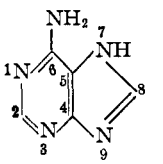
[CONTRIBUTION FROM THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH, NEW YORK]

Degradations in the Purine Series Studied with Isotopes of Nitrogen and Carbon[1]

BY LIEBE F. CAVALIERI, JOHN F. TINKER AND GEORGE BOSWORTH BROWN

Tracer studies concerned with the metabolism of purines have evoked the need for methods suitable for determining the location of isotopes in purines isolated from tissue constituents. Oxidative and hydrolytic degradations of the purines adenine,[2,3,4] guanine,[5,6] xanthine,[7] hypoxanthine[2] and uric acid have been reported but only in the case of guanine[6] has the mechanism been clarified by the degradation of samples labeled with isotopes in known positions. The availability in this laboratory of several synthetic purines variously labeled with the isotopes of nitrogen (N^{15}) and carbon (C^{13} or C^{14}) has permitted the study of a number of such degradations.

The action of concentrated hydrochloric acid on the purines, adenine, guanine, xanthine, hypoxanthine and uric acid leads to the formation of glycine, ammonia, carbon monoxide and carbon dioxide. By analogy to the results with 7-methyl uric acid, which yields sarcosine under these conditions,[8] it has been assumed for both uric acid[9,10] and guanine[11] that the glycine arises from the 4- and 5-carbon atoms and the 7-nitrogen. However, no direct proof of this assumption has been presented.



I

Adenine (I), labeled with C^{13} in the 4- and 6-positions and with N^{15} in the 1- and 3-positions, was hydrolyzed with concentrated hydrochloric acid and the glycine which was isolated contained excess C^{13}. Decarboxylation of a sample of *p*-tosylglycine revealed that the C^{13} was present only in the carboxyl group of the glycine (Table I). Further, since the isotope concentration in the carbon dioxide was equal to that originally present in either the 4- or 6-carbons, dilution had not occurred. That no excess N^{15} was found in the glycine indicates that the amino group must have arisen from the 7- or 9-nitrogens or the 6-amino group of adenine, but not from the 1- or 3-nitrogens. Origin of the amino group of glycine from the 6- or 9-nitrogens would require the carboxyl group to come from the 5-carbon, but since it was the carboxyl carbon of the isolated glycine which contained the excess C^{13}, it follows that the amino group of the glycine originated from the 7-nitrogen.

In the case of guanine a sample was hydrolyzed which contained C^{13} in the 4-position only.[12] This led to the formation of glycine with the C^{13} in the carboxyl group and this decisively confirms the origin of the carboxyl carbon of glycine from this purine.

The acid hydrolysis of guanine, labeled with N^{15} in the 1- and 3-positions and in the 2-amino group, and of xanthine and uric acid, each labeled in the 1- and 3-positions, yielded glycine with no excess N^{15}. This is in conformity with the origin of the glycine from the 4, 5 and 7 atoms.

Under milder conditions, the acid hydrolysis of adenine led to the formation of 4(5)-amino-5(4)-imidazolecarboxamidine (II). Since II exhibits a distribution constant (0.12 *n*-butanol phosphate, *p*H 6.5) different from that of adenine

TABLE I

HYDROCHLORIC ACID HYDROLYSIS

	Starting compound					Degradation product				
			Nitrogen, %		Isotope		Nitrogen, %		Isotope analysis[a]	
Name	Label	Formula	Calcd.	Found	analysis[a]	Formula	Calcd.	Found	Expected	Found
Adenine	N^{15} in 1, 3	$(C_5H_5N_5)_2\cdot H_2SO_4\cdot 2H_2O$	34.9	35.2	N^{15}, 0.36	$C_9H_{11}O_4NS$[b]	6.1	6.3	N^{15}, 0.00	0.01[c]
Adenine	C^{13} in 4, 6		8.1[d]	7.9[d]	C^{13}, 0.14	CO_2[e]			C^{13}, 0.33	0.35
Guanine	N^{15} in 1, 3	$(C_5H_5ON_5)_2\cdot H_2SO_4\cdot 2H_2O$	32.1	31.9	N^{15}, 0.60	$C_9H_{11}O_4NS$	6.1	6.3	N^{15}, 0.00	0.01[c]
Guanine	C^{13} in 4		32.1	32.2	C^{13}, 0.09	CO_2[e]			C^{13}, 0.45	0.43
Xanthine	N^{15} in 1, 3	$C_5H_4O_2N_4$	36.9	36.2	N^{15}, 0.50	$C_9H_{11}O_4NS$	6.1	6.4	N^{15}, 0.00	0.02[c]
Uric acid	N^{15} in 1, 3	$C_5H_4O_3N_4$	33.3	33.7	N^{15}, 0.50	$C_9H_{11}O_4NS$	6.1	6.2	N^{15}, 0.00	0.02[c]

