Fractionation of Oligonucleotides of Yeast Soluble Ribonucleic Acids by Countercurrent Distribution*

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ABSTRACT: Yeast s-RNA was subjected to pancreatic RNAase and the degradation products were fractionated by chromatography on DEAE-cellulose in 7 M urea with a NaCl gradient. Fractions corresponding to hexa- and heptanucleotides were further fractionated by 700- and 400-transfer countercurrent distribution, respectively. Two fractions present in the hexanucleotide appear to be $(Ap)_2(Gp)_3Cp$ and $(Ap)_3(Gp)_2Cp$. Three fractions present in the heptanucleotide fraction appear to be $(Ap)_2(Gp)_4Cp$, $(Ap)_4(Gp)_2Cp$, and a nonanucleotide $(Ap)_5(Gp)_3Cp$, respectively. The Gp content of the fractions appears to decrease with the increase in partition coefficient, whereas the reverse appears to be true for the Ap content of the fractions.

Elucidation of the nucleotide sequence in RNA requires the development of analytical procedures capable of separating oligonucleotides of chain lengths intermediate between mononucleotides and the RNA's themselves. The technique involving DEAE-cellulose anion-exchange chromatography in 7 M urea permits the fractionation of oligonucleotides on the basis of net charge (Tomlinson and Tener, 1962, 1963). This technique has been applied to the determination of end groups in s-RNA (Bell *et al.* 1963, 1964), and the separation of guanine-rich oligonucleotides (Lipsett and Heppel, 1963). Bartos *et al.* (1963) examined the behavior of oligonucleotides of known structure in this system and concluded that fractionation depended not only upon net charge, but also upon the purine/pyrimidine ratio. Fractionations of tetra- and pentanucleotides have been accomplished by means of this technique (Rushizky *et al.*, 1964).

The fractionation of mono- and oligonucleotides up to pentanucleotides has been obtained by several investigators using paper electrophoresis, paper chromatography, column chromatography, or a combination of these techniques. The separation and purification of s-RNA's, on the other hand, have been accomplished successfully by means of a technique such as countercurrent distribution (Holley and Merrill, 1959; Doctor *et al.*, 1961, 1963; Doctor and Connelly, 1961; Apgar *et al.*, 1962). There has been no method described for the fractionation of individual oligonucleotides whose unit chain lengths fall between pentanucleotides and approximately 80-member amino acid–acceptor RNA's. Such methods are quite essential for the successful determination of the nucleotide sequence in RNA. Since countercurrent distribution is capable of separating s-RNA's, there is a good possibility that it would also be effective in fractionation of the longer chain length oligonucleotides.

The present paper describes the preliminary investigation of the development of countercurrent distribution techniques for the fractionation of hexa- and heptanucleotides of yeast s-RNA's.

Experimental Procedures

All spectrophotometric determinations were performed in 1-cm light path silica cells with a Beckman DU spectrophotometer and are expressed as absorbance.

s-RNA. Yeast s-RNA was prepared from baker's yeast (Fleischmann's)[1] according to Holley (1963).

Digestion of s-RNA and Oligonucleotides. Pancreatic ribonuclease (twice crystallized) was purchased from Worthington, Freehold, N.J. Digestions were performed in 0.1 M Tris-acetate buffer, *p*H 7.8, for 8 hours at 37° with 0.03 mg of enzyme per mg of RNA. s-RNA (750 mg) and pancreatic RNAase (20 mg) were dissolved in 20 ml of 0.1 M Tris-acetate, *p*H 7.8. The digestion was carried out as described. Ribonuclease T_2 was a gift from Dr. George W. Rushizky of the National Cancer Institute, National Institutes of Health, Bethesda, Md. Digestions of oligonucleotides to obtain mononucleotides for base composition determinations were performed in 0.1 M sodium acetate, *p*H 7.5, for 6 hours at 37° with sufficient amounts of enzyme to achieve complete hydrolysis (Rushizky and Sober, 1963).

