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Pyrimidines. V. Rearrangement of [*amino*-^{15}N]Cytosine and a Preparation of [$^{15}N_3$]Uracil*

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ABSTRACT: [*amino*-^{15}N]Cytosine (compound II), in refluxing acetic anhydride–acetic acid, undergoes a reversible rearrangement involving an exchange between the exocyclic amine nitrogen and the nitrogen in position 3 of the pyrimidine ring. This rearrangement leads to a mixture of [$^{15}N_3$]cytosine and [*amino*-^{15}N]-cytosine (compound VI) which is easily converted to uracil containing label in the N_3 position. The abnormal values previously obtained for the estimation of the exocyclic amine nitrogen of cytosine by the Van Slyke method are confirmed. Catalytic reductive elimination of the exocyclic amine function provides an elegant method for the determination of the isotope content of the amino nitrogen in labeled cytosines II and VI.

A previous paper in this series (Ueda and Fox, 1964) reported that N_4-methylcytosine, when refluxed with acetic anhydride for prolonged periods, underwent ring opening and rearrangement resulting in a partial conversion to 3-methylcytosine. The reversibility of this reaction was demonstrated and a plausible mechanism for the rearrangement was proposed. In this reversible reaction, the rearrangement was easily discerned by the identification of the final monomethylated cytosines. They suggested that this rearrangement should also occur when *N*-acetylcytosine was treated with acetic anhydride; this reaction should likewise be reversible.

The postulated mechanism (Ueda and Fox, 1964) would involve an intermolecular attack by N_3 of compound III on acetic anhydride (see structure) which would produce the intermediate isocyanate IV which could then recyclize (as indicated by the solid arrows) to regenerate acetylcytosine. Since the nitrogen of the exocyclic[1] amino group and N_3 in intermediate IV are essentially equivalent, the theoretical equilibrium between the two possible products of recyclization should be a 50–50 distribution of the isotope. Therefore, if the exocyclic amino function of compound II were isotopically labeled with ^{15}N and this compound were treated with acetic anhydride–acetic acid for prolonged periods, one should obtain, after deacetylation, a mixture of cytosines labeled in the exocyclic amino group or in the ring N_3 atom.[2] Moreover, deamination of this mixture should yield uracil bearing ^{15}N in the N_3 position, which would constitute conclusive proof that the rearrangement had occurred. Finally since both cytosine and uracil are constituents of nucleic acids, the ready availability of such specifically labeled pyrimidines may be of value in certain biochemical investigations. This paper deals with the synthesis of cytosine labeled in the amino group with ^{15}N and its conversion to N_3-labeled uracil compound through an intermediate mixture of labeled cytosines.

Treatment of 4-methylthiopyrimidone-2 (compound I) (Wheeler and Johnson, 1909) with an alcoholic solution of ^{15}N-enriched ammonia gave [*amino*-^{15}N]-cytosine in good yield.[3] By refluxing a suspension of compound II in acetic anhydride containing a little glacial acetic acid for 24 hours, acetylcytosine (compound V) was obtained.[4] Regeneration of labeled cytosine (compound VI) from the acetyl compound

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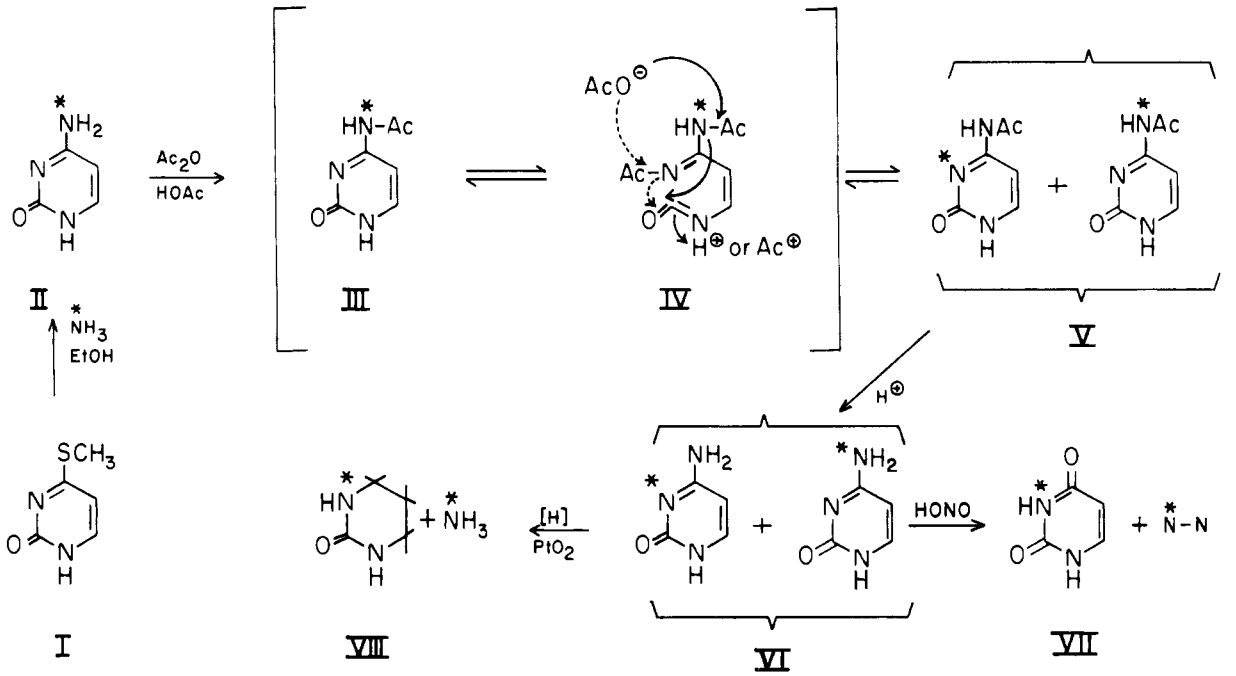
[1] It is understood that although intermediate IV is an open-chain compound, the nomenclature of the pyrimidine ring has been retained for clarity.

