Relative Retention of $H^3$ and $C^{14}$ Labels of Nucleosides Incorporated into Nucleic Acids of Neurospora*

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Reichard and Estborn (1) demonstrated that rats selectively incorporated thymidine-$N^{14}$ as deoxyribonucleic acid thymidine, and their observation has been extended by numerous investigators to include many organisms and several isotopic labels. Tritiated thymidine has been particularly useful as a means of marking DNA for study by high-resolution radioautography (2), and the histological distribution of the radioactivity has followed the pattern expected for selective DNA labeling in all but a few experiments, such as those of Stocking and Gifford (3) and Plaut and Sagan (4). The latter workers deprived their organisms of food and employed DNAse and RNAse in confirmatory tests to obtain evidence that the cytoplasmic radioactivity was associated with DNA and probably did not represent food particles.

When it was found that Neurospora crassa utilized thymidine-$2-C^{14}$ and thymidine-5,6-$C^{14}$ primarily for DNA synthesis (5, 6), it seemed of particular interest to determine the metabolic fate of tritiated thymidine in this organism. The present paper reports that tritium attached to carbon 6 of various pyrimidines was retained during their transformation to DNA thymine, and that Neurospora produced highly radioactive RNA and DNA from biologically synthesized thymidine-$6-H^3$ but not from a commercial preparation of thymidine-methyl-$H^3$ that had been purchased as "thymidine (H3 on C-6)." These results, together with studies of possible intermediates in the utilization of thymidine for RNA synthesis, have been summarized in preliminary papers (7, 8).

RESULTS

In order to minimize uncertainties arising from biological and procedural variations, tritiated and $C^{14}$-labeled preparations of a given nucleoside were generally mixed for testing in a single culture of Neurospora strain 36601. When this procedure was followed with thymidine-$2-C^{14}$ plus the commercial preparation of "thymidine (H3 on C-6)," the $C^{14}$ followed the previously observed pattern of extensive incorporation into the nucleic acids (Table I, Experiment 1), although no more than traces of tritium were incorporated. In a careful recheck of the $H^3$ to $C^{14}$ ratios with the aid of internal standards the tritium levels were below the limits of detection. The sensitivity of these tests was such as to indicate that the thymidine molecules which were converted to RNA pyrimidines lost more than 99.9% of their tritium label in the process and that those utilized for DNA synthesis lost at least 90% of their tritium.

The inability of the tritiated thymidine to label RNA was consistent with evidence from radioautographic studies (2) and allowed some interesting speculations on the nature of the reactions involved in the incorporation of the $C^{14}$ of thymidine into uridine and cytidine. A speculation that seemed worth investigating first, however, was the possibility that the thymidine employed was not tritiated on carbon 6, but in the methyl group or in the deoxyribose, for tritium might logically be lost from both of these moieties during conversion of thymidine to uridine. After acid hydrolysis of the doubly labeled thymidine, the recovered thymine retained approximately 85% of the original tritium label. This indicated that probably less than 15% of the tritium was attached to deoxyribose, for the conditions of hydrolysis may well have caused some isotope exchange between the pyrimidine and the acid solution. Friedkin (9) reported that the label in a commercial preparation of tritiated thymidine was localized exclusively in the methyl group, and thus it seemed...
The results of Experiment 2 could be most simply interpreted as indicating that more than 98% of the original tritium distribution was confirmed by acid treatment, which indicated that the thymidine formed in Experiment 6A was thymidine-6-H^3. In contrast to the difficulties of Experiment 5, then, this first routine test of tritiated deoxycytidine produced enough thymidine-6-H^3 to satisfy the analytical requirements and provide a modest but adequate surplus for use with thymidine-2-C^14 as a doubly labeled substrate. The results (Table I, Experiment 6) amply confirmed Experiment 5B in demonstrating that thymidine tritiated at position 6 can be utilized by Neurospora to produce a high yield of tritiated RNA.

**DISCUSSION**

In general, the present tritium studies have underscored previous results (5-8) which indicated that *Neurospora crassa* utilizes thymidine for nucleic acid synthesis by actually converting it to uridine, or some related RNA precursor, after which it may follow the metabolic pathways open to the usual RNA precursors, presumably including a pathway involving