Chromatography of Ribonuclease Digest of s-RNA on DEAE-Cellulose in 7 M Urea. DEAE-cellulose (DEAE-Selectacel, reagent grade, Brown and Co., Berlin, N.H., 0.9 meq N/g, 60–140 mesh) was washed and equilibrated with the initial buffer (0.05 M NaCl in

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[1] Trade names mentioned here are intended for the convenience of the readers and do not constitute the endorsement of the product or the company.

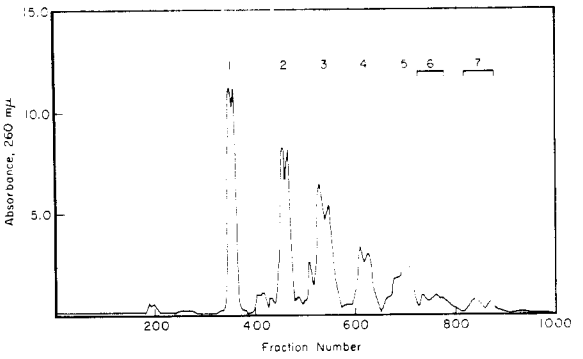

FIGURE 1: Chromatography of pancreatic RNAase digest of 750 mg of yeast s-RNA with DEAE-cellulose column in 7 M urea. Details of the procedure are under Experimental Procedures.

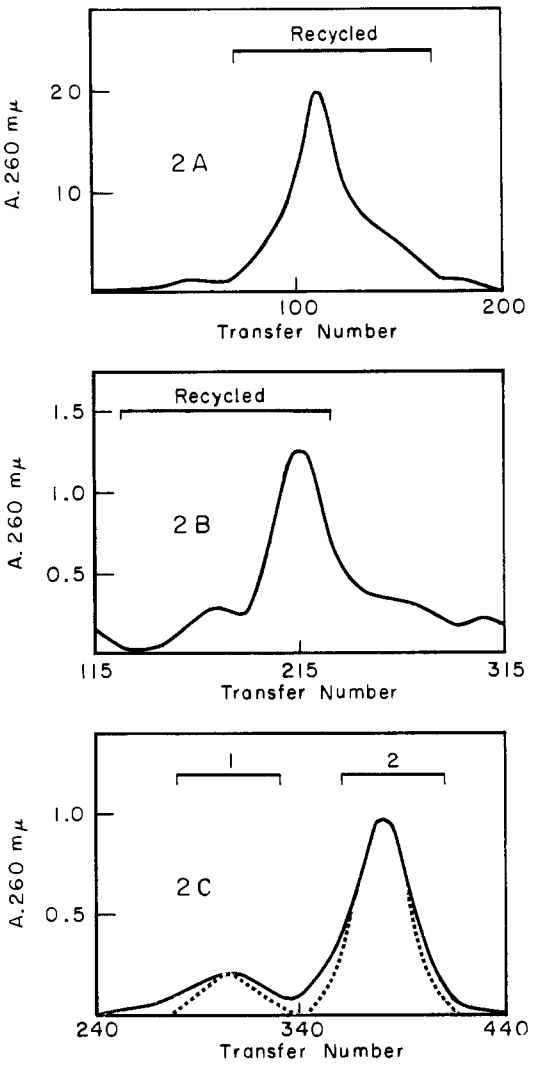

FIGURE 2: Countercurrent distribution of hexanucleotide fraction. (A) Distribution pattern after 200 transfers; (B) materials in tubes 71–166 recycled to a total of 395 transfers; (C) materials in tubes 126–200 and 1–25 (transfer numbers 126–225) recycled to a total of 700 transfers. ⌐——¬, fractions pooled for isolation; ———, $A_{260\ m\mu}$; ---------, theoretical curves calculated for partition coefficients of 0.78 and 1.19, respectively (Craig and Craig, 1956).

