Source of Urinary 8-Hydroxy-7-methylguanine in Man*

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ABSTRACT: The metabolic relationship between urinary 7-methylguanine and 8-hydroxy-7-methylguanine has been studied. 7-[7-^{15}N]Methylguanine was administered to a normal human subject and the presence of isotope in urinary purine bases was followed for 7 days. Most of the ^{15}N given was recovered in the 7-methylguanine and 8-hydroxy-7-methylguanine excreted on the first day. The isotope concentration in these two compounds was quite similar. No excess isotope was detected in xanthine or uric acid. Since only negligible isotope was recovered after the first day, it may be concluded that no appreciable pool of 7-methylguanine exists. A substantial proportion of this compound is rapidly oxidized to 8-hydroxy-7-methylguanine in man by an unknown process. The isotopic 7-methylguanine required was prepared by methylation of guanine in alkaline solution with methyl chloride. 9-Methylguanine and 3-methylguanine were major by-products, and small amounts of 1-methylguanine and N^2-methylguanine were isolable.

Some time ago, it was shown that normal human urine contains small amounts of methylated purine bases (Weissmann *et al.*, 1957a), including N^2-methylguanine, 1-methylguanine, 1-methylhypoxanthine, and 8-hydroxy-7-methylguanine, in addition to 7-methylguanine, long known to be a urinary constituent (Krüger and Salomon, 1898). With the exception of 8-hydroxy-7-methylguanine, the presence of these compounds in urine has since been accounted for, wholly or in part, by their subsequently demonstrated occurrence in the soluble ribonucleic acids (s-RNA) of tissue (Bergquist and Matthews, 1962; Davis *et al.*, 1959; Dunn, 1963; Smith and Dunn, 1959). 8-Hydroxy-7-methylguanine, whose origin has so far remained obscure, as well as 7-methylguanine occur in urine in far higher concentration than the other methylated purine bases (Weissmann *et al.*, 1957b). This circumstance and a structural similarity suggest a product–precursor relationship between the two compounds which is substantiated by the results of the present investigation.

Experimental Section

Materials and Methods. Reference samples of 1-methylguanine, 3-methylguanine, and 7-methylguanine were purchased from Cyclo Chemical Co. Xanthine, grade A, was purchased from Calbiochem, Inc. 9-Methylguanine was a gift from Dr. R. K. Robins. N^2-Methylguanine was a gift from Dr. Gertrude B. Elion. 8-Hydroxy-7-methylguanine was a gift from Dr. David Sprinson (Borowitz *et al.*, 1965).

Ultraviolet spectra were measured with a Beckman DU spectrophotometer or a Cary Model 15 recording spectrophotometer at pH 2, 9, and 14. Infrared spectra were measured with a Perkin Elmer Infracord Model 137B spectrophotometer. Potassium bromide pressings were used for sampling.

Two solvent systems were used for descending paper chromatography. One was butanol–0.6 M ammonia (6:1) (Chargaff *et al.*, 1951) and the papers were developed for 48 hr. The other was butanol–formic acid–water (77:11:12) (Markham and Smith, 1949); the papers were treated with gaseous ammonia before development for 16 hr.

Preparation of 7-[7-^{15}N]Methylguanine. The methylation of guanine as reported by Traube and Dudley (1913) was selected to prepare the needed labeled 7-methylguanine. Because the methylation products found were somewhat different than those reported, the reaction and the separation methods developed for its study are described in some detail. Guanine hydrochloride (0.9 g) was dissolved in 3.0 ml of 4 M sodium hydroxide and the solution was diluted with 5 ml of 95% ethanol. Methyl chloride was slowly passed through the solution for 3 hr while the temperature was maintained at 65–70°. The solution was concentrated *in vacuo* to remove ethanol. No precipitation of alkali-insoluble 1,7-dimethylguanine, reported as a product of this reaction (Traube and Dudley, 1913), was observed. Neutralization with 4 M acetic acid precipitated a mixture of guanine and methylated guanines (0.62 g).

