A synthesis of cytidine-5-^{3}H

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SUMMARY

Cytidine-5-^{3}H was synthesized, by the incorporation of 1.1-diethoxy 2-cyanoethane-2-^{3}H (cyanoacetal-2-^{3}H), using an adaption of the procedure described by Codington [1] for the synthesis of cytidine-2-^{14}C. The final step in this procedure was the coupling of the mercuric compound of the base with the blocked chloro-sugar in a toluene-benzene solvent. The fact that this reaction can be used indicates that mercuric-salt catalyzed hydrogen exchange between benzene-toluene solvent and nucleoside, as described by LePage and Junga [2], does not occur with cytidine at least at the 5-position. Furthermore the present investigation shows that dimethyl acetamide is an excellent aprotic solvent and can be used as such for reactions involving exchange of labile tritium under alkaline conditions.

INTRODUCTION

Fink [3] has shown that in tritium labelled cytidine obtained from several commercial sources, the percentage of tritium located at the 5-position of the pyrimidine nucleus varies between 88 and 98% of the total activity. To fill a need in our laboratory for pure 5-^{3}H-labelled cytidine, this compound was synthesized via two different paths. One of these, the catalytic dehalogenation of 5-iodocytidine, will be described in another paper [4]. The present article describes a synthesis involving cytosine-5-^{3}H as an intermediate.

In applying the procedure described by Codington [1] to this synthesis, two problems were encountered — the preparation of cyanoacetal-2-^{3}H and the choice of a solvent for the coupling of cyanoacetal-2-^{3}H with urea.

In view of the observations of McElvain [5], we assumed that the base-catalyzed addition of ethanol to β-ethoxy acrylonitrile goes to equilibrium and predominantly favours the cyanoacetal addition product. Preliminary experiments showed that this indeed was true and that ethanol, tritiated at the OH-group, could be used for the preparation of cyanoacetal-2-^{3}H by a simple addition to β-ethoxy acrylonitrile:

$$C_2H_5O{-}CH{=}CH{-}CN + C_2H_5OT \underset{}{\overset{NaOC_2H_5}{\rightleftharpoons}} (C_2H_5O)_2.CH.CHT.CN$$

However, because very poor addition yields were obtained in several solvents due to the insolubility of the catalyst, sodium ethylate, the addition was carried out with an excess of tritiated ethanol as solvent.

In the synthesis of the pyrimidine nucleus, 1-butanol could not be used as solvent because of the loosely bound hydrogen atoms which would be expected to exchange rapidly with the tritium in cyanoacetal-2-^{3}H in the presence of a basic catalyst. Most of the usual aprotic solvents could not be used as they reacted with or failed to dissolve the sodium butylate catalyst. Dimethyl acetamide, however, worked well.

All the reactions preceding the purification of the cytosine-5-^{3}H were performed with millimole quantities on a vacuum line. For the purification of the cytosine-5-^{3}H and cytidine-5-^{3}H, simple column chromatographic separations were developed.

The chemical purity of the cytidine-5-^{3}H was checked by the determination of its U.V. spectrum and of its specific rotation. The radiochemical purity was checked by specific substitution for the ^{3}H followed by measurement of the residual activity and by paper radiochromatography. Both methods showed the radiochemical purity to be greater than 99%.

Experimental

All reactions and distillations were carried out *in vacuo*, unless otherwise indicated. The quantities of liquids on the vacuum line were determined by measuring the volumes in calibrated narrow tubes. All activity measurements were done with a scintillation counter * using a toluene-alcohol or a dioxane scintillator solution. Hyamine was sometimes used for dissolving difficult soluble compounds. The starting material, β-ethoxy acrylonitrile, was prepared according to the procedure of Tarsio [6].

Preparation of cyanoacetal-2-^{3}H

Sodium ethylate (44.4 mmol) was prepared in a bulb and dried *in vacuo* at 170 °C. Tritiated water (0.640 ml, s.a. 2.44 μC/mmol) was then distilled into the bulb. After stirring the mixture for 30 minutes ethanol-O-^{3}H (33.80 mmol) was removed by distillation (yield 95%). The s.a. of the ethanol-O-^{3}H obtained was 1.14 μC/mmol).