0.02 M Tris-acetate, *p*H 7.5, in 7 M urea) of the elution gradient. The column (200 × 20 cm² for 300 g of dry DEAE-cellulose) was packed under atmospheric pressure and washed with the initial buffer until the absorbance of the effluent at 260 mμ was constant (<0.1). The sample was mixed with 5 volumes of initial buffer and applied to the column. A nonlinear gradient was produced by a three-chamber system consisting of 3.5 liters of initial buffer (see above) in the first chamber and two chambers containing 3.5 liters each of limit buffer (0.3 M NaCl in 0.02 M Tris-acetate, *p*H 7.5, in 7 M urea) which was connected to the column after the sample was introduced into the column. The gradient was followed by limit buffer until all ultraviolet-absorbing material was eluted from the column. Fractions (15 ml) were collected and absorbance at 260 mμ was measured. Rechromatography of hexa- and heptanucleotide fractions was performed on a smaller column (180 × 7 cm²) with an initial buffer containing 0.2 M NaCl and a limit buffer containing 0.4 M NaCl. The fractions were desalted by DEAE-cellulose column chromatography as described by Rushizky and Sober (1962).

Procedures for Countercurrent Distribution of Oligonucleotides. The solvent system used in this procedure is as follows (McCormick and Doctor, 1963; Doctor and McCormick, 1964); 1200 g of ammonium sulfate was dissolved in approximately 3 liters of distilled water and to it was added 40 ml of glacial acetic acid. The solution was diluted to a total volume of 4 liters with distilled water (*p*H 3.25). To this, 160 ml of formamide (Fisher) and 1600 ml of 2-ethoxyethanol were added and the contents were shaken vigorously. The two-phase solvent system thus formed was allowed to equilibrate overnight at room temperature (23–24°). The 200-tube countercurrent fractionator was charged with 10 ml each of upper and lower phases. To the sample in distilled water was added 30 g of ammonium sulfate and 1 ml of glacial acetic acid and adjusted to 100 ml volume with distilled water. To this was added 4 ml of formamide and 40 ml of 2-ethoxyethanol and the contents were shaken to equilibrate the sample. The solvent system was removed from tubes 1–5 of the apparatus and the sample was introduced in its place (10 ml of each phase per tube). To the portion of the sample to be introduced in tube No. 5 additional amounts of upper and lower phases were added to make a 10-ml volume of each phase. The apparatus was set for the desired number of transfers. The desired fractions were pooled and extracted as described by Doctor *et al.* (1963), and desalted as described here.

Nucleotide Composition of Oligonucleotide Fractions. The samples were subjected to digestion with ribo-

TABLE I: Nucleotide Composition of Oligonucleotide Fractions.

Fraction	Ap (%)	Gp (%)	Cp (%)	Up (%)	Purine/ Pyrimidine
Hexanucleotides					
Unfractionated	45.9	38.1	13.0	3.0	5.2
Fraction 1	35.8 (2.1)	47.3 (2.8)	17.0 (1)[a]	0	4.9
Fraction 2	49.4 (3.0)	33.9 (2.0)	16.6 (1)	0	5.0
Heptanucleotides					
Unfractionated	47.1	38.8	9.7	4.4	6.1
Fraction 1	29.3 (1.9)	55.6 (3.7)	15.1 (1)	0	5.6
Fraction 2	54.4 (3.7)	30.8 (2.1)	14.7 (1)	0	5.8
Fraction 3	54.2 (4.8)	34.4 (3.0)	11.4 (1)	0	7.8

[a] Values in parentheses indicate molar ratios.

nuclease T_2 and the digest was subjected to ion-exchange chromatography on Dowex 1-X8 (formate form) according to Cohn and Volkin (1951). Mononucleotides were characterized by their absorbance ratios at *p*H 2 (250/260, 280/260, and 290/260 mμ) and by their chromatographic profiles.

Results

Fractionation of Ribonuclease Digests of Yeast s-RNA. Figure 1 shows the chromatographic pattern of pancreatic ribonuclease digests of yeast s-RNA obtained on a DEAE-cellulose column with 0.02 M Tris-acetate, *p*H 7.5, in 7 M urea and using a nonlinear gradient of NaCl. The nonlinear gradient was used because a linear gradient has been found to yield an elution profile in which the later peaks tended to be overlapping. The nonlinear gradient also effected a more rapid elution of the early peaks, which better suited the purpose of this study. In order to obtain sufficient material for further studies, a total of four separate column (750 mg s-RNA digest each) chromatography procedures were performed. Since it has been shown that the peaks corresponding to the higher oligonucleotides do not actually contain homogeneous single chain length material (Bell *et al.*, 1963, 1964), the fractions from the sixth peaks and seventh peaks from four columns were pooled and rechromatographed. Fractions from this rechromatography were analyzed for their nucleotide compositions. Fractions having purine/pyrimidine ratios of 5 (from sixth peaks) were pooled as hexanucleotides and purine/pyrimidine ratios of 6 (from seventh peaks) were pooled as heptanucleotides. The nucleotide compositions of these two pooled fractions are given in Table I under Unfractionated.

