THE CONVERSION OF 5-METHYLDEOXYCYTIDINE TO THYMIDINE IN VITRO AND IN VIVO*

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The pyrimidine, 5-methylcytosine, was demonstrated by Wyatt (1) to be a component of plant and animal deoxyribonucleic acid. The deoxyriboside of 5-methylcytosine was isolated from wheat germ DNA by Dekker and Elmore (2), who also characterized this nucleoside. No microorganisms or viruses have yet been shown to contain this base, although the nucleic acids of many of these organisms have been examined for its presence. Its absence in bacteria may be accounted for in several possible ways; for example, (a) the base is not synthesized, or (b) it is metabolized at a rate which does not permit accumulation. Other hypotheses might also be required in some instances since, for example, insect viruses which do contain DNA lacking 5-methylcytosine multiply in nuclei in which 5-methylcytosine is a structural component of DNA.

Little is known of the origin or metabolism of this relatively rare base and its derivatives. The formation of a methyl group possibly proceeds via a hydroxymethylpyrimidine, thereby relating 5-methylcytosine to hydroxymethylcytosine, a pyrimidine found uniquely in certain virus nucleic acids (3). However, it is not evident whether the formation of the methyl

\[
\begin{align*}
\text{NH}_2 & \quad \text{OH} \\
\text{C} & \quad \text{C} \\
\text{N} & \quad \text{C} \\
\text{C} & \quad \text{CH}_2 \\
\text{N} & \quad \text{CH} \\
\text{O} & \quad \text{N} \\
\text{R} & \quad \text{R}
\end{align*}
\]

5-Methylcytosine

\[
\begin{align*}
\text{NH}_3 & \quad \text{OH} \\
\text{C} & \quad \text{C} \\
\text{N} & \quad \text{C} \\
\text{C} & \quad \text{CH}_3 \\
\text{N} & \quad \text{CH} \\
\text{O} & \quad \text{N} \\
\text{R} & \quad \text{R}
\end{align*}
\]

Thymine

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1 The abbreviations used are deoxyribonucleic acid, DNA; deoxycytidine, CDR; 5-methyldeoxycytidine, 5-MCDR; thymidine, TDR; cytosine, C; 5-hydroxymethylcytosine, HMC; 5-methylcytosine, 5-MC; tris(hydroxymethyl)aminomethane, Tris; ribonucleic acid, RNA.
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group occurs on the 6-aminopyrimidines or on the 6-hydroxypyrimidines. In the former instance 5-methylcytosine or a derivative might be deaminated to form the essential DNA component, thymine, as in the accompanying diagram, in which R = H, a deoxyribosyl or a phosphodeoxyribo-
syl moiety. In the latter case it is possible that thymine is aminated to 5-methylcytosine. Thus the problem of the origin of 5-methylcytosine is tied to the problem of the origin of both thymine and hydroxymethyl-
cytosine with which we have been concerned for some years in the study of virus-infected Escherichia coli.

Although the enzymatic deamination of the free base, 5-methylcytosine, to thymine is effected by the cytosine deaminase of yeast (4), we have shown that the cytosine deaminase of E. coli does not act upon the methyl-
pyrimidine (5). Furthermore, E. coli does not normally contain a cytosine deaminase; in this organism it is an inducible enzyme which may be demon-
strated in pyrimidine-requiring strains. Neither the cytosine deaminase of yeast nor that of E. coli is capable of deaminating hydroxymethylcyto-
sine.

However, E. coli does normally contain an active deoxycytidiny deami-
nase, so called because it deaminates deoxycytidine at a rate about 2.5 times greater than cytidine (6). As we have shown, this enzyme is capable of converting hydroxymethylcytosine deoxyriboside to hydroxymethyl-
uracil deoxyriboside, although at a rate which is only 1 to 3 per cent that of the rate of deamination of deoxycytidine. It appeared of interest to determine whether this enzyme deaminates 5-methyldeoxycytidine to thymidine and whether it can function in this manner in intact bacteria.

