The Incorporation of the Carbon Skeleton of Cytidine into the Pyrimidine Nucleosides of Ribonucleic Acid and Deoxyribonucleic Acid by Neurospora

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(Received for publication, October 15, 1957)

Experiments have been carried out with Neurospora which demonstrate that the quantitatively important route for the biosynthesis of the pyrimidine deoxyribonucleosides in the DNA of this organism involves the entire carbon skeleton of a pyrimidine ribonucleoside. A very high labeling of the pyrimidine nucleosides of the RNA and DNA was brought about by feeding uniformly labeled C<sup>14</sup>-cytidine to a pyrimidine-requiring mutant of this organism, grown in the presence of sucrose. The specific activities of the deoxyribose in the deoxycytidine and thymidine of the RNA and the ribose in the cytidine and uridine of the RNA were quite similar, nor were they very different, carbon atom for carbon atom, from that of the corresponding pyrimidine ring. It is clear, therefore, that the carbon skeleton of the pyrimidine nucleosides of the DNA has arisen as such from an intact pyrimidine ribonucleosyl intermediate.

The likelihood of this direct conversion was fully appreciated by Hammarsten et al. (1), and Rose and Schweigert (2) demonstrated that such a conversion can take place in the rat. It is the purpose of this article to show that in Neurospora such a conversion is the important route for the formation of the pyrimidine nucleosides of DNA.

In a subsequent article evidence is presented to the effect that the purine deoxyribonucleosides also arise from an intact purine ribonucleosyl intermediate (3).

**EXPERIMENTAL RESULTS**

**Growth of Organism—Neurospora mutant 36601 was a gift from Dr. H. K. Mitchell. In the usual culture medium (4) which was used, this organism grows some 10 to 60 times better in response to cytidine or uridine than it does to an equimolar amount of uracil. Cytosine is inactive (5).**

The medium (200 ml.) was supplemented with 7.5 mg. of uniformly labeled C<sup>14</sup>-cytidine (2,200,000 c.p.m.) (Schwarz Laboratories) and divided equally into 10 flasks of 125 ml. capacity. Each flask was inoculated with a drop of dilute suspension of conidia and incubated at 25° for 5 days. This concentration of cytidine gives about one-half maximum growth. (Subsequent to the feeding experiments the cytidine preparation was found to have not less than three radioactive contaminants, none of which showed an absorption in ultraviolet light. They were not identified, nor did their presence affect the conclusion reached from these experiments.)

**Separation of RNA and DNA Products—The mycelia from the**

10 cultures were combined, sucked dry on a funnel, and washed well with water, 50 per cent ethanol, 95 per cent ethanol, and ether. The filtrate and washings contained 346,000 c.p.m. (Very little, if any, of this radioactivity was due to remaining cytidine, cytosine, uridine, or uracil.) The dried mycelium (0.264 gm.) was ground thoroughly in a mortar and extracted six times with 5 to 10 ml. portions of 0.1 N KOH at 4°. The insoluble residue (81 mg., 34,000 c.p.m.) was discarded. The extract was adjusted to 1 N KOH (volume = 50 ml.), kept at 35° for 24 hours, and dialyzed for 4 days at 4° against 3 changes of water. The dialysate (9,000 ml.) was neutralized with HClO<sub>4</sub> and evaporated to 100 ml. The filtrate from the KClO<sub>4</sub> containing ribonucleotides (410,000 c.p.m.) was adjusted to pH 3 and treated with 2.5 gm. of Norit A (14,000 c.p.m. unadsorbed). The mononucleotides were eluted from the charcoal with 150 ml. of 30 per cent aqueous pyridine solution (362,000 c.p.m.), and chromatographed on paper. The amounts of the nucleosides subsequently recovered from this fraction after chromatographic purification to constant specific activity were as follows: uridine, 696 µg.; adenosine, 1,160 µg.; cytidine, 672 µg.; and guanosine, 480 µg.

The solution inside the dialysis bag, considered at that time to contain RNA as its principal radioactive substance, was reduced to a small volume at 4°. MgSO<sub>4</sub>·7H<sub>2</sub>O (2.4 mg.); 75 µl. of 1 M tris(hydroxymethyl)aminomethane buffer (pH 7); 0.2 mg. of DNAase (Worthington), and enough water to make 5 ml. were added. After an incubation period of 6 hours at 25°, the solution was dialyzed for 4 days at 4° against four 500 ml. portions of distilled water. The dialysate (250,000 c.p.m.) contained principally ribonucleotides and a fraction of the deoxyribonucleotides.