Countercurrent Distribution of Hexanucleotide Fraction. The hexanucleotide fraction having an absorbance of approximately 900 at 260 mμ was subjected to a 200-transfer countercurrent distribution. The distribution profile is shown in Figure 2A. The contents of tubes 1–70 and 167–200 were discarded and the tubes were refilled with fresh solvents (10 ml upper and 10 ml lower phase each). The apparatus was set to recycle to a total of 395 transfers (an additional 195 transfers). The distribution pattern at the end of 395 transfers is shown in Figure 2B. Roughlyf our peaks were observed. Since peaks 3 and 4 (right-hand side of main peak) contained insufficient amounts of material to attempt an isolation and further studies, they were discarded. The contents of tubes 26–125 (transfer numbers 116–125 and 226–315 in Figure 2B) were thus discarded and these tubes were refilled with fresh solvents. The machine was reset to recycle for an additional 305 transfers. The distribution pattern obtained at the end of 700 transfers is shown in Figure 2C. The fractions were pooled as indicated, extracted, desalted, concentrated to a small volume, and stored in the freezer until further use.

Countercurrent Distribution of Heptanucleotide Fraction. The heptanucleotide fraction having an absorbance of approximately 180 at 260 mμ was subjected to a 200-transfer distribution in the same manner as that performed with the hexanucleotide fraction. The distribution pattern is shown in Figure 3A. The contents of tubes 1–54 and 156–200 were discarded and these tubes were refilled with fresh solvent. The machine was set to recycle for a total of 400 transfers. The distribution pattern at the end of 400 transfers is shown in Figure 3B. Three fractions as indicated were isolated as described for the hexanucleotide fractions.

Absorption Spectra of Hexa- and Heptanucleotide Fractions. Portions of the two hexa- and three heptanucleotide fractions were adjusted to the final concentration of 0.01 M HCl (*p*H 2.0). The concentrations of the oligonucleotides were adjusted to give the same absorbance at 260 mμ for each fraction.The absorption spectra were obtained with a Cary recording spectrophotometer. The results are shown in Figure 4. Fraction 1 of the hexanucleotides shows considerably higher absorbance at wavelengths greater than 260 mμ than does fraction 2 (see Figure 4A). Similarly, in the case of the heptanucleotides, fraction 1 has higher absorbance than fractions 2 and 3. An increase in absorbance in these fractions at wavelengths greater than 260 mμ is

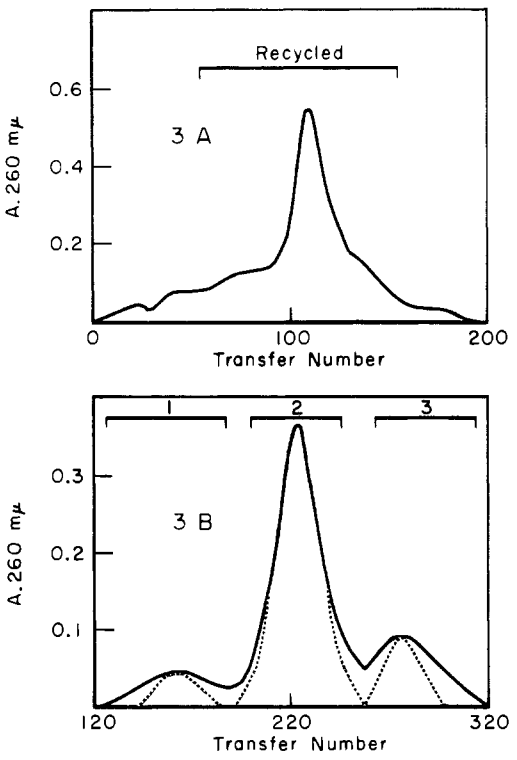

FIGURE 3: Countercurrent distribution of heptanucleotide fraction. (A) Distribution after 200 transfers; (B) materials in tubes 55–155 recycled to a total of 400 transfers. ⌐¬, fractions pooled for isolation; ——— $A_{260m\mu}$; ---------, theoretical curves calculated for partition coefficients of 0.68, 1.26, and 2.23.