The mixture was subjected to chromatography on a column of Dowex 1 x 8 acetate, 200–400 mesh, 5.5 cm^2 × 40.5 cm (Figure 1). The column was prewashed

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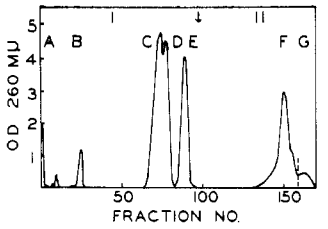

FIGURE 1: Separation of methylguanines on a column of Dowex 1 × 8 acetate, 200–400 mesh, corresponding to 225 ml of settled volume of resin. The column was developed with 0.04 M ammonium acetate buffer, pH 8.9 (I), until peak E was eluted, then it was developed with 0.08 M ammonium acetate buffer, pH 8.9 (II). Fractions of 225 ml were collected. The peak labeled A is unabsorbed material, which was not identified, B is 1-methylguanine, C is 9-methylguanine, D is 3-methylguanine, E is 7-methylguanine, F is guanine, and G is N^2-methylguanine.

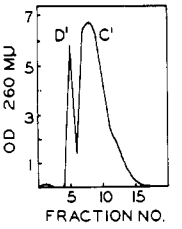

FIGURE 2: Separation of 9-methylguanine and 3-methylguanine (peaks C and D, Figure 1), on a column of Dowex 1 × 8 acetate, 200–400 mesh, similar to column I. This column was developed with 0.04 M ammonium acetate buffer, pH 4.9. Peak D′ is 3-methylguanine and peak C′ is 9-methylguanine.

with 500 ml of 0.02 M ammonium acetate buffer,[1] pH 9.6. A solution of the mixture in 0.02 M sodium hydroxide was added to the column and washed in with 250 ml of 0.02 M ammonium acetate buffer, pH 9.6. The column was first developed with 0.04 M ammonium acetate buffer, pH 9.0; after the 7-methylguanine peak was eluted, the developer was changed to 0.08 M ammonium acetate buffer, pH 9.0. Ultraviolet spectrophotometry and paper chromatography were used for preliminary identification of the components of each peak. For separation of 3-methylguanine and 9-methylguanine which were not resolved, fractions corresponding to peaks C and D (Figure 1) were pooled, adjusted to pH 10 with ammonium hydroxide, and chromatographed again on a Dowex 1 x 8 acetate column similar to the first one. This column was developed with 0.04 M ammonium acetate buffer, pH 4.9 (Figure 2).

The fractions corresponding to each methylguanine peak (Figures 1 and 2) were pooled and concentrated to dryness *in vacuo*. Fractions corresponding to peaks A (believed to be a mixture of dimethylguanines) and F (unreacted guanine) were not investigated further. Each residue was dissolved in a small amount of 1.0 M hydrochloric acid and the methylguanines were precipitated by adjusting the solutions to pH 4–5 with 2 M ammonium hydroxide. The amount of each product isolated is listed in Table I.

All the preparations except N^2-methylguanine showed single spots when subjected to paper chromatography using the two solvent systems described above. The N^2-methylguanine preparation, which was collected from the column in a region corresponding to that occupied by the authentic compound, consisted mainly of guanine with N^2-methylguanine present as a minor component. Ultraviolet spectra and infrared spectra of the preparations and their mobility on paper chromatograms corresponded with those of the authentic materials.

TABLE I: Products Produced by the Methylation of Guanine.

Peak	Compound	Amount (mg) Estd[a]	Recovd
A	Unabsorbed material not identified	56[b]	...
B	1-Methylguanine	14	6.4
E	7-Methylguanine	90	73.7
F	Guanine	101	...
G	N^2-Methylguanine	15	...
C′	9-Methylguanine	129	118.5
D′	3-Methylguanine	42	30.7

[a] These values are based on ϵ_{260} as determined for the pure compounds. [b] This value was based on ϵ_{260} reported for guanine.

