STUDIES ON A RELATIONSHIP OF URACIL AND CYTOSINE NUCLEOSIDES TO BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID THYMINE

BY WILLIAM H. PRUSOFF

(From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut)

(Received for publication, August 29, 1957)

Formate (1), serine (2), formaldehyde (3), and methionine (4) each can serve as the donor of the carbon atom for the biosynthesis of the methyl group of DNA-thymine. The utilization of formate for the biosynthesis of DNA in vitro has been reported previously (5-9), and in most instances the uptake of formate carbon under these conditions has been primarily into the methyl group of thymine (5).

An investigation has now been made of some of the factors which affect the utilization of formate-C\textsuperscript{14} for the biosynthesis of the methyl group of DNA-thymine in vitro.

EXPERIMENTAL

Preparation of Cell Suspensions, Solutions, and Isolation of DNA-Thymine—The separation of thymine, by paper chromatography of a perchloric acid digest of the DNA fraction isolated by a modification of the method of Schmidt and Thannhauser, has been described previously, as has been the preparation of ascites tumor cells (8). Bone marrow cells were prepared by Totter's modification (5) of the method of Marvin et al. (10).

Materials—Uniformly labeled uridine and cytidine were obtained from the Schwarz Laboratories, Inc., and the specific activity of both nucleosides was adjusted to 0.54 μc. per mg.

Results

Studies with Rabbit Bone Marrow—It has been reported previously that uracil deoxyriboside (UdR) increased the incorporation of formate-C\textsuperscript{14} into DNA-thymine of rabbit bone marrow cells in vitro (8). This observation has been extended to include the effect of cytosine deoxyriboside.

* A preliminary report has been presented before the Forty-seventh annual meeting of the American Society of Biological Chemists at Atlantic City, April 16-20 (1956).

1 The following abbreviations are used: DNA, deoxyribonucleic acid; UR, uridine; UdR, uracil deoxyriboside; CR, cytidine; CdR, cytosine deoxyriboside; TdR, thymidine; RNA, ribonucleic acid.
NUCLEOSIDES AND THYMINE

(CdR) as well as the corresponding ribosides, cytidine and uridine (Table I) (11).

The ribosides are as effective as the corresponding deoxyribosides, presumably because of a rapid conversion of the riboside to the corresponding deoxyriboside (12–14). The cytosine nucleosides are significantly more efficient than the uracil nucleosides in increasing the appearance of C\textsuperscript{14} from formate-C\textsuperscript{14} into DNA-thymine.

**Studies with Mouse Ehrlich Ascites Carcinoma Cells; Effect of Nucleosides**—In these cancer cells, *in vitro*, UdR and CdR exert effects similar to those observed with rabbit bone marrow, as has been reported previously.

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Relative specific activity* of DNA-thymine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1.0</td>
</tr>
<tr>
<td>Uridine</td>
<td>2.4</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>2.8</td>
</tr>
<tr>
<td>Cytidine</td>
<td>4.2</td>
</tr>
<tr>
<td>Deoxythymidine</td>
<td>4.0</td>
</tr>
</tbody>
</table>

The reaction mixtures consisted of packed cells (0.25 ml.), formate-C\textsuperscript{14} (5 μc., 5 μmoles), nucleoside (10 μmoles), pig serum (0.1 ml.), Totter's modified Chambers solution (5) to 2.6 ml. The incubations were conducted in 20 ml. beakers, in duplicate, in a Dubnoff metabolic shaker at 37° under air and agitated at 90 cycles per minute for 3 hours.

* Specific activity of DNA-thymine in the control vessel equated to 1.00.

(8). The corresponding ribosides also are active (Table I), and the cytosine nucleosides are appreciably more active than the corresponding uracil derivatives (UR, UdR) in increasing the utilization of formate-C\textsuperscript{14} for DNA-thymine biosynthesis. A recent report by Kit (15) is in agreement with these observations. The marked increase in the uptake of formate-C\textsuperscript{14} which resulted from the addition of deoxyuridine supports the findings of Friedkin and Roberts (16) and of Reichard (17) which demonstrate the utilization of deoxyuridine-2-C\textsuperscript{14} for the biosynthesis of DNA-thymine by the rat, chick embryo, or in rabbit or chicken bone marrow.