The contents remaining inside the dialysis bag (250,000 c.p.m.) had further amounts of deoxyribonucleotides and ribonucleotides. The amounts of the deoxyribonucleotides subsequently obtained from these two latter fractions after chromatographic purification to constant specific activity were as follows: thymidine, 57 µg.; deoxyadenosine, 46 µg.; deoxycytidine, 47 µg.; and deoxyguanosine, 18 µg.

Of the original radioactivity fed (2,200,000 c.p.m.) 1,500,000 c.p.m. or 70 per cent was recovered in the various fractions.

1 In apology for the awkward way in which the RNA and DNA products were separated it should be stated that these experiments and those described in the subsequent article (3) were carried out at a time when I did not realize the extent to which nucleotides may be retained inside membranes during dialysis (6, 7).
The ribonucleotides were separated into adenylic acid, cytidylic acid, and a mixture of uridylic and guanylic acids by paper chromatography on Whatman No. 3 paper in the system of Magasanik et al. (8). In all chromatograms the solvent moved from 35 to 42 cm. The eluate from each band was adjusted to pH 5 and treated with 4 mg. of acid phosphatase (Nutritional Biochemicals). Adenosine (RF = 0.76) and cytidine (RF = 0.00) were chromatographed in the isobutyric acid-water-NH₃ system of Löfgren (9) and in the butanol-water-acetic acid system of Whitby (10). Adenosine (RF = 0.50), cytidine (RF = 0.38), uridine (RF = 0.68), and guanosine (RF = 0.55) were separated in n-butanol, 45 ml.; morpholine, 15 ml.; ethylene glycol, 10 ml.; and water, 20 ml., and in Whitby's system (10) (uridine RF = 0.31, guanosine RF = 0.22).

As an example, the determination of the specific activity of cytidine will be given. The cytidine band and a corresponding area of paper from the chromatogram were eluted into 10 ml. volumetric flasks. An aliquot of the eluate from blank paper (0.5 ml.) was placed in the reference cuvette and 0.5 ml. of the cytidine solution was placed in the experimental cuvette. After diluting to 3 ml., concentrated HCl (2 μl.) was added and the spectrum was taken. Each solution was neutralized with concentrated NH₃OH (10 μl.) and the spectrum was taken again. The solution showed the maxima and minima in acid and base characteristic of cytidine. It analyzed 22 μg. per ml. in acid at 280 ma and 22.7 μg. per ml. in base at 270 ma or an average of 22.4 μg. per ml. Aliquots of this solution had an average radioactivity of 348 c.p.m. per 100 μl. The specific activity was therefore 378 c.p.m. per 10⁻⁴ mole of cytidine.

The specific activities of the other ribonucleosides and deoxynucleosides were similarly determined.

The combined fractions containing deoxynucleotides contaminated with ribonucleotides were reduced to about 2 ml. The solution was buffered at pH 9.1 with 100 μl. of 1 N NaOH-glycine, 3 mg. of dehydrated venom (Agkistrodon piscivorus; Ross Allen's Reptile Institute) were added, and the solution was covered with benzene and incubated at 35° for 10 hours. Upon chromatographing in butanol-water-ammonia (11) the deoxynucleotides migrate, whereas the ribonucleoside 3'-phosphates, which are unattacked by the specific 5'-nucleotidase of snake venom, remain at the base line. Each deoxynucleoside band was rechromatographed in the morpholine system, followed by chromatography in butanol-water-ammonia (11). The specific activity was determined again from its spectra. 503 c.p.m. per 10⁻⁴ mole were found.

A portion of this purified material (2760 c.p.m.) was diluted with 30 mg. of carrier cytidine, and the cytidine was crystallized from water-ethanol, m.p. = 212.5-214.5°. (All melting points were determined in tubes unless otherwise stated, and are corrected.) If all of the radioactivity were due to cytidine, the specific activity after dilution should be: 2760 x (2.43/30) = 224 c.p.m. per 10⁻⁴ mole. 215 c.p.m. per 10⁻⁴ mole (4 per cent error) were found.

**Comparative Specific Activities of RNA and DNA Components**—The specific activities of the nucleosides from RNA and DNA and the administered cytidine are presented in Table I.

It may be seen from the table that the administered cytidine was incorporated specifically into the pyrimidine nucleosides of the nucleic acids, undergoing a dilution of only about 30 per cent in the process. Hence, it is evident that the labeling cannot be due merely to the incorporation of the pyrimidine portion of the cytidine into these sites, for were this the case, the dilution would be 56 per cent. The remarkable similarity in the molar specific activities of the pyrimidine ribonucleosides and deoxyribonucleosides suggests that all four of these substances arose from a common intermediate. Furthermore, the specific activities of the pyrimidine nucleosides and deoxyribonucleosides, although having a much lower value, are similar as to make one think that all four of those also have a common origin.