EXPERIMENTAL

Preparations and Properties of 5-Methyldeoxycytidine—An approximately equimolecular mixture of deoxycytidylic and 5-methyldeoxycytidylic acids was kindly provided by the California Foundation for Biochemical Re-
search. A solution containing 6.6 mg. of the 5-methyldeoxycytidylic acid and 0.72 ml. of \( \text{M} \) MgCl₂ was adjusted to the phenolphthalein end point with \( \text{NH}_4\text{OH} \) in a final volume of 12 ml. To the mixture was added 0.05 ml. of purified alkaline phosphatase (7) containing 12 units of enzyme. After 4 hours incubation at 37°, the mixture was stored at 4° overnight and centrifuged to remove \( \text{MgNH}_4\text{PO}_4 \). The supernatant fluid was concen-
trated and chromatographed at room temperature on three large sheets of Whatman No. 1 paper in butanol-N\( \text{H}_2\text{OH} \). Deoxycytidine, 5-methyldeoxycytidine, and thymidine could be separated readily in this solvent (Table I). However, if \( \text{NH}_3 \) is permitted to escape from the solvent, the separation of the first two nucleosides is less satisfactory. In Table I are given \( R_f \) values for the compounds in a number of solvent mixtures.
The ultraviolet absorption spectra of the compound eluted from paper were determined on a Beckman DU spectrophotometer in acid, neutral

**Table I**

<table>
<thead>
<tr>
<th>Solvent mixtures</th>
<th>CDR</th>
<th>5-MCDR</th>
<th>TDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopropanol-HCl*</td>
<td>0.64</td>
<td>0.70</td>
<td>0.87</td>
</tr>
<tr>
<td>Ethyl acetate-PO₄†</td>
<td>0.0</td>
<td>0.0</td>
<td>0.13</td>
</tr>
<tr>
<td>Butanol-NH₄OH‡</td>
<td>0.21</td>
<td>0.29</td>
<td>0.48</td>
</tr>
<tr>
<td>Butanol-H₂O§</td>
<td>0.19</td>
<td>0.24</td>
<td>0.45</td>
</tr>
</tbody>
</table>

* 65 ml. of isopropanol, 16 ml. of concentrated HCl, 19 ml. of H₂O.
† Ethyl acetate saturated with 0.05 M phosphate buffer, pH 6.0 (8).
‡ 150 ml. of n-butanol equilibrated with 50 ml. of 5 per cent NH₄OH.
§ 86 per cent by volume of n-butanol.

**Fig. 1.** Ultraviolet absorption spectra of 5-methylcytosine deoxyriboside at various pH levels. O, pH 7; •, pH 13; ▲, pH 1.
presented by those authors (2). An isosbestic point at 272 m\(\mu\) was observed by Cohn (9) for the 5-methyldeoxycytidylic acid and is closer to the value predicted by Fox and Shugar by analogy to other 5-methylcytosine derivatives (10).

In common with other cytosine derivatives, 5-MCDR formed a crystalline picrate when mixed in aqueous solution with 1 per cent picric acid. Generally, the picrates of the free pyrimidines were more insoluble than those of the nucleosides. In Fig. 2 is presented a comparison of the crystal forms of the picrates precipitated from aqueous solution containing 2 to 4 \(\mu\)moles of cytosine derivative per ml. In some instances it was necessary to concentrate the solution before crystallization occurred. It can be seen in Fig. 2 that the picrates of 5-chlorocytidine, 5-bromocytidine, and 5-hydroxymethylcytosine formed relatively thick crystals in contrast to the others. As noted below, the rates of deamination of these nucleosides are markedly slower than the rates of deamination of cytidine, CDR, and 5-MCDR which form needle-like picrates.

Other Cytosine Derivatives—Hydroxymethylcytosine was kindly given to us by Dr. C. Miller of Merck and Company, Inc. The isolation of hydroxymethylcytosine deoxyriboside from the DNA of T6r+ bacteriophage has been described (5). The nucleosides, 5-chlorocytidine and 5-bromocytidine, were kindly given to us by Dr. D. Visser of the University of Southern California. Cytosamine is a cytosine-containing degradation product of the antibiotic amicetin (11), which was provided by Dr. J. Hinman of The Upjohn Company; as estimated by the perchloric acid-tryptophan reaction (12), the substituent at N\(_3\) does not contain deoxypentose. Cytosine, 5-methylcytosine, deoxycytidine, and cytidine were obtained from the California Foundation for Biochemical Research. Cytosine glucopyranoside and 5-methylcytosine glucopyranoside were gifts of Dr. James Hunter of The Upjohn Company.