When this preparation was repeated, using [7-^{15}N]-guanine as a starting material, the resolution in the first Dowex 1 x 8 acetate chromatogram was less sharp than in the run described. To ensure the purity of the 7-[7-^{15}N]methylguanine isolated, this product was recrystallized as the hydrochloride. Absence of other methylated guanines was verified by subjecting a sample to vigorous acid hydrolysis. Under these conditions 7-methylguanine is known to yield sarcosine as a degradation product, while the other methylated guanines yield glycine (Weissmann and Gutman, 1957). Sarcosine and a trace of glycine were produced in the present hydrolysis. The glycine, barely detectable on paper chromatograms, was estimated to correspond to contamination of the 7-[7-^{15}N]methylguanine with less than 0.3% of the other methylguanines.

Observations made in the course of synthesis of labeled 7-methylguanine merit brief comment. 7-Methylguanine was obtained in expected quantity

[1] Concentrations given for ammonium acetate buffers are based on acetate ion concentration.

TABLE II: Ultraviolet Absorption Data and Nitrogen Analyses for Compounds Isolated from Urine and Values from the Literature.

Sample	Day	λ_{max} mμ, pH 2.0	$\epsilon \times 10^3$ max	% N
Uric acid	1	283	11.1	32.6
Cavalieri and Bendich (1950)		283	11.5	33.3
Xanthine	1	266.5	9.8	34.1
	2	267	9.3	33.1
	3	267	*a*	36.0
	5	267	10.1	34.5
	7	266.5	*a*	36.8
Volkin and Cohn (1954)		267.5	9.7	36.8
7-Methylguanine	1	250	10.2	41.9
	2	249.5	9.9	40.0
	3	249.5	10.7	41.0
	4	249.5	10.2	39.8
	5	250	10.3	39.0
	7	249.5	10.3	40.2
Gulland and Story (1938)		250	10.3	42.4
8-Hydroxy-7-methylguanine	1	248	*a*	38.4
	2	248.5	*a*	37.1
	4	248	*a*	38.7
	7	248	*a*	39.7
Weissmann and Gutman (1957)		248	11.1	38.6

[a] Values in the literature were used for calculating the per cent N.

(18%) in the methylation of guanine with methyl chloride; production of 9-methylguanine as the major product (33%) and 3-methylguanine as a substantial minor product (11%) was unexpected, however, since neither of these compounds were reported products of this reaction (Traube and Dudley, 1913). These compounds may have escaped notice previously because of their appreciable solubility in water, and their detection has been facilitated by modern methods of separation and ultraviolet spectrophotometry. 3-Methylguanine has apparently not been isolated previously as a product of methylation of guanine, and the procedure described affords a simplified route for its preparation.

Preparation of [7-^{15}N]Guanine. For preparation of [^{15}N]potassium nitrite (*cf.* Vaughan *et al.*, 1951), an intimate mixture of powdered potassium nitrate (3.21 g, 63.7 atom % excess ^{15}N, Eastman) and lead (18 g, about 200 mesh) was heated without stirring in a test tube of 25-mm diameter, immersed in a metal bath maintained at 400°. After a few minutes the mixture suddenly became red hot; it was allowed to cool after 5 min more. Extraction with water and removal of lead ions by addition of sulfuric acid to pH 6 yielded a solution which contained 24.4 mmoles of potassium nitrite (77% yield), as shown by colorimetry (Snell and Snell, 1949). The isotopic potassium nitrite solution (12.0 ml, 1.00 M) was added slowly and with stirring to a solution of 12.5 mmoles of 2,4-diamino-6-oxypyridine (Francis Earle Laboratories) in 30 ml of 0.5 M hydrochloric acid, and the mixture was then heated briefly (Cain *et al.*, 1946). The resulting crude nitrosopyrimidine was reduced with sodium hydrosulfite to 2,4,5-triamino-6-oxypyrimidine; this was isolated as the bisulfite (Cain *et al.*, 1946), and purified as the sulphate monohydrate (88% yield from potassium nitrite). This compound was transformed into [7-^{15}N]guanine by condensation with formic acid (Traube, 1900). The purity of the product, isolated as the hemisulfate monohydrate in 82% yield, was verified by ultraviolet measurements.