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<table>
<thead>
<tr>
<th>Exp-No.</th>
<th>Radioactive supplement*</th>
<th>% Recovery of Isotope in nucleic acid components</th>
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<tr>
<td>1</td>
<td>Thymidine-2-C^14</td>
<td>Cytidine Uracil Deoxy- cytidine Thymidine</td>
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<tr>
<td></td>
<td>(commercial)</td>
<td>13 13 1.5 1.5</td>
</tr>
<tr>
<td>2</td>
<td>Uridine-2-C^14</td>
<td>30 10 17 5.7</td>
</tr>
<tr>
<td></td>
<td>(acid-stable)</td>
<td>30 10 17 5.7</td>
</tr>
<tr>
<td>3</td>
<td>Uridine-2-C^14</td>
<td>70 16 17 2.3</td>
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<tr>
<td></td>
<td>(acid-stable)</td>
<td>70 16 17 2.3</td>
</tr>
<tr>
<td>4</td>
<td>Cytidine-2-C^14</td>
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<tr>
<td>5A</td>
<td>Cytidine-2-C^14</td>
<td>2,000 12 0.02 0.03</td>
</tr>
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<td>(from 5A)</td>
<td>2,000 12 0.02 0.03</td>
</tr>
<tr>
<td>5B</td>
<td>Thymidine-2-C^14</td>
<td>0.6 14± 15±</td>
</tr>
<tr>
<td></td>
<td>(from 5B)</td>
<td>0.6 14± 15±</td>
</tr>
<tr>
<td>6</td>
<td>Deoxythymidine-H3</td>
<td>8,000 10 9 1.9</td>
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<tr>
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<tr>
<td>6B</td>
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<td>2 10 9 1.9</td>
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The results had a high probable error, they indicated that thymidine did not lose tritium from C-6 during its transformation to uridine, or some related RNA precursor, after which it may follow the metabolic pathways open to the usual RNA precursors, presumably including a pathway involving highly desirable to test some thymidine-6-H^3 prepared by an independent procedure.

A biosynthetic approach to the preparation of thymidine-6-H^3 seemed logical, although not necessarily frugal, for the yield of C^14 thymidine from C^14 ribonucleosides administered to *Neurospora* had averaged only approximately 1%. When doubly labeled uridine was administered to *Neurospora* (Table I, Experiment 2), the two isotopes showed similar patterns of incorporation except that DNA thymidine contained 0.1% of the added C^14 but only approximately 0.01% of the added tritium, definitely not an encouraging result from the standpoint of the biosynthesis. The results of Experiment 2 could be most simply interpreted as indicating that more than 98% of the original tritium label was attached to uridine at carbon 5 and thus subject to displacement during introduction of the methyl group of thymidine. This interpretation was supported by the finding that approximately 98% of the tritium in the doubly labeled uridine was acid-labile (1 N HSO_4 at 100° for several days) whereas the remainder, presumably on C-6, was much more stable. As a further check, a large sample of doubly labeled uridine was subjected to the acid treatment, chromatographically separated from the uracil by-product (which showed the same H^3 to C^14 ratio), and administered to *Neurospora*. The results (Table I, Experiment 3) clearly showed that conversion of uridine to thymidine did not involve displacement of tritium from carbon 6.

Results with doubly labeled cytidine (Table I, Experiment 4) were similar to those with untreated uridine (Experiment 2), except that the cytidine molecules which were converted to thymidine lost only approximately 90% of their tritium, and a similar percentage was subsequently found to be lost during long acid treatment which converted the cytidine to a mixture of uridine and uracil. This indicated that tritiated cytidine, with nearly 10% of its label on C-6, might be a feasible starting material for biosynthesis of thymidine-6-H^3 for use as a substrate in a subsequent experiment.

A new preparation of tritiated cytidine was procured, mixed with cytidine-2-C^14, and administered to *Neurospora* (Table I, Experiment 5A). A very low yield of thymidine-6-H^3 was obtained, probably due to a combination of several adverse factors. Growth of the culture was poor, possibly as a result of radiation damage; the yield of DNA was very poor, due possibly to radiation or the use of a shortcut isolation procedure or both; and the new cytidine preparation turned out to have less than 2% of its label attached at C-6, rather than the hoped-for 10%. In spite of the disappointing yield, the weakly radioactive thymidine-2-C^14-6-H^3 isolated in Experiment 5A was fed back to *Neurospora* (Table I, Experiment 5B). Although the analytical results had a high probable error, they indicated that thymidine did not lose tritium from C-6 during its transformation to uridine.

While Experiment 5B was underway, a sample of tritiated deoxycytidine was tested with the *Neurospora* mutant (Table I, Experiment 6A). The results were of both theoretical and practical interest in showing a utilization pattern such as might have been expected if the original tritium label were equally distributed between C-5 and C-6 and if the deoxycytidine were converted to a ribose derivative before utilization. The postulated tritum distribution was confirmed by acid treatment, which indicated that the thymidine formed in Experiment 6A was thymidine-6-H^3. In contrast to the difficulties of Experiment 5, then, this first routine test of tritiated deoxycytidine produced enough thymidine-6-H^3 to satisfy the analytical requirements and provide a modest but adequate surplus for use with thymidine-2-C^14 as a doubly labeled substrate. The results (Table I, Experiment 6B) amply confirmed Experiment 5B in demonstrating that thymidine tritiated at position 6 can be utilized by *Neurospora* to produce a high yield of tritiated RNA.

