

The Isolation of Nitrogen 1 and 3 as Methylamine and Ammonia from Pyrimidine Ribosides

ULF LAGERKVIST

Biochemical Department, Karolinska Institutet, Stockholm, Sweden

During investigations on pyrimidine metabolism with the aid of N^{15} it became desirable to study the turnover of the nitrogen atoms separately.^{1,2}

As a first attempt in this direction a method has been worked out that permits the preparation of the nitrogen in position 1 and 3 in pyrimidine ribosides separately from each other.

In order to make the separation of the two nitrogen atoms possible one of them had to be labelled in some way. This was done by methylating the pyrimidine riboside in position 1 with diazomethane as described by Bredereck for purine ribosides³.

The methylation was preceded by acetylation of the sugar with ketene.

The ribose was then split off with formic acid according to Vischer and Chargaff⁴ and the methylated pyrimidine separated from uracil and from degradation products by chromatography on a starch column with butanol-water.

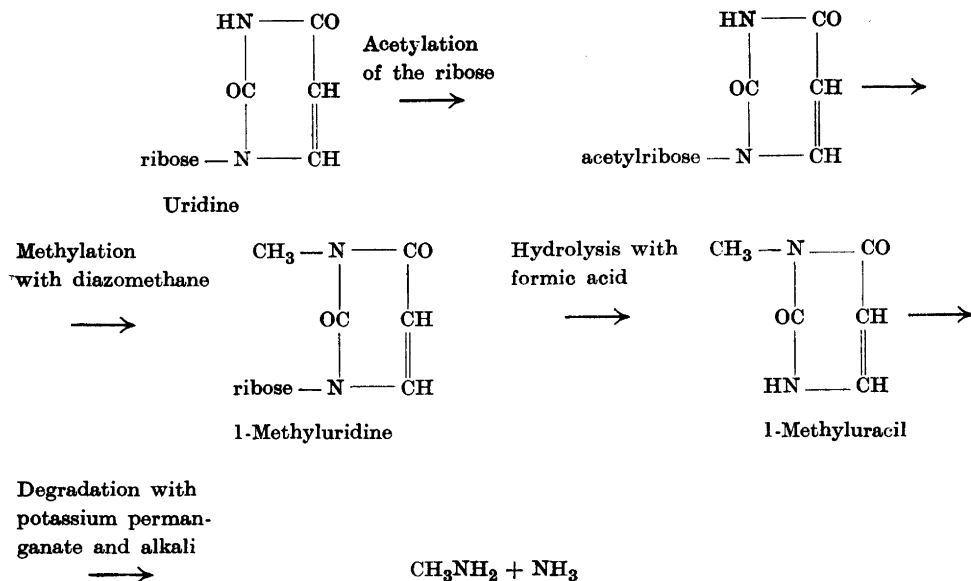
The pyrimidine ring was opened by means of potassium permanganate in neutral solution and then degraded by distillation with 50 % sodium hydroxide solution. The nitrogen in position 1 was split off as methylamine and that in position 3 as ammonia.

Methylamine and ammonia were separated by chromatography on a starch column with propanol — 0.5 *N* HCl, 2 : 1 as described by Moore and Stein for the amino acids^{5,6}.

The method has been worked out for uridine using an amount of 20 mg.

EXPERIMENTAL

Acetylation. The uridine (20 mg) was dissolved in 2 ml water + 20 ml acetone and acetylated for 2 hours with ketene.



Methylation. After acetylation the solution was evaporated to dryness *in vacuo*. The acetylated riboside was dissolved in 5 ml metanol + 10 ml acetone. A solution of diazomethane in ether prepared from 5 g nitrosomethylurea was poured into the riboside solution. After 3 hours the remaining diazomethane was destroyed with glacial acetic acid, the solution transferred to a bomb tube and evaporated to dryness on a steam bath.

Hydrolysis. The ribose was split off by hydrolysing for 6 hours with 5 ml conc. formic acid (98–100 %) at 175° C.

Chromatography of hydrolysate of methylated uridine. The hydrolysate was washed out with 5 ml + 3 ml + 3 ml water, centrifuged and the supernatant repeatedly evaporated to dryness *in vacuo* to remove the formic acid. Before the last evaporation the solution was neutralized with a few drops of 0.1 N NaOH. It was taken up in 10 ml + 2 ml + 2 ml of butanol-water (13.5 parts water + 86.5 parts butanol) and added to a starch column, length 200 mm diam. 40 mm. The chromatogram was developed with the same solvent. The size of each fraction was approximately 10 ml.

A chromatogram of the hydrolysate showed a high peak in the position expected for 1-methyluracil and a small one in the position expected for uracil (Fig. 1).

A chromatogram of synthetic 1-methyluracil (F. 174°–175° C)⁹ and uracil is shown in Fig. 2.