Action of Deoxycytidine Deaminase—The deaminase was prepared from extracts of E. coli strain B and was purified to eliminate the nucleoside phosphorylase. The bacteria were grown with aeration in a mineral medium containing glucose as the sole carbon source (13) and were harvested by centrifugation while in their exponential phase. All subsequent steps were carried out at 4\(^\circ\). The wet pellet was ground with 2.5 times its weight of Alumina A-301, and the sticky paste was extracted with 10 ml. of 0.05 M Tris buffer, pH 7.0, per gm. of bacteria. After centrifugation at 5000 r.p.m. to remove alumina and at 40,000 r.p.m. for 2 hours, 0.1 volume of 0.25 M MnCl\(_2\) was added to the supernatant fluid. The mixture was centrifuged for 30 minutes at 5000 r.p.m., and to the supernatant fluid was added an equal volume of saturated \((\text{NH}_4)_2\text{SO}_4\) adjusted to pH 7.6. After standing for an hour, the precipitate was removed by centrifugation. 2
volumes of saturated $(\text{NH}_4)_2\text{SO}_4$ were added to the supernatant fluid, and the precipitate which formed in 1 hour was collected by centrifugation.

The precipitate which formed in the range of 50 to 75 per cent saturated $(\text{NH}_4)_2\text{SO}_4$ contained at least 85 per cent of the deaminase. It was readily soluble in water and could be stored for months in the frozen state without loss of activity. Dialysis against distilled water produced only a slight decrease in activity.
Upon incubation of the enzyme with deoxycytidine at 36° in 0.05 N Tris buffer at pH 7.5 in quartz cuvettes in the Beckman spectrophotometer, deamination was followed by observing the change in absorption at 282 mμ. The molecular extinction coefficients at 282 mμ of deoxycytidine and deoxyuridine are 6000 and 2700, respectively; those for 5-methyldeoxycytidine and thymidine are 7800 and 5600, respectively, or a decrease of 28 per cent at 282 mμ as compared to a 55 per cent decrease obtained in the deamination of deoxycytidine. The procedure employed was essentially that of Wang (14).

In Fig. 3, A is presented a comparison of the deamination rates of the two deoxyribosides at an initial concentration of 0.1 μmole per ml. At the end of the reactions, the conversions estimated from the extinction coefficients are seen to be within several per cent of the theoretical values. The spectra of the final products determined in 0.1 N HCl were those of deoxyuridine and thymidine, as presented in Fig. 3, B. The positions of the peaks did not shift in 0.1 N NaOH, revealing that the products were present solely as deoxyribosides.

To characterize the reaction products more adequately, the two deoxyribosides (0.5 μmole) were incubated in 1 ml. of 0.05 M Tris buffer, pH 7.0, containing an amount of enzyme which produced complete deamination in 30 minutes at 37°. Before addition of enzyme and at 40 minutes after
incubation, the aliquots were placed in a boiling water bath for 1 minute. The coagulated protein was removed by centrifugation and the solution was concentrated. Paper chromatography in \( n \)-butanol-\( H_2O \) demonstrated the complete conversion of deoxycytidine to deoxyuridine and of 5-methyldeoxycytidine to thymidine. The spots revealed under ultraviolet light were eluted in water in yields of approximately 80 per cent. The spectra of the eluted initial products in 0.1 \( N \) HCl were those of deoxycytidine and 5-methyldeoxycytidine; the final products possessed spectra of deoxyuridine and of thymidine, respectively.