**Distribution of Labeling within Pyrimidine Nucleosides**—The labeling in the glycosyl group and in the aglycone was determined after dilution of the nucleoside with a carrier. The specific activity of the ribose was determined as its toluene-p-sulfonylhydrazone, and the deoxyribose as the 2,4-dinitrophenylhydrazone of the levulinic acid derived therefrom. The specific activities of uracil and thymine were determined upon the crystalline substances, and that of cytosine was determined from the optical density and radioactivity of a solution, or on a sample of the picrate.

**Administered Cytidine**—After chromatography in Löfgren's system (9), the cytidine band was eluted with water. To a portion of the solution 300 mg. of carrier cytidine were added, and the cytidine was crystallized from water-ethanol, m.p. = 212-214°. 100 mg. of this material were hydrolyzed with 1 ml. of 60 per cent HClO₄ at 100° for 11 hours. The solution was diluted with water (50 ml.), filtered, and treated with 3.5 gm. of Norit A. The washed charcoal was eluted with 100 ml. of 30 per cent pyridine-water. The solvent was distilled off, and the

| RNA constituents | Cytidine | 378 | 9 [396]  |
| DNA constituents | Deoxyctydine | 351 | 339  |

| RNA constituents | Uridine | 333 [367] |
| DNA constituents | Thymidine | 342 [349] |

| RNA constituents | Adenosine | 8.9 | 7.2  |
| DNA constituents | Deoxyadenosine | 8.5 | 13.0  |

| RNA constituents | Guanosine | 9.7 | 16.8 |
| DNA constituents | Deoxyguanosine | 9.1 | 14.5  |

*Bracketed figures represent a second experiment in which C14-cytidine was administered at a concentration sufficient to give about one-third maximal growth.*
residue was taken up in 5 ml of water and boiled for 15 minutes with 80 mg of picric acid and 0.5 ml of 2 M HCl. Upon cooling, light yellow clumps formed which upon recrystallization from hot water (4 ml) gave long, chrome-yellow needles of cytosine picrate. It melted with decomposition around 276–279° (placed on the stage at 240°). Lefevre (13) reports 275°.

A second portion of the cytidine was reduced chemically and hydrolyzed with Dowex 50 (14). The toluene-p-sulfonylhydrazone of p-ribose melted at 162–163° (decomposition point). Easterby et al. (15) report 164° (uncorrected).

Cytidine from RNA—The nucleoside (82,000 c.p.m.) was diluted with 330 mg of carrier and crystallized, m.p. = 213–215°. The specific activity was 630 c.p.m. per 10⁴ mole (calculated value, 650 c.p.m.). It was degraded as described above.

Uridine from RNA—The uridine, diluted with carrier and crystallized from water-ethanol, melted at 166–167.5°. One portion was hydrolyzed as for cytidine, and the uracil was adsorbed on charcoal, eluted, and crystallized twice from hot water. Also the specific activity was determined from the optical density and radioactivity of a solution of the uracil after paper chromatography. The toluene-p-sulfonylhydrazone of ribose was obtained as described above.

Thymidine from DNA—The nucleoside was diluted with 26 mg of thymidine and crystallized from water, m.p. = 168°. Diluted thymidine (11.5 mg) was hydrolyzed with 3 ml of 6 M HCl for 3 hours at 100°. The HCl was removed in vacuum over KOH. The residue was washed well with ether, taken up in hot water, decolorized with a small quantity of charcoal and crystallized twice from water. The thymine sublimed at about 270° on a melting point stage. The ethereal solution of levulinic acid was evaporated, taken up in water, and decolorized with charcoal. The 2,4-dinitrophenylhydrazone of levulinic acid melted on the stage at 201–202°. Mixture with an authentic sample (m.p. = 202–203°) gave a m.p. of 202–203°.

Deoxycytidine from DNA—The nucleoside diluted with 26 mg of carrier and crystallized from ethanol, melted at 205–207.5°. The deoxycytidine was hydrolyzed and the levulinic acid was obtained as described for thymidine. The cytosine was estimated from the optical density of a solution in acid, at neutrality, in base, and from its radioactivity after chromatography on paper.

The results (Table II) show the very similar pattern of labeling within the pyrimidine nucleosides of RNA and DNA and the administered cytidine. There is, therefore, little doubt that the administered cytidine contributed its intact carbon skeleton to these groupings in the nucleic acids.