[2] A cytosine labeled with ^{15}N in both N_1 and N_3 has been synthesized from ^{15}N-enriched urea and cyanoacetal (Bendich *et al.*, 1949).

[3] 4-Thiouracil itself was only partially aminated under the conditions used.

[4] A minor contaminant, presumably the diacetate, was also formed as revealed by paper chromatography, but this material was not investigated further.



was carried out and the total ^{15}N content measured was found to be the same as that of compound II or V, i.e., there had been no loss of label. Deamination of compound VI was performed with either nitrous acid or hydrochloric acid, the latter being the method of choice. The resulting uracil (compound VII) was purified by ion-exchange chromatography. Determination of the ^{15}N content showed that in the 24-hour acetic anhydride treatment, interchange of the exocyclic amino- and N_3 nitrogens had occurred to the extent of 25%. Since a 100% equilibrium should be theoretically possible, aliquots of the 24-hour product were retreated in the refluxing acetic anhydride–acetic acid system for a total time of 72 and 108 hours, respectively. The latter reaction mixture became very dark owing to extensive decomposition. The products of these extended reaction periods were subjected to exactly the same procedures used for the 24-hour reaction and the ^{15}N content of the resulting labeled uracils was measured. The percentage rearrangement was determined to be 43% for the 72-hour reaction product (see Table I) and about 40% for that derived from the 108-hour reaction. Thus it is demonstrated that the exchange between the exocyclic amino group and N_3 in fact does occur to a large extent. All of the following discussion and the data included in Table I (except compound II) are confined to the product of the 72-hour treatment.

The proportional amount of the total ^{15}N content of the starting material II which was found in the [$^{15}N_3$]-uracil was 21.3%. Therefore 42.5% of the possible equilibrium had been reached and *ca.* 78% of the total label should still remain in the exocyclic amino group. In order to confirm these values, a nitrous acid deamination of compound VI was performed. The resulting value for the ^{15}N content of the nitrogen evolved (see Table I) was 55% of the calculated value for the exocyclic amino nitrogen. These data indicate that a dilution with 45% of additional nitrogen had occurred, presumably from degradation of the pyrimidine ring. To demonstrate that this dilution was real, the same nitrous acid deamination was carried out on the un-

TABLE I: Isotope Analyses on Products from 72-Hour Experiment.

Compounds	Atom % Excess		
	Found[a]	Calcd. for	
Cytosine (II)	1.95, 1.94	NH_2	5.85
Acetylcytosine (V)	1.97, 1.96	$NH_2 + N_3$	5.89
Cytosine, regenerated (VI)	1.96, 1.98	$NH_2 + N_3$	5.91
Uracil, by HCl (VII)	0.59, 0.64	N_3	1.24
Uracil, by HONO (VII)	0.62, 0.64	N_3	1.26
N_2, from VI	1.27, 1.30	NH_2	2.57
N_2, from II	1.29, 1.28	NH_2	2.52
NH_3, by reduction of VI	4.58		4.58

[a] The ^{15}N values were determined with a Consolidated-Nier isotope ratio mass spectrometer, Model 21-201. NH_3 was obtained from compounds II–VII by Kjeldahl digestion and distillation; the resulting ^{15}N-enriched ammonium chloride was oxidized by sodium hypobromite to nitrogen gas, which was then introduced into the mass spectrometer (Rittenberg, 1946; Nier, 1946; Francis *et al.*, 1954; Kamen, 1957). The details of the preparation of the gas samples from the last three compounds are discussed in the experimental section.

