type nucleoside. It was rechromatographed in System *E* with several methylated guanosine nucleosides cochromatographed as standards. Compound X had an R_f identical to that of N_2-dimethylguanosine as indicated by the cochromatographed authentic sample. This material was eluted, and a UV spectrum obtained that was identical to that of N_2-dimethylguanosine (Table I). Chromatography of this compound in Systems *B*, *C*, *D*, and *F* showed it to have an R_f identical to that of N_2-dimethylguanosine (Table II). Electrophoresis of this purified material revealed one spot whose mobility was identical to that of an authentic sample of N_2-dimethylguanosine (Table III).

Nucleoside Y, the second orcinol-reacting substance found in Fraction VI, was isolated and identified in a manner similar to that for Nucleoside X (N_2-dimethylguanosine). Preliminary UV spectrophotometry of this material suggested a resemblance to 1-methylinosine. Rechromatography in solvent System *E* along with several standards revealed the presence of a major UV absorbing substance with the same R_f as that of 1-methylinosine. Further chromatography of this compound in Systems *B*, *C*, *D*, and *F* confirmed its chromatographic mobilities as identical to those of 1-methylinosine (Table II). UV spectra of this material were identical with those of an

Table I—Comparison of the Ultraviolet Absorption Characteristics of Authentic Nucleosides with the Compounds Isolated from Urine

Compd.	pH[a] Acidic	Sh[b]	Neutral	Sh	Basic	Sh
Nucleoside X	264,	295	260,	283	262,	280
N_2-Dimethylguanosine	264,	295	260,	283	262,	280
Nucleoside Y	250	—	249	—	250	—
1-Methylinosine	250	—	249	—	250	—
Nucleoside Z	259,	282	253,	282	257,	275
N_2-Methylguanosine	259,	282	254,	282	258,	275
Acid hydrolysis product of X	257,	283	250,	283	280,	252
N_2-Dimethylguanine	257,	285	252,	282	280,	255
Acid hydrolysis product of Y	248	—	250	—	260	—
1-Methylhypoxanthine	248	—	250	—	260	—

[a] Acidic pH 1.6–1.8; neutral pH 6.3–6.5; basic pH 11.6–11.8. [b] Sh = shoulder.

authentic sample of 1-methylinosine (Table I). Additional evidence was provided by the electrophoretic mobility of Nucleoside Y, which was identical to that of 1-methylinosine (Table III).

Acid Hydrolysis of Nucleoside X (N_2-Dimethylguanosine)—To 100 μl. of the sample (0.095 μmole) was added 10 μl. of 0.5 *N* HCl, and the solution was heated in a sealed tube at 100° for 30 min. The resulting reaction mixture was streaked on a 20.3-cm. (8-in.) Whatman No. 1 paper and was developed in System *E* for 7 hr. The main UV-absorbing band was eluted with water, and the eluate was concentrated to a small volume. Its UV spectra and paper chromatography in Systems *A*, *E*, and *G* were identical to those of N_2-dimethylguanine (Tables I and II).

Another aliquot was chromatographed in System *G*, along with ribose as a standard. After development and drying, the chromatogram was sprayed with aniline hydrogen phthalate. An elongated pink spot opposite the ribose marker indicated the presence of ribose in the hydrolysis mixture. The identification of Nucleoside X as N_2-dimethylguanosine has thus been conclusively demonstrated.

Acid Hydrolysis of Nucleoside Y (1-Methylinosine)—To 200 μl. of the sample, containing 0.44 μmole of 1-methylinosine, was added 20 μl. of 0.5 *N* HCl. The solution was heated in a sealed tube at 100° for 60 min. The entire sample was streaked on a 20.3-cm. (8-in.) Whatman No. 1 paper and chromatographed in System *A* for 18 hr. The UV-absorbing band was eluted and concentrated to a small volume. The UV spectra and paper chromatographic behavior of this hydrolysis product were identical to those of 1-methylhypoxanthine (Tables I and II).

An aliquot of the hydrolysate was chromatographed in System *G*, along with a ribose marker. Spraying the paper with aniline phthalate revealed the presence of ribose in the hydrolysate. Nucleoside Y was thus identified as 1-methylinosine.