Administration of Labeled Material and Isolation Procedures. 7-[7-^{15}N]Methylguanine hydrochloride (6.2 mg, corresponding to 5.0 mg of free base) was dissolved in 10 ml of 0.01 M hydrochloric acid and diluted to 200 ml with 0.15 M sodium chloride solution buffered with sodium monohydrogen phosphate to pH 7.1. This solution was administered intravenously to a normal adult male human subject over a 2-hr period. Following isotope administration, urine was collected over chloroform for 7 days. Using methods described previously, a purine concentrate was prepared from each 24-hr urine collection and chromatographed on a column of Dowex 50 x 12 H^+, 200–400 mesh (Weissmann *et al.*, 1957a). In each case the fractions corresponding to the xanthine peak, the 7-methylguanine peak and the 8-hydroxy-7-methylguanine peak were pooled and concentrated to dryness *in vacuo*.

7-Methylguanine was purified by reprecipitation from hydrochloric acid solution with ammonium hydroxide. 8-Hydroxy-7-methylguanine was purified

TABLE III: Conversion of 7-Methylguanine into Urinary Purine Bases.

Day	Compound	24-Hr[a] Excretion	^{15}N (atom % excess)	^{15}N (mg excess in total sample)
...	7-Methylguanine[b]	5.0	12.0	0.254
1	7-Methylguanine	10.0	2.90	0.123
	8-Hydroxy-7-methylguanine	3.8	2.72	0.040
	Xanthine	14.9	0	...
	Uric acid	...	0	...
2	7-Methylguanine	6.6	0.04	0.001
	8-Hydroxy-7-methylguanine	2.8	0.07	0.001
	Xanthine	11.6	0	...
3	7-Methylguanine	6.4	0	...
	8-Hydroxy-7-methylguanine	2.6	*c*	...
	Xanthine	6.7	0	...
4	7-Methylguanine	6.8	0	...
	8-Hydroxy-7-methylguanine	3.0	0	...
	Xanthine	10.3	*c*	..
5	7-Methylguanine	6.6	0	...
	8-Hydroxy-7-methylguanine	3.2	*c*	...
	Xanthine	7.6	0	...
7	7-Methylguanine	8.7	0	...
	8-Hydroxy-7-methylguanine	3.4	0	...
	Xanthine	10.3	*c*	...

[a] Value based on column data. [b] Values for compound administered. [c] Insufficient or inadequately pure material isolated for isotopic analyses.

by precipitation on dilution of a solution in hot 6 M hydrochloric acid (Weissmann and Gutman, 1957). Xanthine was purified by the procedure of Hitchings and Fiske (Hitchings and Fiske, 1941). Uric acid, for the first day only, was isolated by the procedure of Geren *et al.* (1950). The compounds isolated were characterized by their ultraviolet spectra. Their molar extinction coefficients and the results of analyses for nitrogen are given in Table II. The nitrogen analyses were carried out using a Kjeldahl micromethod and the samples were digested for 17 hr (Sprinson and Rittenberg, 1949).

Measurements of Isotope Abundance (Sprinson and Rittenberg, 1949). Nitrogen gas was generated from the ammonia available from the Kjeldahl analyses, using alkaline hypobromite. The isotope ratios in the nitrogen samples were measured with a Hitachi-Perkin Elmer RMU-6D mass spectrometer equipped with an MD-1020 double collector. Measurements were controlled by frequent determinations of the natural abundance in air and by measurements on previously analyzed amino acid samples kindly donated by Dr. D. Shemin. Also, to exclude possible errors in measurements resulting from air contamination or from incomplete combustion of the compounds, peak heights at mass 32 (oxygen) and mass 30 (methylamine) were measured for each sample and compared with the peak heights at mass 29 ($^{14}N^{15}N$). The estimated accuracy of the isotopic abundance measurements was 0.02 atom % excess ^{15}N. Samples showing less enrichment than this value were considered to have the natural abundance.