The observed specific activity of DNA-thymine reflects the utilization of that portion of the administered metabolite which has successfully reached the site of utilization in spite of the various cellular and particulate
membranes, the degrading enzymes, and the competing anabolic reactions. Thus, it is extremely difficult to ascertain unequivocally with intact cells whether there is a difference in the degree of penetration of the nucleosides to the site of utilization.

That the present results do indeed reflect utilization of the nucleosides for DNA-thymine biosynthesis and not merely an indirect stimulatory effect is indicated by the observations with totally labeled C14-uridine and C14-formate.

**Table II**

*Relationship between Incorporation In Vitro of Totally Labeled Nucleosides and of Formate-C14 in Presence of Equal Amount of Unlabeled Nucleoside*

<table>
<thead>
<tr>
<th>Nucleoside added</th>
<th>Relative specific activity of DNA-thymine of Ehrlich ascites cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleoside-C14</td>
</tr>
<tr>
<td>Uridine</td>
<td>1.00</td>
</tr>
<tr>
<td>Cytidine</td>
<td>1.54</td>
</tr>
</tbody>
</table>

* Details of incubation conditions are described in Table I.

**Table III**

*Incorporation In Vitro of Totally Labeled Ribosides* into DNA and RNA of Mouse Ehrlich Ascites Tumor Cells

<table>
<thead>
<tr>
<th>Nucleoside added</th>
<th>Specific activity of isolated base</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RNA</td>
</tr>
<tr>
<td></td>
<td>Uracil c.p.m. per µmole</td>
</tr>
<tr>
<td>Uridine-C14</td>
<td>5270</td>
</tr>
<tr>
<td>Cytidine-C14</td>
<td>174</td>
</tr>
</tbody>
</table>

* Specific activities of both UR and CR were adjusted to 0.54 µc. per mg.
† Details of incubation conditions are described in Table I.

cytidine. The data in Table II clearly demonstrate that the radioactive nucleosides are incorporated into DNA-thymine in a manner identical to that observed with radioactive formate.

The amount of incorporation of UR-C14 into RNA-uracil is approximately equivalent to the amount of CR-C14 incorporated into RNA-cytosine (Table III). Thus, in terms of RNA biosynthesis there appears to be no difference in the rate of penetration of the two nucleosides into the cell.

When a comparison is made of the relative incorporation of the radioactive ribonucleosides into the pyrimidine bases of RNA and DNA (Table
III), one observes a ratio of RNA-uracil to DNA-thymine of 27:1 with UR-C\textsuperscript{14} as the precursor in contrast to that of 0.53:1 with CR-C\textsuperscript{14} as the precursor. If cytidine were deaminated before conversion to the deoxyribose, then one would expect that the relative specific activities of RNA-uracil to DNA-thymine would be equal to or greater than 1.0. Thus, it appears that the conversion of cytidine to the corresponding deoxyribose occurs more rapidly than its deamination to uridine. After the conversion to the deoxyribose, the problem to be resolved is whether CdR penetrates to the site of formate utilization at a greater rate than UdR and then

![Graph](image1.png)

**Fig. 1.** The effect of pH on the incorporation of formate-C\textsuperscript{14} into DNA-thymine of mouse Ehrlich ascites tumor cells. The details of the incubation conditions are described in Table I.

**Fig. 2.** The effect of uridine and cytidine on the incorporation of formate-C\textsuperscript{14} into DNA-thymine of mouse Ehrlich ascites tumor cells at various times of incubation. The details of the reaction mixtures are given in Table I.

is deaminated before the acceptance of the precursor of the methyl group, or whether CdR also is a primary acceptor of the single carbon unit.

*Effect of pH*—In Fig. 1 is shown the effect of pH on the incorporation of formate-C\textsuperscript{14} into DNA-thymine. The uptake of formate-C\textsuperscript{14} as influenced by either UR or CR is the same at pH 8.2 as at pH 7.0. On the other hand, at pH 5.8, cytidine exerts a marked effect on the incorporation of formate-C\textsuperscript{14} into DNA-thymine, while uridine is quite inactive. These results are suggestive of a primary role for CdR as a formate acceptor in the biosynthesis of thymine.