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1 Detailed data on rates of isotope exchange to be published.
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phosphorylations, reduction to the deoxyribonucleotide form, (re)-methylation, and eventual incorporation into DNA as a thymidylate residue.

Based on the lack of RNA labeling by the commercially tritiated thymidine used in Experiment 1, and the efficient labeling effected by thymidine that had been biologically synthesized to contain tritium at C-6, it would appear that the commercially tritiated preparation had practically no tritium at C-6. Since it also had little tritium in its deoxyribose, it must have been essentially thymidine-methyl-H\(^3\). In this light, the absence of a detectable amount of tritium in the DNA thymidine isolated in Experiment 1 apparently constitutes a highly sensitive confirmation of previous experiments (5, 6, 10) which were interpreted as indicating that Neurospora cultures failed to utilize exogenous thymidine directly by phosphorylation and incorporation into DNA. The loss of methyl-bound tritium in Experiment 1 also indicates that Neurospora's pathway for incorporation of thymidine into DNA via ribonucleotide intermediates provides no means by which the methyl label can be retained intact through formation, phosphorylation, and subsequent reduction of metabolites such as 5-methyluridine (11, 12) or 5-hydroxymethyluridine (13).

The data on thymidine-2-C\(^{14}\)-6-H\(^3\) agree with previous results (6) which indicated that thymidine was transformed to RNA pyrimidines without fragmentation of the pyrimidine ring. The stability of the tritium attached to carbon 6 would also appear to rule out any intermediate metabolic step involving formation of a barbituric acid derivative (9, 14), and render unlikely any step involving a dihydroxyphosphoryl (15, 16), which would probably (although not necessarily) lose tritium during a subsequent dehydrogenation. Such a process of elimination strengthens the possibility that the metabolic pathway for conversion of thymidine to RNA pyrimidines will prove to involve the stepwise oxidation of the methyl group of thymine noted in chemical studies (13) in liver slices (12, 13) and in Neurospora (7, 8).

The experiment with tritiated deoxycytidine yielded an incorporation pattern comparable to those obtained with C\(^{14}\)-labeled cytidine, uridine, and thymidine, and showed little evidence of direct phosphorylation for selective incorporation into DNA. This deoxyribonucleoside, like thymidine, may accordingly have been utilized by the organism for nucleic acid synthesis only after a preliminary transformation to the ribonucleotide form.

It may be noted that the pyrimidine interconversions carried out by Neurospora, when used in conjunction with C\(^{14}\)-labeled substrates, provide a quite sensitive means for determining the amount of tritium attached to carbon 6 in tritiated pyrimidine nucleosides. Although acid treatment\(^1\) may prove to be more convenient and sufficiently precise for use with the non-methylated compounds, use of Neurospora for localization of tritium in thymidine is relatively simple due to the high yield of labeled RNA and the ease with which the RNA nucleotides may be isolated. Relatively few of the published studies which have utilized isotopic thymidine would have clearly revealed a minor conversion of thymidine to RNA, and most of the studies have probably involved thymidine methyl-H\(^3\), which would not have detected even major conversions of this type. Accordingly, it is difficult to judge from presently available data whether the ability to convert thymidine to RNA pyrimidines represents a unique characteristic of some Neurospora strains, an occasional genetic adaptation that may be found in other organisms lacking a mechanism for phosphorylating thymidine, or a common capability that is largely suppressed by most species under normal conditions.

**SUMMARY**

With recovery of appropriate C\(^{14}\)-labeled nucleosides used as a baseline it was found that (a) a commercially tritiated uridine sample appeared to have approximately 98\% of its label attached at C-5, displaced by conversion to thymidine or by isotope exchange with aqueous acid, and approximately 2\% at C-6, retained through acid treatment and subsequent biological methylation. (b) Two tritiated cytidine preparations showed 90\% and 98\%, respectively, as the fraction of the tritium label that was eliminated by acid treatment or methylation at C-5. (c) Thymidine-6-H\(^3\), biologically prepared from tritiated cytidine or deoxycytidine, retained its label during conversion to uridylate and cytidylate residues of Neurospora ribonucleic acid. (d) A preparation purchased as "thymidine (H\(^3\) on C-6)," appeared actually to be thymidine-methyl-H\(^3\). This preparation was ineffective in labeling either type of nucleic acid in Neurospora, which probably cannot phosphorylate thymidine but must demethylate it for use as a ribonucleotide precursor. (e) Tritiated deoxycytidine, not doubly labeled, provided a nucleic acid labeling pattern which suggested that this deoxyribonucleoside, like thymidine, was utilized primarily as a precursor of ribonucleotides.

**REFERENCES**

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