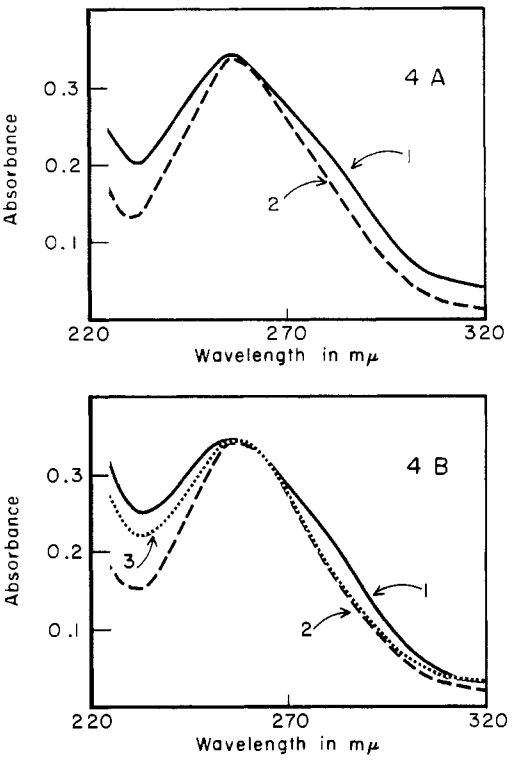

FIGURE 4: Absorption spectra of hexa- and heptanucleotide fractions. (A) Absorption spectra at *p*H 2 of fractions isolated from 700-transfer countercurrent distribution of hexanucleotides (see Figure 2C); (B) absorption spectra at *p*H 2 of fractions isolated from 400-transfer countercurrent distribution of heptanucleotides (see Figure 3B).

an indication of high guanylic acid content. Since the increased guanylic acid content is found in the fractions having lower partition coefficients in each case, this further supports the apparent relationship between adenylic acid content and guanylic acid content and partition coefficient in this solvent system, as was observed in the case of yeast RNA "core" (McCormick and Doctor, 1963).

Nucleotide Composition of Hexa- and Heptanucleotide Fractions. Table I shows the nucleotide contents of two hexa- and three heptanucleotide fractions. Figure 5 shows a typical chromatographic pattern obtained on a Dowex 1 (formate) column. No peaks were obtained that would correspond to the methylated derivatives that are present in s-RNA. Methylated adenylic acid would be located immediately after adenylic acid while methylated guanylic precedes guanylic acid (in 1 N formic acid; Nihei and Cantoni, 1963). A peak in one of these positions would have been detectable if it had contained approximately $0.15A_{260\ m\mu}$ unit, assuming a reasonable shape for the curve. Thus, in the range of sample quantity employed (approximately $25A_{260\ m\mu}$ units), the levels of those methylated derivatives could not have exceeded approximately 1% of the total. None of the countercurrent distribution fractions contains any detectable amounts of uridylic acid; it is apparent from this that the fractions discarded during the fractionation process must have contained oligonucleotides terminated with uridylic acid.

Results shown in Table I indicate the purine/pyrimidine ratios of fractions 1 and 2 of hexanucleotides are 4.9 and 5.0, respectively, indicating a chain length of six nucleotides in each case. In the case of fractions 1, 2, and 3 of heptanucleotides, the ratios are 5.6, 5.8, and 7.8, respectively, which would indicate a chain length of seven nucleotides for fractions 1 and 2, and of nine nucleotides for fraction 3. Fraction 3 is apparently a nonanucleotide. The molar ratios of the nucleotides in the case of each fraction are almost whole numbers and, on this basis, one can assign empirical formulas for each fraction. Thus, hexanucleotide fractions 1 and 2 correspond to $(Ap)_2(Gp)_3Cp$ and $(Ap)_3(Gp)_2Cp$. Heptanucleotide fractions 1 and 2 correspond to $(Ap)_2(Gp)_4Cp$ and $(Ap)_4(Gp)_2Cp$, respectively, and the nonanucleotide (fraction 3) corresponds to $(Ap)_5(Gp)_3Cp$.