*Rate of DNA-Thymine Biosynthesis*—Since both UR and CR exert a marked stimulatory effect on DNA-thymine synthesis at pH 7.6, it is assumed that at this hydrogen ion concentration both nucleosides not only are readily transported across the cell membrane but also reach the site of
formate utilization (Fig. 2); consequently, the effect of UR and CR on the rate of DNA-thymine biosynthesis was investigated at pH 7.6. The stimulatory effect of uridine after 5 hours of incubation was less than that observed after only 2 hours of incubation with cytidine. In another experiment in which measurements were made after incubations of up to 8 hours and the results at pH 5.8 were compared to those at pH 7.6, the greater effectiveness of CR, as compared to that of UR, was again clearly demonstrated. At pH 7.6, in the presence of CR, the specific activity of DNA-thymine (46,400 c.p.m. per μmole) was 2.3 that observed in the presence of UR, whereas at pH 5.8 this ratio was increased to 8.1. It is remarkable that under these conditions, in vitro, the biosynthesis of DNA apparently continues even after 8 hours of incubation.

**Effect of Time of Addition of Formate or Nucleosides**—The effect of deoxycytidine on formate uptake into DNA-thymine was investigated when these compounds were added at varying intervals of time during the incubation period. No stimulation of formate utilization for the synthesis of DNA-thymine was observed when there was a delay of 1 hour in the addition of formate or CdR or both to the incubation vessel (Fig. 3). The results are shown in Fig. 3, Curves A, B, and C. A marked decrease in the uptake of formate into DNA-thymine was observed not only in the absence of deoxycytidine (Curve A) but also in its presence, whether the nucleoside

![Figure 3](image-url)
NUCLEOSIDES AND THYMINE

was added simultaneously with the formate (Curve C) or included in the incubation vessels initially, before the addition of formate (Curve B). The addition of deoxycytidine and formate-C\(^{14}\) at zero time resulted in the expected high specific activity of DNA-thymine; however, a delay in the addition of formate resulted in a marked decrease. This is not because of a loss of the formate acceptor (CdR), since a similar effect was observed when the nucleoside was added simultaneously with the formate.

When formate was added to the incubation vessels and the addition of CdR was delayed for 1 hour, the observed specific activity of DNA-thymine was essentially that of its zero time control, whereas, when CdR was added initially, a 2- to 3-fold increase in the specific activity was observed. Hence, within an hour, the nucleoside fails to exert any stimulatory effect, yet, when present initially with radioactive formate, a stimulatory effect on the biosynthesis of DNA-thymine continues for at least 8 hours.

Several possibilities may be considered in elucidation of the above. The ability of metabolites to enter the Ehrlich ascites tumor cells may be rapidly decreased after the cells are removed from the host animal. The enzymes or coenzymes involved in the activation of formate or of the nucleoside, or in the condensing of these two activated components into a non-dialyzable component, may be rapidly dialyzed out of the cell under the conditions used. This possibility is supported by the demonstration by Wu\(^2\) that several enzymes concerned with carbohydrate metabolism rapidly diffuse out of Ehrlich ascites cells during their incubation in vitro. A third possibility is that the enzymes may be protected from inactivation by the addition of the nucleoside and formate substrates. Another consideration is that the non-radioactive formate "pool" may increase during the incubation procedure; however, when the amount of added formate-C\(^{14}\) was varied from 0.3 to 10 \(\mu\)moles in the incubation vessel, it was apparent from the constancy of the observed specific activity of DNA-thymine that the added formate-C\(^{14}\) was probably in excess of the cellular formate pool.

Role of Phosphate—The effectiveness of added phosphate on the biosynthesis of DNA-thymine was investigated. There is no apparent effect in either the presence or absence of a riboside or a deoxyriboside.

DISCUSSION

Both UdR and CdR (or derivatives of them) have been implicated as primary acceptors of the single carbon unit for the biosynthesis of the methyl group of thymine. Friedkin and Roberts (16) and Reichard and Estborn (17, 18) have demonstrated that radioactive UdR and CdR are incorporated into DNA-thymine, and Friedkin (19) has shown that deoxyuridine 5'-phosphate can be enzymatically methylated to thymidine 5'-phosphate.