rearranged [^{15}N]cytosine (compound II) and a similar excessive dilution was found. The agreement between the two values (Table I) suggests that the opening of the cytosine ring by nitrite occurred and that the amino and N_3 are the nitrogens diazotized under these reaction conditions. These data confirm the abnormal yield of nitrogen noted previously (Wilson, 1923) in the Van Slyke amino analyses on cytosine. It was obvious therefore that a proper ^{15}N content of the exocyclic amino group of compound VI could not be obtained by this procedure.[5]

Fortunately, an alternative method was discovered recently by Iwasaki (1962), who found that when an aqueous solution of cytosine was subjected to hydrogenation with platinum oxide as catalyst at ordinary temperature and pressure, reductive elimination of the amino group occurred to yield trimethylene urea (Fox and Van Praag, 1960; Fischer and Koch, 1886) and ammonia. Therefore such a reduction was carried out on the rearranged cytosine (compound VI) with dilute hydrochloric acid as the ammonia acceptor. After reduction was completed, as confirmed by complete loss of ultraviolet absorption, the reduction mixture was treated with strong alkali to release the ammonia, which was caught in dilute acid. The ^{15}N content of this ammonia, 4.58 atom % excess, demonstrated that 55.7% of the equilibrium value (2.94) remained in the amino group, in excellent agreement with the 42.5% entering the N_3 of uracil.

Experimental

[amino-^{15}N]Cytosine. A solution of 28 g. NH_4NO_3 (containing $^{15}NH_4$, *ca.* 6 atom % excess) in 100 ml of water was heated to 95° and treated dropwise with 60 ml of a 30% NaOH solution (w/v). The gas evolved was swept by nitrogen through a drying tower filled with soda lime and then introduced through a gas-sparger into ethanol (60 ml) previously chilled to *ca.* −70° in a dry-ice–chloroform bath. After *ca.* 15 minutes, the ethanolic ammonia was transferred to a glass-lined bomb tube containing 2 g (0.014 mole) of 4-methylthiopyrimidone-2. The sealed bomb was heated at 150° for 22 hours. After chilling, the bomb was opened and the solid was filtered and washed with absolute ether. The yield of crude product was 1.38 g (89%). From evaporation of the mother liquor, another 60 mg was obtained. The product was recrystallized three times from boiling water with use of decolorizing charcoal for the first recrystallization. The product was pure by spectrophotometric and chromatographic examination (2-propanol-HCl-H_2O, 17:4:4) when compared with authentic material. The ^{15}N content of the product, determined by the mass spectrometer (see footnote to Table I) was found to be 5.85 atom % excess.

Rearrangement of [amino-^{15}N]Cytosine (Compound V). One g of the [*amino*-^{15}N]cytosine was added to a mixture of 10 ml of acetic anhydride and 2 ml of glacial acetic acid and the resulting suspension was refluxed for 24 hours. The cooled reaction mixture was filtered and the solid was freed of acid by washing with cold alcohol followed by ether. The yield of *N*-acetylcytosine was 1.22 g (88%). Paper chromatography showed the presence of authentic *N*-acetylcytosine and a second minor spot of high R_F which is probably some diacetylated material. The ^{15}N content of the product was identical with that of the starting material.

Two aliquots of the acetylcytosine from the above rearrangement were each re-treated with the acetic anhydride–acetic acid system for a total period of 72 and 108 hours, respectively, and the resulting products together with that from the 24-hour reflux period were subjected separately to deamination, isolation of the uracil, and a determination of the ^{15}N content of each.

Regeneration of [^{15}N]Cytosine after Rearrangement (Compound VI). A solution of 400 mg *N*-acetyl[^{15}N]-cytosine in 2 N HCl was allowed to stand overnight at room temperature. The shift in the ultraviolet-absorption maximum in acid solution from 304 to 275 mμ indicated that deacetylation was complete. The solution was evaporated *in vacuo* and the residue was recrystallized from water. The product, 235 mg (82%), was chromatographically pure (2-propanol-H_2O, 7:3).

[$^{15}N_3$]Uracil (Compound VII). (A) FROM 72-HOUR REARRANGEMENT. (1) HCl deamination. A solution of 0.4 g (0.0026 mole) of *N*-acetyl[^{15}N]cytosine in 10 ml of 2 N HCl was heated in a glass-lined bomb at 155° for 24 hours. When cool, the bomb was opened and the solid was collected. The filtrate was neutralized with ammonium hydroxide and evaporated to a small volume, and additional product was collected, washed with a small amount of ice water, then with alcohol and ether. Yield of crude product was 215 mg (74%). Spectrophotometric examination showed the conversion of cytosine to uracil was almost complete. In order to remove any traces of cytosine remaining after the deamination, the solid was dissolved in 80 ml of water, applied to a column of Dowex 50 (H^+) (2 × 20 cm; 200–400 mesh), and washed with water. The uracil was collected in the first four fractions (50 ml each) which were combined and evaporated *in vacuo* to a small volume. The solid, filtered and dried, weighed 140 mg and was demonstrated to be pure uracil by spectrophotometric and chromatographic comparison (2-propanol-HCl-H_2O, 17:4:4) with authentic material.