Discussion

The purpose of this investigation has been to explore the possibility of developing procedures for the frac-

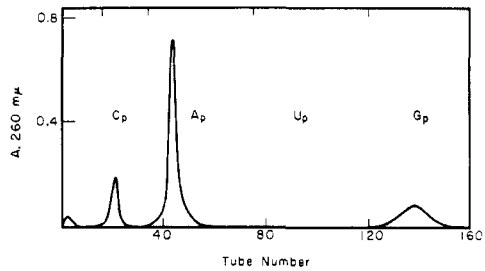

FIGURE 5: Chromatography of RNAase T_2 digest of oligonucleotides on Dowex 1 (formate) column (7 × 0.75 cm^2). Fractions contained 4 ml each. First fifteen fractions were eluted with water; 16–35 with 0.02 M formic acid; 36–70 with 0.15 M formic acid; 71–120 with 0.01 M formic acid in 0.1 M ammonium formate; and 121–160 with 0.15 M formic acid in 0.3 M ammonium formate.

tionation and purification of oligonucleotides of chain lengths greater than five. The information presented here demonstrates that countercurrent distribution is one of the procedures which can be used successfully to this end. Using a *p*H 3.25 solvent system, it has been possible to demonstrate separations of two hexanucleotide fractions in one case and two heptanucleotide fractions and a nonanucleotide fraction in another case. One of the bases of separation appears to be variation in the Gp and Ap content of oligonucleotides obtained by pancreatic ribonuclease digestion. It is quite possible that, in addition to this variation in oligonucleotides, the chain length and the nucleotide sequence may also be factors contributing to the separation. The latter appears to be the case in the fractionation of heptanucleotides, whereas the former has not been demonstrated in the present investigation. The possibility cannot be ruled out that the fractions obtained by countercurrent distribution in such an investigation may contain oligonucleotides of the same nucleotide composition but different sequence. This, however, may be unlikely since the peaks appear to follow theoretical curves closely, thus indicating their homogeneity.

It is quite interesting to note that the Ap content of the fractions obtained from the countercurrent distribution of various oligonucleotides increases with the partition coefficient. The reverse trend is observed with respect to the Gp content of these fractions. One can visualize from these observations that any enzymes with a specificity for Cp, Up, or both (i.e., pancreatic RNAase) would act upon RNA to yield oligonucleotides that can be separated by countercurrent distribution procedures. The variation of Cp and Up contents with the partition coefficient appears to be very small (McCormick and Doctor, 1963). However, this may be misleading, since in pancreatic RNAase digests of RNA one obtains oligonucleotides with pyrimidines in terminal positions only. Thus the oligonucleotides under investigation here do not possess any pyrimidine-pyrimidine linkages. In view of this fact, it will be quite interesting to observe the pattern of countercurrent distribution of oligonucleotides obtained after the digestion of RNA with RNAase T_1. These oligonucleotides contain pyrimidine-pyrimidine linkages as well as pyrimidine-adenine, and vice versa, in such experiments. The variation of Cp and Up content with the variation in partition coefficient would furnish important additional information about the basis of separation by countercurrent distribution.

The oligonucleotides mentioned here cannot constitute all the oligonucleotides of this nature existing in yeast s-RNA. In order to demonstrate and develop a method for fractionating oligonucleotides of the same chain length, many fractions were discarded at various stages of the procedure. This was deemed necessary for two reasons: First, the fractions that were discarded contained such small quantities of material that their isolation and further characterization was not practicable. Second, the main purpose of this investigation was to demonstrate that countercurrent distribution procedures can be employed successfully for the fractionation of oligonucleotides of the same chain lengths.

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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.