[a] Atom % excess. [b] In all cases glycine was isolated as the *p*-tosyl derivative. [c] These values represent about 1% of the N^{15} present in either the 1- or 3-positions. This N^{15} probably arises from *p*-toluenesulfonamide which is formed from traces of ammonia remaining in the residue of the reaction mixture. [d] Sulfur analysis. [e] This carbon dioxide was obtained by the decarboxylation of the *p*-tosylglycine.

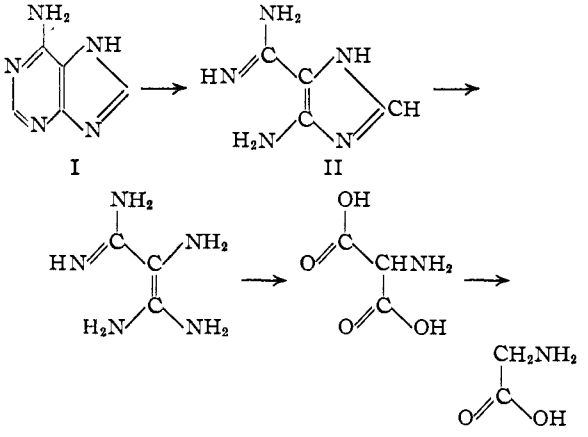
(1) This work was supported by grants from the National Cancer Institute of the United States Public Health Service, the Office of Naval Research, United States Navy and the James Foundation of New York, Inc.

(2) Krüger, *Z. physiol. Chem.*, **16**, 160 (1892).
(3) Kossel, *ibid.*, **12**, 241 (1888).
(4) Jolles, *J. prakt. Chem.*, [2] **62**, 61 (1900).
(5) Wulff, *Z. physiol. Chem.*, **17**, 468 (1893).
(6) Plentl and Schoenheimer, *J. Biol. Chem.*, **153**, 203 (1944).
(7) Schmidt, *Ann.*, **217**, 308 (1883).
(8) Fischer, *Ber.*, **32**, 435 (1899).
(9) Shemin and Rittenberg, *J. Biol. Chem.*, **167**, 875 (1947).
(10) Sonne, Buchanan and Delluva, *J. Biol. Chem.*, **173**, 69 (1948).
(11) Abrams, Hammarsten and Shemin, *J. Biol. Chem.*, **173**, 429 (1948).

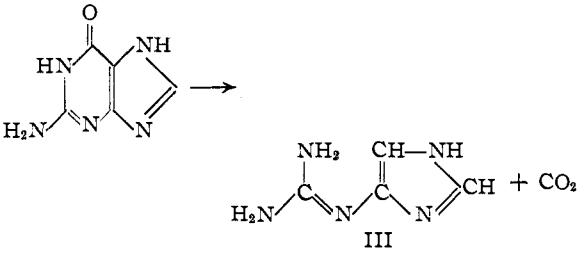
(12) Lowy, unpublished.

(2.2) reaction mixtures were readily analyzed and optimum conditions for the formation of II from adenine were thus easily found. The production of this carboxamidine is consistent with the observed production of formic acid in the hydrolysis of adenine or of nucleic acids.[13] This degradation permits a differentiation between the 2- and 8-carbons of adenine.