Results

The distribution of excess isotope in the urinary purine bases examined is shown in Table III. Most of the excess isotope recovered was excreted on the first day. The 7-methylguanine from that period contained 48% of the excess ^{15}N administered and the 8-hydroxy-7-methylguanine contained 16%. In the urine excreted on the second day, these compounds each contained 0.5% of the ^{15}N given. No significant excess of isotope was found in the compounds isolated from urine collected on subsequent days. Uric acid and xanthine contained no excess isotope in any of the samples analyzed. It should perhaps be emphasized that daily excretion data and recoveries of ^{15}N have been computed on the basis of ultraviolet measurements made in the column chromatography, rather than on weight of crystalline materials finally isolated. (The amounts of crystalline compounds were less than the

analytical values derived from ultraviolet measurements, and the yields were variable.)

Discussion

The observed incorporation of isotope from 7-methylguanine into 8-hydroxy-7-methylguanine (Table III) clearly establishes a precursor–product relationship between them. The nearly identical isotopic abundance in these compounds indicates that 7-methylguanine must be the sole source of 8-hydroxy-7-methylguanine in man. An independent origin from s-RNA is thus precluded. The similar isotopic abundance also shows that the observed oxidation of 7-methylguanine must be extremely rapid. Absence of appreciable excess isotope in the 7-methylguanine and 8-hydroxy-7-methylguanine excreted on the second day (Table III) indicates that there are no pools of any consequence of these compounds. Thus, it appears that the 7-methylguanine which is not oxidized to 8-hydroxy-7-methylguanine is excreted as rapidly as it is formed. Supporting evidence for the rapid excretion of endogenously produced 7-methylguanine is the finding (Table III) of increased excretion immediately following administration of this compound.

The rapid oxidation of 7-methylguanine to 8-hydroxy-7-methylguanine seems to indicate that the reaction must be enzymatically catalyzed. The enzyme responsible for this oxidation is unknown and may not be present in other mammals since unpublished experiments indicate the absence of detectable quantities of 8-hydroxy-7-methylguanine in rat urine. Unlike guanine and N^2-methylguanine, 7-methylguanine is not a substrate for milk xanthine oxidase (Weissmann and Gutman, 1957; Wyngaarden, 1957). The human enzyme might conceivably have a different substrate specificity; however, a limited comparison of human liver xanthine oxidase with milk xanthine oxidase has shown no significant differences in substrate specificity (Bergmann and Dikstein, 1956). Involvement of enzymes other than xanthine oxidase in the oxidation of purine rings has been suspected previously. For example, theophylline, theobromine, and caffeine, which are not substrates for xanthine oxidase (Coomb, 1927), are nevertheless known to be oxidized in mammals (Brodie *et al.*, 1952). The enzyme catalyzing these oxidations has not been characterized. With respect to the oxidation of 7-methylguanine, this may perhaps be a substrate for a nonspecific enzyme, similar to one reported to occur in liver microsomes; this catalyzes nonspecific hydroxylations of aromatic compounds (Mitoma *et al.*, 1956). Quinoline, the only heterocyclic compound tested with the microsomal enzyme, was oxidized to 3-hydroxyquinoline (Mitoma *et al.*, 1956). The possible agency of a more specific enzyme in the oxidation of 7-methylguanine cannot be excluded. Such an enzyme might be analogous to the quinoline-oxidizing enzyme; this enzyme is specific for heterocyclic compounds such as quinoline, isoquinoline, and substituted pyridines with available α-hydrogen atoms (Knox, 1946).