In another bulb sodium ethylate (0.53 mmol) was similarly prepared and dried. Ethanol-O-^{3}H (29.80 mmol) and β-ethoxy acrylonitrile (9.43 mmol) were distilled into this bulb and the mixture stirred for 18 hours at room temperature. Afterwards 2.6-dimethyl benzoic acid (1.95 mmol) was added from a side-arm to neutralize the sodium ethylate. After stirring the mixture for 3-5 minutes all

* PANAX scintillation counter SC/LP.

the liquid products were removed by distillation. The distillate was fractionated into two portions in the following way: ethanol-O-^{3}H was distilled into a trap cooled to −20 °C (obtained 21.17 mmol, s.a. 0.89 μC/mmol) and cyanoacetal-2-^{3}H was distilled into a trap cooled to −195 °C (obtained 9.47 mmol, s.a. 1.65 μC/mmol). The cyanoacetal-2-^{3}H had to be kept at low temperature to prevent polymerization, which is probably due to the β-radiation of the incorporated tritium [7].

The addition reaction was found to be quantitative. The s.a. found for both substances agrees with the assumption that both exchange and addition take place. The calculated s.a. for the cyanoacetal-2-^{3}H is 1.71 μC/mmol (found 1.65 μC/mmol) and for the excess ethanol-O-^{3}H 0.86 μCmmol (found 0.89 μC/mmol).

Preparation of cytosine-5-^{3}H

In a bulb equipped with a side-arm, sodium butylate (9.08 mmol) was prepared and dried *in vacuo* at 150 °C. Dimethyl acetamide (4.56 ml, purified by vacuum distillation from KOH pellets and P_2O_5) and cyanoacetal-2-^{3}H (7.74 mmol) were distilled into the bulb. Urea (8.12 mmol) was added from the side-arm. The mixture was stirred for 3 hours at 140-145 °C under a nitrogen atmosphere. Distillation to dryness of the dark brown solution yielded 5.61 ml of a colourless liquid (total activity 9.78 μC). The brown-yellow residue was treated with 2N sulfuric acid (11 ml) at 70 °C for 4-5 minutes. The resulting greenish foaming solution was immediately cooled and placed on a column of Dowex-50Wx12 (H^+-form, 2×32 cm). After washing the column with 500 ml of 20% ethanol (eluted activity 1.17 μC), cytosine-5-^{3}H was isolated by elution with 2N hydrochloric acid. A fraction collector, connected to a U.V. flow spectrophotometer, sampled the eluate and the absorption at 275 mμ was continuously recorded. The fractions containing cytosine-5-^{3}H were collected and the yield spectrophotometrically determined (3.52 mmol = 45%).

A U.V. spectrum taken in 0.1 N hydrochloric acid was identical to the spectrum of a pure sample of cytosine [1] (λ_{max} = 275 mμ, λ_{min} = 238.5 mμ), but the ratio E_{max}/E_{min} was smaller (5.4 instead of 8), probably due to some hydrolyzed Dowex resin. The cytosine-5-^{3}H was further purified by means of an additional chromatographic separation. For this purpose the collected cytosine-5-^{3}H containing fractions were evaporated to dryness *in vacuo*, stirred with 15 ml 25% ammonium hydroxide solution at room temperature, and again evaporated to dryness *in vacuo*. A white residue resulted and this, after suspension in 15 ml of a mixture of butanol-1 and water (86:14 v/v), was placed on a cellulose powder column * (4.2×31 cm). The cytosine-5-^{3}H was eluted by washing with

* Schleicher and Schuell cellulose powder for chromatography, purified by a 24 hour soxhlet extraction with a mixture of petroleum ether and methanol (1:1 v/v).

the same butanol-water mixture at a flow rate of 31 ml/hour. Again use was made of the fraction collector and automatic U.V. absorption recording (at 267 mμ).