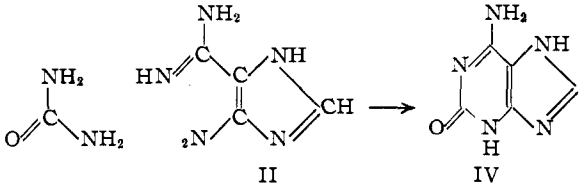
On the basis that the amidine (II) is an intermediate, it can be postulated that the formation of glycine from adenine proceeds according to the scheme shown.



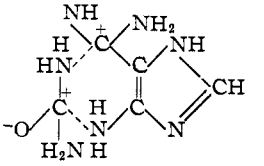
The sequence of steps involving the degradation of II may not necessarily be the one shown, but the over-all mechanism is consistent with the data. It is of interest to note that 4(5)-guanidinoimidazole (III) has been isolated by Hunter[14] after subjecting guanine to very nearly the same hydrolytic conditions.



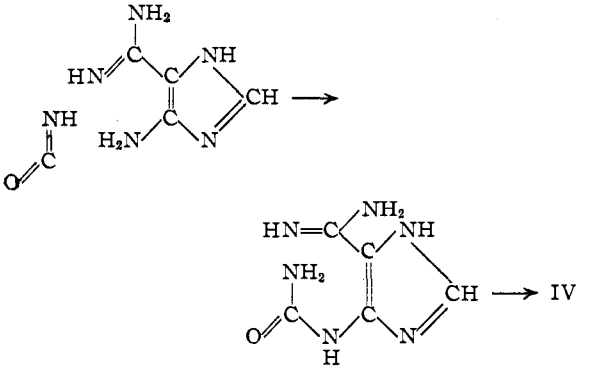
Proof of structure of the amidine (II) is found in the conversion to isoguanine (IV) by the



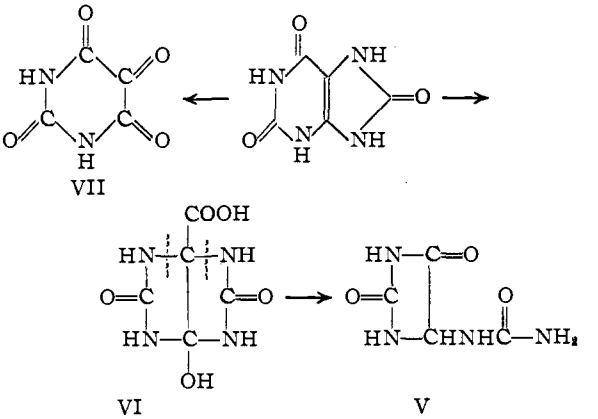
action of either urea or phosgene. When the fusion with urea was carried out on a sample of II containing N^{15} in both the amino and amidine groups (prepared from 1,3 labeled adenine), the N^{15} of the resulting isoguanine (III)[15] was 88% of that originally present in the II. Since it is likely that the 4(5)-amino group is a nucleophilic center attacking the electrophilic carbon of the urea, it is reasonable to assume that the N^{15}



was not eliminated from the amino substituent, but rather from the amidine grouping which can act both as a nucleophilic and electrophilic entity. Indeed, the 12% loss of N^{15} may be taken as a measure of the electrophilic character of the amidine unit in this molecule. An alternative but similar mechanism involves the addition of cyanic acid which is present in the reaction mixture.



Oxidative degradations of the purines have been carried out employing acid and alkaline permanganate, perchlorate,[6] nitric acid,[16,17] and various other oxidizing agents. The oxidation of uric acid with alkaline permanganate yields allantoin (V) via a symmetrical intermediate (VI), while



(13) Stevens, *J. Biol. Chem.*, **120**, 751 (1937).

(14) Hunter, *Biochem. J.*, **30**, 1183 (1936)

(15) Bendich, Tinker and Brown, THIS JOURNAL, **70**, 3109 (1948).

(16) Wöhler and Liebig, *Ann.*, **26**, 256 (1838).

(17) Biltz and Heyn, *ibid.*, **413**, 60 (1917).