Some 35% of the isotope administered was not accounted for in the excretion products. It is believed that the entire discrepancy may readily be accounted for by shortcomings of the isolation methods. These have previously been shown to give incomplete recoveries, especially for 8-hydroxy-7-methylguanine (Weissmann *et al.*, 1957b). However, fates for the administered compounds other than those discussed are not completely excluded by the data. For example, rough calculation shows that if the 35% of labeled 7-methylguanine in question had suffered demethylation on the first day with the ultimate generation of uric acid, the uric acid sample isolated would contain about 0.02 atom % excess of ^{15}N. Such a value would be only marginally detectable under the circumstances of measurement.

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Studies on the Nucleotide Arrangement in Deoxyribonucleic Acids. XI. Selective Removal of Cytosine as a Tool for the Study of the Nucleotide Arrangement in Deoxyribonucleic Acid*

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ABSTRACT: When calf thymus deoxyribonucleic acid (DNA) is treated with nitrous acid at pH 3.35, complete deamination takes place. The resulting product is not dialysable and retains about 96% of the original DNA phosphorus. It contains the initial proportions, characteristic of the starting DNA, of thymine and uracil (from cytosine), and about 80% of the original adenine in form of hypoxanthine, whereas most of the xanthine (from guanine) is found as the free purine. Apurinic acid and oligonucleotides derived from DNA can be treated similarly. From such preparations of deamino-DNA or of deaminated apurinic acid, uracil can be eliminated selectively by treatment with hydroxylamine at pH 10 as shown by the analysis of several products. The application of these procedures to the study of the arrangement of thymidylic acid and of its oligonucleotides in DNA is illustrated by a few examples, namely, the determination of the proportion of position isomers in a dinucleotide consisting of thymidylic and deoxycytidylic acids and the liberation of the thymidylic acid runs of lengths 1–5 occurring in calf thymus DNA.

An unambiguous solution of the problem of the nucleotide sequence of a cellular deoxyribonucleic acid (DNA) is not likely to occur in the near future. The very great length of the chains composed of a very small number of different building blocks, the absence of enzymes of a narrow specificity, the lack of generally applicable methods for the determination of end groups, and the existence of many lacunas in our understanding of even the primary structure of these high polymers all combine to render practical only the most general statistical interpretation of the nucleotide arrangement. This has been discussed before (Chargaff, 1963; Shapiro *et al.*, 1965).

This communication attempts to remove one of the difficulties encountered in the study of the pyrimidine nucleotide runs in DNA. A naturally occurring polynucleotide chain is composed of alternating runs of purine and pyrimidine nucleotides of different length and composition. Differential degradative procedures permit the separate isolation of either the purine or the pyrimidine sequences of a DNA. Thus, the acid degradation of DNA or the alkaline degradation of apurinic acid afford a mixture of pyrimidine isostichs (Shapiro and Chargaff, 1964) of the general structure[1] Py_np_{n+1}, whereas by the alkaline degradation of apyrimidinic acid the corresponding purine isostichs, Pu_np_{n+1}, become available (Chargaff *et al.*, 1963, 1965). In all instances, either the purines flanking the pyrimidine runs or the pyrimidines flanking the purine runs must be removed before the intact sequences are liberated by a series of β-elimination reactions (Shapiro and Chargaff, 1957).

The oligonucleotides, which result from the chromatographic separation of the hydrolysates, will in many cases consist of mixtures of position isomers. For instance, the fraction CT_2p_4 can be prepared (Spencer and Chargaff, 1963; Shapiro and Chargaff, 1963); but in most cases it will presumably comprise the three

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[1] The following abbreviations are used: Pu, purine deoxyribonucleoside; Py, pyrimidine deoxyribonucleoside; A, deoxyriboadenosine; I, deoxyriboinosine; G, deoxyriboguanosine; C, deoxyribocytidine; U, deoxyribouridine; T, thymidine; O, deoxyribose. Esterified phosphate is indicated as p, placed on the right of the symbol when linked to the 3′-hydroxyl and on the left when linked to the 5′-hydroxyl. When no specified sequence of an oligonucleotide fraction is implied, the appropriate symbols are followed by the number of phosphate groups in the particular fraction. Thus, CTp_3 can indicate a mixture of the position isomers pCpTp and pTpCp.