\(^2\) Personal communication from Dr. R. Wu.
phosphate. Phear and Greenberg (29) observed that UdR is a better acceptor of radioactive formaldehyde than CdR or the corresponding nucleotide for the biosynthesis in vitro of acid-soluble thymine derivatives by a cell-free preparation of thymus tissue. On the other hand, cytosine nucleosides exert a markedly greater stimulatory effect on the synthesis of DNA-thymine than do the uracil nucleosides in two systems in vitro, normal rabbit bone marrow and mouse Ehrlich ascites carcinoma cells. The experimental data of Friedkin and Roberts (16) and of Reichard (17) do not eliminate the consideration of CdR as a primary acceptor of the single carbon unit in addition to that of UdR, but do rule out the conversion of UdR into cytosine of DNA. However, uracil in a riboside form can be aminated and subsequently utilized for the biosynthesis of RNA- or DNA-cytosine. Lieberman (21) has observed that this amination reaction occurs in Escherichia coli at the triphosphate level (UTP → CTP). Cohen and Barner (22) have presented evidence compatible with a methylated cytosine derivative, 5-methyl CdR, being on the pathway of TdR biosynthesis in E. coli. Thus, it is possible that UdR may be converted into a derivative containing cytosine, which, although not incorporated as such into DNA, may be "formylated," deaminated, and then incorporated in such a form as to introduce a thymine-containing unit into DNA. That cytosine derivatives probably can accept a single carbon unit is indicated by the presence of 5-methylcytosine in plant and animal DNA (23) and of 5-hydroxymethylcytosine in the DNA of T-even coliphages (24). Kit (15) observed that CR and CdR, but not UR or UdR, increased the utilization of formate-C¹⁴ for the biosynthesis of 5-methylcytosine of DNA of rat thymus and tumor E-9514A in vitro.

Thus, it is not yet clear whether UdR or CdR or both (or their derivatives) act as the primary acceptor for the single carbon unit in the synthesis of DNA-thymine. In an investigation of intermediates involved in the biosynthesis of DNA-pyrimidine nucleotides, Hecht and Potter (25) could not evaluate the importance of the interconversion of CdR to TdR. That several pathways may exist for the metabolism of a particular compound is not an untenable hypothesis.

Studies with radioactive uridine and cytidine eliminate the possibility of a more favorable transport into the cells of the cytosine nucleoside. However, no evidence is available concerning the possible subsequent preferential kinetic treatment of cytosine nucleosides along the pathway to and at the specific cellular site where deamination to UdR (or derivatives) may occur before the acceptance of the single carbon unit. Observations of Cohen and Barner (22), of Kit (15), and those in our laboratory (8, 11) indicate strongly that a cytosine derivative may indeed be a primary acceptor of the single carbon unit. The importance of this pathway in
NUCLEOSIDES AND THYMICELATION to the established UdR → TdR mechanism should be elucidated. Studies of cell-free systems are in progress.

SUMMARY

Uridine and cytidine are as effective as the corresponding deoxyribosides in increasing the utilization of radioactive formate for the biosynthesis of deoxyribonucleic acid thymine in rabbit bone marrow and mouse Ehrlich ascites carcinoma cells in vitro, presumably because of a rapid conversion of the riboside to the deoxyriboside. The cytosine nucleosides are markedly more efficient than the corresponding uracil nucleosides at various pH levels and times of incubation. Studies with radioactive uridine and cytidine reveal no preferential transport into the cell and subsequent utilization of cytidine for ribonucleic acid pyrimidine biosynthesis. This evidence is presented to support the proposed role of deoxycytidine, in addition to deoxyuridine, as a primary acceptor of the single carbon unit during the biosynthesis of the methyl group of DNA-thymine.

The relative incorporation of radioactive cytidine and uridine into nucleic acid thymine of mouse Ehrlich ascites cells in vitro is identical with the relative utilization of radioactive formate for the biosynthesis of the methyl group of this thymine in the presence of non-radioactive cytidine and uridine. Cytidine is utilized better than uridine as a precursor of deoxyribonucleic acid pyrimidines; however, uridine is a better precursor of ribonucleic acid cytosine than is cytidine of ribonucleic acid uracil.

Whereas the biosynthesis of nucleic acid thymine by Ehrlich ascites tumor cells in vitro may proceed for 8 hours, a delay of 15 to 30 minutes in the addition of formate or of nucleoside to the incubation vessel results in a marked decrease in formate-C14 incorporation into the methyl group of thymine. After a delay of 1 hour, no formate is utilized whether in the presence or absence of nucleoside.

The author wishes to express his appreciation to Dr. A. D. Welch for his interest and encouragement in the development of this study, to Dr. L. G. Lajtha for many discussions of various aspects of this problem, and to Mrs. Angela Scimone Sleddon for technical assistance.

This study was supported by grants from the American Cancer Society, as recommended by the Committee on Growth of the National Research Council, and from the National Institutes of Health, United States Public Health Service.

BIBLIOGRAPHY