TABLE II

OXIDATION OF ADENINE

Position of tracer	Adenine: Elementary analyses, % Calcd.	Found	Isotope analysis[a]	Dixanthydrol urea: Calcd.	Elementary analysis, % Found	Isotope analysis[a]	NH_3 Isotope	CO_2 analysis[a]
1,3-N^{15}	S, 8.3	8.2	0.43	N, 6.7	6.7	0.50	0.42	
4,6-C^{13}	S, 8.3	7.9	0.14					0.14
8-C^{13}	N, 28.9	28.7						
	S, 13.2[b]	13.7	0.54	N, 6.7	6.9	0.01		
4,6-C^{14}	N, 51.8	52.2	126 cpm/mg.[c]	N, 6.7	6.1 cpm/mg.[d]			

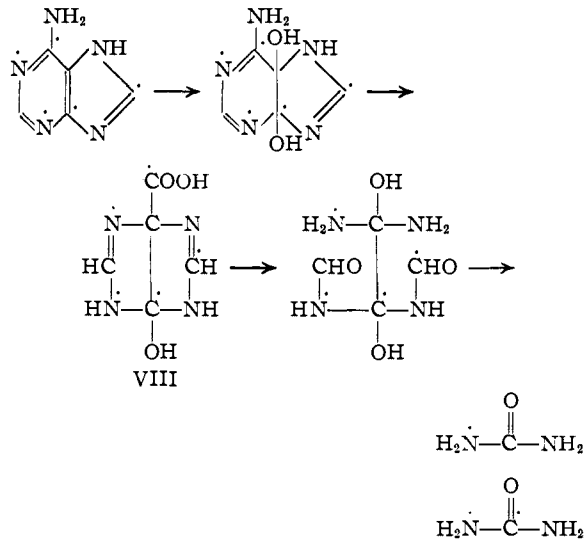
[a] The C^{13} and N^{15} values are expressed as atom % excess over the normal abundance. [b] Adenine may be obtained as the mono sulfate depending upon the rate of crystallization. [c] Sufficient adenine was burned to give a value of 4500 c. p. m. over background. [d] Sufficient dixanthydrol urea was burned to give a value of 150 c. p. m. over background.

with nitric acid the imidazolone ring is cleaved to produce alloxan (VII).[18]

The oxidation of adenine with permanganate in acid solution has been reported[3,4] to yield one mole of glycine, two moles of urea, and one mole of carbon dioxide. In our hands, this reaction did not result in the formation of glycine. The mechanism of this reaction was studied employing adenine which was labeled with N^{15} in the 1- and 3-positions, C^{14} in the 4- and 6-positions and C^{13} in the 8-position.[19] The oxidation was carried out by the use of potassium permanganate in sulfuric acid solution. Urea was isolated as dixanthydrol urea, and the ammonia and carbon dioxide collected. Since the isotope values of the ammonia and carbon dioxide evolved were essentially equal to those of the parent adenine, it is clear that complete degradation of a large portion of the sample occurred.

The isotope analyses (Table II) reveal that the N^{15} content of the urea isolated was appreciably higher than the average N^{15} content of the adenine and was one-half (47%) of that contained in either the 1 or 3 nitrogens. Since only 0.01 atom % of C^{13} was found in the urea, the 7-, 8- and 9-atom unit contributed but to a small extent to the urea found. The ratio of C^{14}:C^{12} in the urea was 0.6:1.0 and it was constant upon repeated recrystallization of the dixanthydrol urea.

Upon examination of the adenine molecule it is evident that, if no rearrangement occurs, the urea can arise in four ways, *viz.*, from the (1,3), (1,6), (3,9) or (7,9) positions. No one of these four possibilities or any combination of them is consistent with the isotope analyses. On this basis it appears necessary to postulate a rearrangement similar to that occurring in the permanganate oxidation of uric acid in alkaline solution. In this case, however, it is required that preponderant cleavage of the proposed intermediate (VIII) takes place as shown, although the C^{13} value, together with the low C^{14}:C^{12} ratio, indicates some cleavage similar to that of VI to V. Since a sample of urea under the conditions of the experiment was oxidized to the extent of only 5 to 10%, it can be assumed that once the urea is formed in the reaction it remains for the most part intact. Thus each molecule of the urea



VIII

contained one atom of N^{15} originally present in the 1- or 3-position and one normal nitrogen from the 7- or 9-position. Appreciably less than half of the urea molecules must have contained C^{14}, arising chiefly from the 4-carbon.