The *R*-value for synthetic 1-methyluracil was 1.63 and that for uracil 0.83². The substance that was formed during the hydrolysis had an *R*-value of 1.60 while the compound that was thought to be unmethylated uracil had *R* = 0.79. The light absorption curves for 1-methyluracil and the unknown compound had identical shapes with E_{max} at 2580 Å. For these reasons the substance formed during hydrolysis was thought to be identical with 1-methyluracil.

The overall yield calculated as uridine was 70 %.

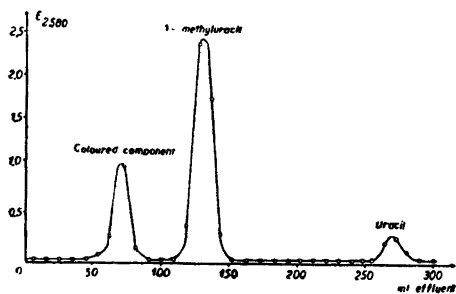


Fig. 1. Chromatography of hydrolysate of methyluridine. Length of column: 204 mm, diameter: 36 mm.

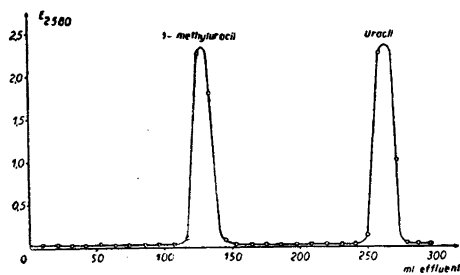


Fig. 2. Chromatographic separation of 1-methyluracil and uracil. Length of column: 205 mm, diameter: 36 mm.

Degradation. The fractions from the chromatogram that contained the methyluracil were evaporated to dryness *in vacuo*. The methyluracil was dissolved in 20 ml water and about 50 mg of Ba Cl_2 were added. A solution of KMnO_4 in 20 ml water was added to the solution of methyluracil. The amount of KMnO_4 used was such that the ratio $\frac{\text{mg KMnO}_4}{\text{mg methyluracil}} = 2$. The amount of methyluracil was calculated from the light absorption read in the Beckman spectrophotometer at 2580 Å. $E_{\text{max}2580 \text{ Å}} = 1.000$ for 17.2 γ methyluracil/ml. After about 30 min. the permanganate-colour had disappeared. The MnO_2 was centrifuged off and washed twice with a few ml water. The centrifugate and the washing were transferred to the reaction flask and the solution was made to be 50 % with respect to NaOH.

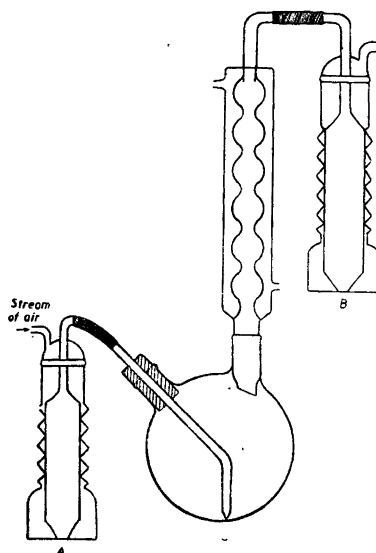


Fig. 3. Apparatus for the distillation with alkali.

- A. Washbottle containing HCl for removal of ammonia from air stream.
- B. Washbottle containing 0.1 N HCl for collection of methylamine and ammonia.
- C. Degradation vessel.

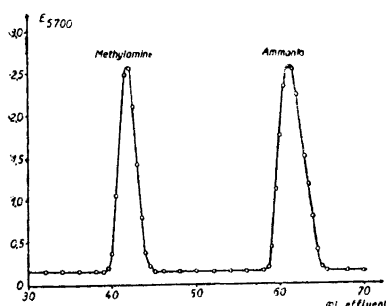


Fig. 4. Chromatographic separation of methylamine and ammonia. Length of column: 300 mm, diameter: 10 mm.

The solution was now distilled for 6 hours in the apparatus shown in Fig. 3. A stream of air which had been freed from ammonia by passing through a washbottle filled with HCl (A) was lead through the apparatus.

Methylamine and ammonia were collected in washbottle (B) which contained 0.1 N HCl.

Chromatography of methylamine and ammonia. Methylamine and ammonia were separated by chromatography on a starch column with propanol — 0.5 N HCl, 2 : 1. The technique was the same as that described by Moore and Stein^{5,6} for the aminoacids. The solution of methylamine hydrochloride and ammonia chloride obtained after distillation was evaporated to dryness *in vacuo*. The residue was taken up in 5 ml + 2 ml + 2 ml of propanol — 0.5 N HCl, 2 : 1 and added to a starch column, length 200 mm diam. 40 mm. The chromatogram was developed with the same solvent. The positions of the peaks were determined by means of ninhydrin. A chromatogram of synthetic methylamine and ammonia is shown in Fig. 4.