The fractions comprising the first half of the eluted cytosine-5-^{3}H peak were combined (I) as were those for the second half (II). The total amount of cytosine-5-^{3}H was spectrophotometrically determined and found to be 3.22 mmol = 41.6% relative to cyanoacetal-2-^{3}H. The U.V. spectra of the cytosine-5-^{3}H in I and II, taken in 0.1 N hydrochloric acid, were identical (λ_{max} = 275 mμ, λ_{min} = 238 mμ, E_{max}/E_{min} = 7.2) and the s.a. was in each case 0.27 μC/mmol. A second purification with a cellulose powder column did not change the s.a. Thus the purification technique employed yields a product of reasonable radiochemical purity.

Preparation of the mercuric salt of N-acetyl cytosine-5-^{3}H

Cytosine-5-^{3}H (2.72 mmol, s.a. 0.27 μC/mmol) and acetic anhydride (60 ml) were refluxed together for 4 hours. The resulting dark brown solution was concentrated *in vacuo* to about 7 ml and kept at 3 °C for 2 days. The brown-yellow precipitate was filtered and washed with water. Then the crude N-acetyl cytosine-5-^{3}H, suspended in 60 ml of water, was brought into solution by slowly adding 4.7 ml of 1.ON sodium hydroxide solution at 50 °C with stirring. After filtration of the light yellowish solution (pH = 9) mercuric chloride (2.72 mmol, dissolved in 5 ml of ethanol) was added, with stirring. The white suspension of mercuric derivative formed, was treated as described by Codington [1]. The yield of Hg-N-acetyl cytosine-5-^{3}H was 2.23 mmol, equal to 82% with respect to cytosine-5-^{3}H. The s.a. of this compound was not determined, but the total loss of activity was found by measurements of the activities of all the solvents and solutions used during its synthesis. This loss was found to be 0.13 μC, equal to 18% of the total activity of cytosine-5-^{3}H used (0.734 μC), indicating that only mechanical losses occurred, not exhange of tritium.

Preparation of cytidine-5-^{3}H

This synthesis is carried out exactly as described by Codington [1], with tetra-O-acetyl-D-ribofuranoside (3.14 mmol) being converted to the chloro compound and reacted with Hg-N-acetyl cytosine-5-^{3}H (1.45 mmol). Activity measurements of the solvents used (benzene, toluene, petroleum ether, chloroform, and the wash-solution 30% potassium iodide in water) revealed no activity. After deacylation of the reaction product with ammonia in ethanol, the yellow solution was evaporated to dryness *in vacuo* and the yellowish-brown residue dispersed in 15 ml 0.2 N hydrochloric acid. The turbid liquid was placed on a Dowex 50Wx12 column (H^+-form, 2 × 31 cm) and the column washed with 325 ml water.

The absorption of the effluent (at 280 mμ) was recorded during the elution

with 2N hydrochloric acid. Besides cytidine-5-^{3}H, a considerable amount of cytosine-5-^{3}H was washed out, the absorption peaks having been well separated (retention volumes resp. 640 and 890 ml effluent). The fractions containing cytidine-5-^{3}H and cytosine-5-^{3}H were then separately collected and each compound was purified with a cellulose powder column (4.2 × 31 cm) as described for cytosine-5-^{3}H. The yield of each product was spectrophotometrically determined and found to be 0.16 mmol (11%) for both compounds. From the activity measurements the s.a of cytidine-5-^{3}H was found to be 0.27 μC/mmol and that of cytosine-5-^{3}H 0.26 μC/mmol. This result indicates that no tritium exchange or loss occurred during the conversion of cytosine-5-^{3}H to cytidine-5-^{3}H.

In two more syntheses the observations on the change of s.a. from ethanol-O-^{3}H to cytidine-5-^{3}H were verified. The only difference found was that the yield of the cytidine synthesis reached 35% instead of the above mentioned 11%, accompanied by a simultaneous decrease of the side product, cytosine, to about 1%. The low yield of cytidine-5-^{3}H in the experiment described may be due to some moisture having been present during the deacylation reaction leading to the relatively large amount of cytosine-5-^{3}H recovered.