Experimental

Isotope Analyses.—All N^{15} and C^{13} determinations were carried out with a Consolidated–Nier mass spectrometer, Model 21-201. The average error in the N^{15} determinations was ±0.002; in the C^{13} determinations ±0.005. For the C^{14} measurements the samples were converted to barium carbonate and the analyses were carried out on carbon dioxide.[20]

Materials.—Adenine containing N^{15} in the 1- and 3-positions and C^{13} (or C^{14}) in the 4- and 6-positions was synthesized according to Cavalieri, Tinker and Bendich[21] and adenine labeled with C^{13} in the 8-position was prepared according to Cavalieri and Brown.[19] Guanine, xanthine[2,22] and uric acid[23] with N^{15} in the 1- and 3-positions were prepared as in previous communications. C^{13} was introduced into the 4-position of guanine by employing methyl cyanoacetate ($C^{13}NCH_2COOCH_3$) obtained by a previous procedure.[21]

(18) Cavalieri and Brown, THIS JOURNAL, **70**, 1242 (1948).

(19) Cavalieri and Brown, *ibid.*, **71**, 2246 (1949).

(20) Eidinoff, *Science*, **108**, 535 (1948).

(21) Cavalieri, Tinker and Bendich, THIS JOURNAL, **71**, 533 (1949).

(22) Getler, Roll, Tinker and Brown, *J. Biol. Chem.*, **178**, 259 (1949).

(23) Cavalieri, Blair and Brown, THIS JOURNAL, **70**, 1240 (1948).

4(5)-Amino-5(4)-imidazolecarboxamidine Hydrochloride (II).—Adenine sulfate (2 g.) was heated in a sealed tube containing 30 cc. of 6 *N* hydrochloric acid at 150 ± 2° for two hours. The solution was evaporated to dryness and extracted with two 10-cc. portions of warm concentrated hydrochloric acid. To this filtrate was added 30 cc. of ethanol and the solution cooled to induce crystallization. The crystalline deposit was recrystallized three times by dissolving it in warm concentrated hydrochloric acid (*ca.* 2 cc.) and adding alcohol (15 cc.). The yield of pure material was 200 mg. (10%); distribution constant, 0.12 (*n*-butanol–phosphate buffer, *p*H 6.5); ultraviolet absorption spectrum: λ_{max} 287 mμ, log ε 4.03.

Anal. Calcd. for $C_5H_7N_5 \cdot 2HCl$: N, 35.3. Found: N, 35.3.

Conversion of II to Isoguanine. (a) Phosgene Method.—Fifty milligrams of II was dissolved in 5 cc. of *N* sodium hydroxide and phosgene passed through the solution for one hour, during which time three 4-cc. portions of 40% sodium hydroxide were added. The mixture was then aerated for one hour. One cubic centimeter of 2 *N* sulfuric acid was added and the solution heated, decolorized and filtered. On cooling 14 mg. (26%) of material was obtained which was recrystallized from 2 *N* sulfuric acid. The ultraviolet absorption spectrum[24] at various *p*H values was identical with that of isoguanine prepared by another synthesis.[15]

Anal. Calcd. for $(C_5H_5ON_5)_2 \cdot H_2SO_4 \cdot H_2O$: S, 7.6. Found: S, 7.7.

(b) Urea Fusion Method.—Seventy-five milligrams of II (containing 0.97 atom % excess N^{15}) was fused with 410 mg. of urea at 180° for one hour. The cooled melt was extracted with 5 cc. of boiling water and the extract discarded. The residue was recrystallized twice from 2 *N* sulfuric acid; yield 37 mg. (47%). The N^{15} content of this isoguanine sulfate was 0.85 or 88% of that present in II.

Anal. Calcd. for $(C_5H_5ON_5)_2 \cdot H_2SO_4 \cdot H_2O$: S, 7.6. Found: 7.8.