R for methylamine was 0.52 and for ammonia 0.36. This value for ammonia agrees with that found by Moore and Stein⁶.

A chromatogram of the degradation products revealed two peaks in the same position as those in the chromatogram of synthetic methylamine and ammonia. *R* for the first peak was 0.52 and for the second 0.35.

Characterization of the compounds as free from foreign nitrogen

Methylamine. Methylamine was determined quantitatively according to Alexander⁷, the only difference being that different proportions of methylamine and chromotropic acid were used. After the methylamine had been oxidized to formaldehyd, distilled over and diluted to 10 ml, 0.5 ml of the solution was diluted to 5 ml with chromotropic acid in 21 N H₂SO₄ (2 mg/ml) and heated in a steam bath for 30 min. The colour was read in the Beckman spectrophotometer at 5700 Å. The ratio $\frac{\text{mg N from CH}_3\text{NH}_2}{\text{mg total N}}$ was determined and found to be 0.98–0.99.

Ammonia. Ammonia was determined by nesslerization⁸. The colour obtained was read in the Beckman spectrophotometer at 4000 Å and compared to the colour given by

a standard solution of NH_4Cl . The ratio $\frac{\text{mg N from NH}_3}{\text{mg total N}}$ was determined and the value 0.96 was obtained.

The overall yield of the method from uridine to methylamine and ammonia was 45–50 % of the theoretical.

DISCUSSION

Acetylation. In order that methylation with diazomethane in ether could be performed, the pyrimidine riboside had to be made soluble in a mixture of methanol and acetone. This was accomplished by acetylation of the ribose. Bredereck in his work on methylation of purineribosides³ used acetic anhydride in pyridine as an acetylating agent, but the present author preferred to use ketene. Contamination with foreign nitrogen from the pyridine could thus be avoided.

Methylation. Diazomethane was thought to be the most selective methylating agent as Bredereck states that it only methylates the NH -group in the neighbourhood of a CO -group. Methylation of the sugar did not occur and the possibility that methyl groups might be transferred from the sugar to position 3 in the pyrimidine when the ribose was split off with formic acid could be neglected.

Hydrolysis. Chargaff states that 2 hours hydrolysis is sufficient⁴ but later experiments by Reichard and Estborn at this laboratory (unpublished) have shown that the ribose is not completely split off after such short time.

Degradation. Before degrading with alkali the pyrimidine ring was opened with KMnO_4 in neutral solution. If this was not done more methylamine than ammonia was formed while theoretically they should appear in equal proportions. This suggested either that methylamine is formed to some extent from the nitrogen in position 3 as well as from that in position 1 or that under these conditions the nitrogen in position 3 is split off more slowly than that in position 1 and the reaction has not yet gone to completion.

As an experiment in which uracil was degraded in NaOH in the absence of permanganate showed that no methylamine is formed from the nitrogen in position 3 the second possibility seemed more likely. Thus the function of the KMnO_4 is believed to be the speeding up of the release of nitrogen from position 3. On the other hand if an excess of KMnO_4 was used more ammonia than methylamine was formed making it probable that ammonia was also formed from the nitrogen in position 1. If the treatment with KMnO_4 was carried out at room-temperature and if just so much KMnO_4 was used that no permanganate colour was present after 30 min. methylamine and ammonia after subsequent degradation with alkali appeared in the same amounts.

When just so much KMnO_4 was used that the ratio $\frac{\text{mg KMnO}_4}{\text{mg methyluracil}} = 2$ the change in the light absorption indicated that 90 % of the ring was opened. The oxidation was more readily achieved in the presence of Ba-ions.

SUMMARY

A method has been described that permits the isolation of the nitrogen in position 1 in pyrimidineribosides as methylamine and the nitrogen in position 3 as ammonia.

BIBLIOGRAPHY

1. Bergstrand, A., Eliasson, N., Hammarstem, E., Norberg, B., Reichard, P., and v. Ubisch, H. *Cold Spring Harbor Symp.* **13** (1948) 22.
2. Reichard, P. *Acta Chem. Scand.* **3** (1949) 422.
3. Bredereck, H. *Chem. Ber.* **80** (1947) 401.
4. Vischer, E., and Chargaff, E. *J. Biol. Chem.* **176** (1948) 715.
5. Moore, S., and Stein, E. H. *J. Biol. Chem.* **176** (1948) 367.
6. Moore, S., and Stein, E. H. *J. Biol. Chem.* **178** (1949) 53.
7. Alexander, B., Landwehr, G., and Selyman, A. *J. Biol. Chem.* **160** (1945) 51.
8. Kolthoff, I., and Sandell, E. *Textbook of quantitative inorganic analysis*. New York (1947) p. 669.
9. Heyl, F. W., and Johnson, T. B. *Am. Chem. J.* **37** (1905) 633.

Received February 3, 1950.