Purity checks on cytidine-5-^{3}H

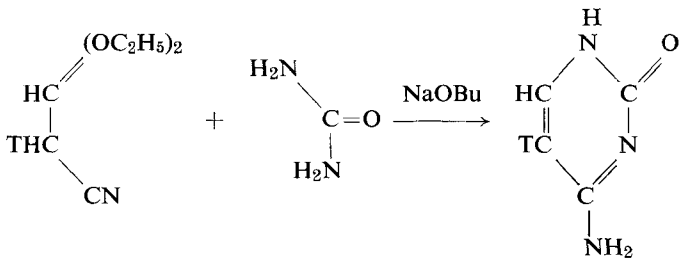
Both the U.V. spectrum [1, 8] (λ_{max} = 280 mμ, λ_{min} = 241 mμ, E_{max}/E_{min} = 6.6, taken in 0.1 N hydrochloric acid) and the optical rotation [9] ($[\alpha]_D^{20} = +32.8°$, c = 0.2, in water) match the values in the literature. The R_f-values determined with butanol-1:water mixture (86:14 v/v) and with butanol-1:water:ethanol mixture (50:35:15 v/v) as solvent on Whatman 1 paper (R_f resp. 0.12 and 0.42) agree with the values given by Markham [10]. In both cases only a single spot could be detected by either U.V. or radiochromatogram scanning.

As a final check on the position of the tritium, some of the cytidine-5-^{3}H was converted to 5-iodocytidine according to the procedure of Chang [11]. The 5-iodocytidine was isolated, purified [4], and tested for activity, but none was found (activity less than 0.005 μC/mmol). This result shows the cytidine-5-^{3}H to be labelled at least 99% specifically at the 5-position.

Discussion

The low s.a. of the cytosine-5-^{3}H (0.27 μC/mmol), compared to that of the cyanoacetal-2-^{3}H (1.65 μC/mmol) can be understood as follows. Assuming that the s.a. of the cyanoacetal-2-^{3}H which ultimately produces the cytosine-5-^{3}H is twice as high (0.54 μC/mmol) as that of the cytosine-5-^{3}H obtained, T-H exchange with urea must have occurred before the coupling reaction took place. Taking into account the amounts of materials used, calculation shows that the s.a. of the cyanoacetal-2-^{3}H must decrease from 1.65 μC/mmol to 0.533 μC/mmol, in agreement with the above assumption. T-H exchange with

the solvent dimethyl acetamide could not have been occurring, since if it had, the s.a. of the cyanoacetal-2-^{3}H would have decreased to 0.13 μC/mmol.



Concerning the reaction conditions for the synthesis of cytosine, the following observations were made :

1. Substitution of sodium ethylate for sodium butylate had no effect on the yield.
2. The reaction temperature is critical:

Solvent	Reaction period	Reaction temp.	Yield
dimethyl acetamide	3 hours	100 °C	13%
dimethyl acetamide	3 hours	110 °C	14%
dimethyl acetamide	2 hours	120 °C	17%
dimethyl acetamide	3 hours	120 °C	26%
dimethyl acetamide	2 hours	125 °C	22%
dimethyl acetamide	3 hours	140 °C	40%
dimethyl acetamide	3 hours	155 °C	35%
butanol-1	2 hours	117 °C	46%

3. Temperature and duration of hydrolysis are critical:

Temp. 2N H_2SO_4	Hydrolysis period	Hydrolysis temp.	Yield
80 °C	15 min	80 °C	19%
70 °C	10 min	65 °C	26%
70 °C	3-5 min	70 °C	40-44%

Conclusion

It is found that cytidine-5-^{3}H can be synthesized from cytosine-5-^{3}H via the mercuric intermediate without a change in the s.a. This result is in contrast with the results of LePage and Junga [2] for the same type of reaction with a purine nucleoside.

Taking into consideration Fink's work on the isotope distribution in tritiated pyrimidines, [3] we must conclude that it is most unlikely that LePage and Junga's method can be applied for a labelling of the base moiety in cytidine.

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