Hydrolytic Degradation.—The hydrolyses of adenine, guanine, xanthine and uric acid were carried out by heating about 1 g. of the purine in 15 cc. of concentrated hydrochloric acid at 180° in a sealed tube for eighteen hours (Table I).

Isolation of Glycine.—The mixture from the above hydrolysis was evaporated to dryness and to the residue was added 10 cc. of 5% sodium hydroxide. The ammonia was removed by aeration, while warming the mixture. One gram of *p*-toluenesulfonyl chloride was added to the alkaline solution and the mixture stirred for six hours at room temperature. The solution was filtered and acidified. The precipitation of *p*-tosylglycine was complete in two hours and the product was collected by filtration. Two recrystallizations from ethyl acetate–petroleum ether gave 30–50 mg. of pure *p*-tosylglycine (Table I).

Decarboxylation of *p*-Tosylglycine.—*p*-Tosylglycine (20 mg.) was mixed intimately with copper powder and heated to 200 ± 5° for one-half hour in a stream of nitrogen. The carbon dioxide was collected in saturated barium hydroxide; yield $BaCO_3$, 14–16 mg. (81–92%).

Oxidation of Adenine (Table II).—Adenine sulfate (400 mg.) was dissolved in 80 cc. of water containing 4 cc. of concentrated sulfuric acid. To the boiling solution was added dropwise over a period of five minutes 2.6 g. of potassium permanganate in 35 cc. of water. Carbon dioxide was collected in saturated barium hydroxide during this time. After the outlet tube had been disconnected the excess permanganate was destroyed with oxalic acid. The solution was filtered, and 160 cc. of glacial acetic acid was added followed by 2 g. of xanthydrol (in 20 cc. methanol). Precipitation of dixanthydrol urea began after one-half hour and was complete after standing overnight. The product was recrystallized three times from glacial acetic acid; yield 120 mg. A sample of ammonia was obtained from the original oxidation mixture (after treatment with oxalic acid) by adding excess alkali and collecting the ammonia in boric acid.

Acknowledgment.—The authors wish to thank Dr. Maxwell Leigh Eidinoff for performing the C^{14} determinations, Roscoe C. Funk, Jr., and John Deonarine for the microanalyses and the N^{15} and C^{13} determinations.

Summary

The acid hydrolysis of purines has been studied by means of tracer elements, and it has been confirmed that glycine arises from the 4,5- and 7-atoms. The oxidation of adenine with potassium permanganate produces urea which is postulated to arise from the 1,7- and 3,9-nitrogen atoms.

The preparation and characterization of 4(5)-amino-5(4)-imidazolecarboximidine are described.

A new synthesis of isoguanine is described.

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NEW YORK, N. Y. RECEIVED JUNE 6, 1949

(24) Cavalieri, Bendich, Tinker and Brown, THIS JOURNAL, **70**, 3875 (1948).

[CONTRIBUTION FROM THE RESEARCH LABORATORY OF GALAT CHEMICAL DEVELOPMENT, INC.]

A New Method for the Isolation of Histamine

BY ALEXANDER GALAT AND HARRIS L. FRIEDMAN

Histamine is usually prepared by the bacterial decarboxylation of histidine in solution, and it is isolated in the form of its dipicrate. The decarboxylation of histidine proceeds readily and in good yield but the isolation of pure histamine by the picrate method offers a number of disadvantages. The purification of the dipicrate by recrystallization from water or dilute alcohol requires large volumes of solvent since the compound is sparingly soluble even at the boiling point. The conversion of the dipicrate into histamine salts, such as the dihydrochloride, involves the treatment of the dipicrate with an excess of hydrochloric acid, removal of the bulk of picric acid by filtration and extraction with an organic solvent to free the product from the last traces of this reagent. Because of the low solubility of the dipicrate these operations must be conducted in hot, dilute solutions which finally must be evaporated to dryness. This method is quite inconvenient for the preparation of relatively large amounts of histamine.

Recently, Vickery described a method for the isolation of histidine involving the precipitation of