Table II—Comparison of Chromatographic Mobilities of the Authentic Nucleosides and Bases with the Compounds Isolated from Urine and Their Hydrolysis Products

	$R_f \times 100$ Various Solvents						
	A	*B*	*C*	*D*	*E*	*F*	*G*
Nucleoside X	39	32	61	37	18	11.5	—
N_2-Dimethylguanosine	39	32	61	37	18	11.5	—
Nucleoside Y	45	39	62	38	20	18	—
1-Methylinosine	45	39	62	38	20	18	
Nucleoside Z	32	35	59	—	—	—	—
N_2-Methylguanosine	32	35	59	—	—	—	—
1-Methylguanosine	58	30	57	—	—	10	—
7-Methylguanosine	35	29	36	—	—	4	—
Acid hydrolysis product of X	45	—	—	—	44	—	40
N_2-Dimethylguanine	45	—	—	—	45	—	41
Acid hydrolysis product of Y	47	—	—	—	49	—	40
1-Methylhypoxanthine	49	—	—	—	50	—	40
Acid hydrolysis product of Z[a]	40	—	—	—	—	—	—
N_2-Methylguanine	42	—	—	—	—	—	—

[a] Further comparison of the hydrolysis product, presumably N_2-methylguanine, could not be done, because of the limited availability of the unknown Nucleoside Z.

Table III—Comparison of Electrophoretic Mobilities of Authentic Nucleosides with the Compounds Isolated from Urine

Compd.	Distance Traveled, cm.	vol./cm.
Nucleoside X	−13.6[a]	54
N_2-Dimethylguanosine	−13.8	
Nucleoside Y	−14.7[b]	71
1-Methylinosine	−14.6	
Nucleoside Z	−12.2[a]	54
N_2-Methylguanosine	−12.2	

[a] Electrophoresis was carried out in a mixture of 1.0 *M* acetic acid, 0.75 *M* formic acid at pH 2, 3,000 v. for 1 hr., and on Savant flat-bed apparatus. [b] Electrophoresis was carried out in 0.05 *M* ammonium formate buffer at pH 3.5, 4,000 v. for 4 hr., in an electrophorator tank.

Isolation and Identification of Nucleoside Z (N_2-Methylguanosine)—Initial paper chromatographic examination of Fraction VII from the column revealed that it also contained at least five UV-absorbing compounds. The fourth band from the solvent front gave a positive orcinol reaction and was designated Nucleoside Z. It was isolated and purified in a manner similar to that for Nucleosides X and Y. This material was chromatographed in Systems *B*, *C*, *D*, *E*, and *F*. In all systems, one major UV absorbing spot was observed having the same R_f as N_2-methylguanosine (Table II). The UV spectra of the purified Nucleoside Z were identical to those of N_2-methylguanosine (Table I).

Acid Hydrolysis of Nucleoside Z (N_2-Methylguanosine)—A sample of 0.4 absorbance units of Nucleoside Z was hydrolyzed in 50 λ of 0.5 *N* HCl at 100° for 30 min. in a sealed tube. The reaction mixture, when examined by paper chromatography in System *A*, revealed a spot corresponding to the marker N_2-methylguanine (Table II). The UV spectra of this material were identical to those of N_2-methylguanine (Table I). The electrophoretic mobility of this hydrolysis product coincided with that of N_2-methylguanine. The aniline hydrogen phthalate reaction, performed on an aliquot of the hydrolysate, indicated the presence of ribose. The Nucleoside Z is thus characterized as N_2-methylguanosine.

Table IV lists the five subjects whose urines were analyzed and the quantities of the three isolated nucleosides/24 hr. In three of four normal subjects (A, B, D), N_2-dimethylguanosine was found. Subjects A, B, and C, all normal, also excreted 1-methylinosine. N_2-Methylguanosine was found in extremely small quantities in one normal subject (A). Subject F had carcinoma of the ovary with metastases, but Subject E was normal. Inability to detect the methylated nucleosides in these subjects is probably not significant. The possibility of missing these components during the complex purification process cannot be excluded.