The theoretical ^{15}N value for a 100% rearrangement equilibrium between the exocyclic amino group and the N_3 position of cytosine is 2.94 atom % excess. The total ^{15}N content of the uracil obtained from the foregoing deamination was found to be 1.24 atom % excess (see Table I), which demonstrates that a 42.5% rearrangement had occurred.

[5] A further complicating factor in the use of nitrous acid deaminations to release nitrogen gas for mass spectrometry was the presence of a very large amount of another gas with a mass of 30 which is not absorbed by the base. This gas was obviously NO and there was no equilibration of ^{15}N between the N_2 and the NO, as evidenced by a mass 31:30 ratio of 0.00362, essentially normal for ordinary nitrogen.

(2) HONO deamination. A solution of 100 mg of [^{15}N]cytosine, 15 ml glacial acetic acid, and 600 mg of $NaNO_2$ was shaken for 1 hour and allowed to stand overnight at room temperature. The reaction mixture was evaporated *in vacuo* to a small volume, neutralized with dilute ammonium hydroxide, and applied to a Dowex column as in (1). The ultraviolet-absorbing fractions were evaporated and the residue was recrystallized from water; yield, 9 mg.

The total ^{15}N content of the uracil obtained by HONO treatment was found to be identical with that of the uracil resulting from the HCl deamination, 1.26 atom % excess (Table I).

(B) FROM A 24-HOUR REARRANGEMENT. A solution of 0.4 g (0.0026 mole) of *N*-acetyl[^{15}N]cytosine (product of 24-hour reflux of [^{15}N]cytosine with acetic anhydride–acetic acid) in 10 ml of 2 N HCl was subjected to the same treatment as in (A, 1). The resulting uracil was found to contain a total ^{15}N content of 0.75 atom % excess which indicated that rearrangement had occurred to the extent of 25%.

(C) FROM 108-HOUR REARRANGEMENT. The same deamination procedure was performed on 80 mg of the dark *N*-acetyl[^{15}N]cytosine which resulted from the 108-hour reflux under rearrangement conditions. The resulting uracil was analyzed for ^{15}N content which was found to be 1.17 atom % excess, representing a 40% rearrangement.

Determination of the ^{15}N *Content of the Exocyclic Amino Group.* (A) BY NITROUS ACID TREATMENT. A solution of [^{15}N]cytosine (regenerated from the acetylated product of 72-hour rearrangement) in 2 ml of glacial acetic acid was put into one leg of a three-legged tube equipped with a stopcock and adapter to the mass spectrometer system. Into the second leg was placed a solution of 300 mg of sodium nitrite dissolved in 2 ml of water. Into the third leg was placed 2 g of potassium hydroxide pellets. The solutions were degassed on a mercury diffusion pump with chilling to −75°, and evacuated to a final vacuum of 12 μ. The tube was now warmed until the frozen solutions became fluid. The two solutions were thoroughly mixed, care being taken not to dislodge the potassium hydroxide pellets. The reaction tube was now allowed to stand at room temperature for 48 hours. The acid solution was thoroughly mixed with the KOH pellets to absorb unused nitrous acid and the tube was rechilled to −75° for 20 minutes; it was then attached to the inlet of the mass spectrometer and the ^{15}N content of the nitrogen gas evolved in the deamination was determined.[5] Theory: 4.63 atom % excess; found: 2.57 atom % excess (Table I). There was therefore *ca.* a 45% dilution with unlabeled nitrogen.

The same deamination procedure as that described was carried out on unrearranged [^{15}N]cytosine (compound II). Results of analysis showed practically identical values with those given by the rearranged compound (Table I).

(B) HYDROGENOLYSIS OF REARRANGED [^{15}N]CYTOSINE. A solution of 68 mg of [^{15}N]cytosine (compound VI) in 15 ml of 0.1 N hydrochloric acid was treated with 74 mg of platinum oxide (monohydrate) and subjected to hydrogenation at normal temperature and pressure until uptake of hydrogen ceased. The entire reduction mixture was transferred to a Kjeldahl distillation apparatus, treated dropwise with 30% sodium hydroxide solution (w/v), and the liberated ammonia was swept by a stream of air into 20 ml of 0.1 N HCl. The resulting solution of [^{15}N] ammonium chloride was evaporated to dryness to remove excess acid, and the residue was diluted in 2 ml of water and converted to nitrogen gas with sodium hypobromite (see footnote to table); ^{15}N content, 4.58 atom % excess (Table I), calcd 4.63 atom % excess.

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