Distribution of Labeling within Purine Nucleosides—As may be seen from Table I, the specific activities of the purine nucleosides, although only about 4 per cent as radioactive as the pyrimidine nucleosides, are quite similar to each other. Adenine, guanine, and deoxyadenosine were degraded, and the radioactivity of the purine was determined. As the amount of radioactivity was extremely small in the case of deoxyadenosine, the estimate is only a rough one. (For a detailed description of the degradation of purine nucleosides see the subsequent paper (3).) The adenine.HCl.H₂O (m.p. = 232–233°, stage) and guanosine.H₂O (m.p. = 234–238°) were obtained after dilution with carrier (25 to 30 mg) and crystallization from water. After hydrolysis the purines were counted as adenine.HCl·½ H₂O and guanine. Deoxyadenosine was diluted with 500 μg of carrier and chromatographed. The specific activity of the diluted compound was determined from its molecular extinction coefficient; it was hydrolyzed, and the adenine was determined similarly from its absorption spectra at neutrality and in acid after paper chromatography. The specific activity of the adenine from the undiluted deoxyadenosine was also determined. The results (Table III) show a similar pattern of labeling in the purine ribonucleosides and in the deoxyadenosine. Although the radioactivity of these substances was of a very low order and may have arisen from impurities in the cytidine preparation rather than the breakdown producte of cytidine itself, the similar specific activities and distribution of labeling in these substances suggest that the purine nucleosides of RNA and DNA might also arise from a common intermediate (see the subsequent article (3) for a conclusive experiment on this point.)

**DISCUSSION**

An interesting point which these experiments emphasize is that the deoxyribose of the DNA originates from the ribose of a nucleoside. Although the enzymatic synthesis of deoxyribose-5-phosphate from d-glyceraldehyde-3-phosphate plus acetalde-
hyde has been demonstrated with preparations from Escherichia
coli (16) and the guinea pig (17), it is not known whether this
conversion is of any consequence to the biogenesis of the deoxy-
ribose of DNA. Even in E. coli, the organism from which Racker
obtained this enzyme (16), such a scheme does not account for
the deoxyribose in the DNA (18). On the contrary, the ribose
of RNA and the deoxyribose of DNA were similarly labeled
from the glucose-1-C\textsuperscript{14} fed.

In the metabolism of the rat also, as Hammarsten et al. (1)
suggested, the deoxyribose of the pyrimidine nucleosides of DNA
can arise, at least in part, from the ribose of cytidine (2) and of
cytidilic acid (19). With the mutant of Neurospora dealt with
here it is certain that this is the conversion which is of major
importance in the biogenesis of DNA.

There is the question whether this conversion is general to
Neurospora or represents a special case in this mutant. As this
mutant responds in growth to pyrimidine ribonucleosides 10
to 60 times better than it does to uracil, it might be supposed
that little splitting of the nucleoside occurs, thus making possible
a clear demonstration of this conversion. But can the conversion
be demonstrated in the other pyrimidine-requiring mutants of
Neurospora? Certain of these grow as well or better in response
to the free pyrimidines as they do in response to the nucleosides
or nucleotides. The relative responses may be altered in
certain cases, also, if the organism is grown in different media.

With these mutants, also, the question one wishes to answer is
whether the labeling within the pyrimidine nucleosides of RNA
agrees with the labeling within the pyrimidine nucleosides of
DNA. If such is the case, it is reasonable to suppose that the
differences in growth-response of the mutants to pyrimidines or
deoxyribonucleic acid is also known to occur (21). Crude enzymatic preparations
from this mutant have been tested for their ability to convert
the ribose in cytidine, adenosine-5-phosphate, and the 5'-mono-
di-, and triphosphates of uridine into a deoxyribose containing
product, so far without success.

**SUMMARY**

1. Uniformly labeled C\textsuperscript{14}-cytidine, when fed to a mutant of
Neurospora, was incorporated equally into the pyrimidine nucleo-
sides of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA)
with very little dilution.

2. The distribution of labeling between the aglycone and the
glycosyl group in the pyrimidine nucleosides of both RNA and
DNA showed that the entire carbon skeleton of the administered
cytidine was incorporated as such into these groupings.

3. The purine nucleosides of RNA and DNA were labeled to
only a very slight extent.

4. It is concluded that in this mutant of Neurospora the princi-
pal pathway for the biogenesis of the carbon skeleton of the
pyrimidine nucleosides of DNA involves a ribonucleosyl inter-
mediate.

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