DISCUSSION

There have been few specific reports regarding the presence of nucleosides in normal human urine. Pseudouridine, a nucleoside in which the ribose moiety is attached to a carbon atom, is found in large quantities in normal urine (6, 7). Inosine (6) has been isolated from the urine samples of leukemic subjects. Occasionally traces of guanosine (6), uridine (9, 17), and adenosine (9) have been detected in normal human urine. Fink *et al.* (10) have characterized N_2-

Table IV—Urinary Nucleoside Excretion, mg./24 hr.[a]

	Subjects[b] A	B[c]	C	D[c]	E[c]	F
N_2-Dimethylguanosine	2.07	1.68	—	0.71	—	1.04
1-Methylinosine	3.0	2.14	1.5	—	—	—
N_2-Methylguanosine	0.19	—	—	—	—	—

[a] Purified nucleosides were quantitated by UV spectrophotometry, using the ϵ_{max} values reported for synthetic materials (28, 29). [b] A, B, C, D, and E were normal subjects. F had carcinoma of the abdomen. [c] The urine samples from Subjects B, D, and E were processed by ion-exchange and partition chromatography. After absorbing the acidified lyophylizate on the ion-exchange resin (Dowex 50W H+) column and then washing with water, the column was stripped with 1 *N* NH_4OH. The ammonia lyophylizate was then fractionated on a diatomaceous earth partition column, using the customary procedure.

dimethylguanosine from one urine sample, and the base N_2-dimethylguanine from several normal and leukemic human urine specimens. Weissmann *et al.* (3) have reported the isolation of the bases N_2-methylguanine and 1-methylhypoxanthine, part of which might have arisen from the hydrolysis of corresponding nucleosides during the isolation procedures.

The authors' isolation of N_2-dimethylguanosine, 1-methylinosine, and N_2-methylguanosine from normal human urine suggests the possibility of the general occurrence of methylated nucleosides in human urine. The presence of these methylated nucleosides in normal human urine raises some important questions regarding the origin and metabolism of these compounds. The occurrence of methylated nucleosides in tRNA from various sources is very well documented. Dunn and Hall (18) have compiled a list of minor nucleosides and bases, giving their content in various biological sources. Smith and Dunn (19) have reported the presence of N_2-dimethylguanosine and N_2-methylguanosine in tRNA from wheat germ and rat liver. The isolation and characterization of N_2-dimethylguanosine, N_2-methylguanosine, and 1-methylinosine from yeast tRNA has been reported by Hall (20). N_2-Dimethylguanosine and 1-methylinosine have also been detected in the tRNA of human leukemia cells grown in tissue culture (21).

These three nucleosides isolated from urine cannot be of dietary origin, since Subjects A, B, and C were kept on purine-free diets, and other subjects were kept on low-purine diets before and during the urine collection. Other workers have found that the urinary levels of N_2-methylguanosine, 1-methylguanine, 7-methylguanine, and 1-methylhypoxanthine are clearly not of dietary origin (4).

It appears fairly certain that these methylated nucleosides found in urine arise as a result of the metabolic turnover of tRNA. Mandel *et al.* (14) showed that when ^{14}C-methylmethionine was injected into rats, the commonly occurring methylated bases excreted in rat urine were radioactive. It is known that methylation of tRNA occurs at a preformed polynucleotide level (22, 23), and that the methylated bases cannot be incorporated directly into tRNA (24). These observations indicate that the methylated nucleosides isolated from urine must arise primarily from metabolism of human tRNA and in small measure from ribosomal RNA (13).

Not all the known minor nucleosides have been found as yet in normal urine. Little is known about the metabolism of the 2′-*O*-methyl nucleosides. The metabolism of 6-methylamino purine by a rat liver homogenate is known to give hypoxanthine and uric acid (25), but methylated guanines are resistant to dealkylation and deamination (5, 26). It appears that 7-methylguanine is a precursor of 7-methyl-8-hydroxyguanine (27); but an enzyme other than xanthine oxidase would be required, since it has been demonstrated that naturally occurring methylated guanines are not acted upon by xanthine oxidase (5, 26). Nucleoside phosphorylases, commonly present in many tissues, may not be efficient enough to cleave the glycosidic bond, and, as a result, portions of the methylated nucleosides are excreted unchanged in urine. The action of enzymes, such as deaminases, nucleoside phosphorylases, and xanthine oxidase, which are involved in the conversion of common purine nucleosides to uric acid, remains to be studied systematically on the methylated nucleosides.

Systematic exploration of normal human urine for the presence of unusual nucleosides may provide a basis for the study of nucleoside metabolism in certain disease states.

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