The thymidine generated enzymatically was characterized as follows: After elution from the chromatogram, 0.29 \( \mu \)mole was concentrated and incubated in 4 ml. of 0.025 \( \mu \) phosphate at pH 7.8. After removal of an initial 1 ml. aliquot, 0.075 ml. of a complete water extract of \( E. coli \) strain B containing a nucleoside phosphorylase was added to the remaining 3 ml. After incubation for 95 minutes at 37\(^\circ\), sufficient to give almost maximal cleavage of known thymidine under comparable conditions, the mixture was heated in a boiling water bath for 2 minutes and sedimented. The supernatant fluid was concentrated and chromatographed on paper in ethyl acetate-phosphate, a solvent mixture which cleanly separates thymine and thymidine (8). The initial aliquot contained no detectable thymine. A portion of the spot possessing the \( R_F \) of the thymine was extracted in water and contained 0.09 \( \mu \)mole of thymine (absorption maximum 265 \( m\mu \)) as estimated spectrophotometrically in 0.1 \( N \) HCl.

When the rates of deamination were estimated with CDR and 5-MCDR, it was observed that for a fixed low enzyme concentration, \( i.e. \) that amount sufficient to deaminate 0.1 \( \mu \)mole of deoxycytidine per ml. completely in about 15 minutes, the initial rate of deamination of 5-methyldeoxycytidine was 0.55 that of deoxycytidine, at 0.05 \( \mu \)mole of substrate per ml. At lower concentrations of substrate the initial rate of deamination of deoxycytidine fell off more rapidly than that of the 5-methyl nucleoside and the rates were substantially equal at 0.02 \( \mu \)mole of nucleoside per ml. For deoxycytidine and 5-methyldeoxycytidine, the rates at this concentration were 32 and 60 per cent of the maximal rates, respectively. It may be noted that this concentration (0.02 \( \mu \)mole per ml.) is approximately the lowest optimal level for fulfilling the thymine requirement of \( E. coli \) in permitting growth to a level of \( 10^9 \) organisms per ml. Thus, in terms of the physiological thymine requirement of the organism, the deoxycytidine deaminase might be termed a 5-methyldeoxycytidine deaminase.

Studies have also been carried out with the deaminase and a number of other cytosine nucleosides at 0.1 \( \mu \)mole per ml. As noted earlier, cytidine and 5-hydroxymethylcytosine deoxyriboside were deaminated, although at lower rates than deoxycytidine. 5-Chlorocytosine riboside was
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deaminated to chlorouridine at a rate approximately a quarter that of deoxycytidine. The initial rate of deamination of 5-bromocytosine riboside was about a third that of the enzyme on chlorocytidine. In the latter instance a complete cessation of deamination occurred when the reaction had proceeded 40 to 60 per cent. Cytosine glucopyranoside, 5-methylcytosine glucopyranoside, and cytosamine were completely inert to the enzyme.

Bacterial Experiments—A thymine-requiring bacterium, E. coli strain 15T−, has been studied for several years in this laboratory (5, 15). Among the unusual properties of the organism is the phenomenon of "unbalanced growth" observed when it is permitted to metabolize in a medium complete in all essential metabolites except thymine (16, 17). As has been described, cells which have a specific deficiency with respect to this nuclear requirement synthesize cytoplasmic constituents such as proteins and RNA and fail to synthesize DNA. The result of such unbalanced growth is an irreversible loss of the ability to multiply, as detected by plating on thymine-containing media.

The organism grows on thymidine as well as on thymine. Since the killing effect of 5-bromouracil is overcome by thymidine better than by thymine, and 5-bromodeoxyuridine is far more toxic than bromouracil in the presence of thymidine (17), it is inferred that in this organism thymine is converted initially into thymidine, which is then phosphorylated to the nucleotide level.

The effect of a compound on strain 15T− is tested routinely in the following manner: Bacteria are grown at 37° in the aerated mineral medium containing glucose as sole carbon source (13) and thymine at 2γ or 0.016 μmole per ml. At approximately 3 × 10⁶ per ml., the bacteria are centrifuged and washed twice in cold mineral medium. The organisms are resuspended in mineral medium, glucose, and the test compound at an equimolar concentration of pyrimidine and aerated at 37°. The turbidity of the culture is followed in the Klett photoelectric colorimeter with a No. 420 filter (18), and viable count is determined by plating an appropriate dilution on nutrient broth agar. In Fig. 4 are presented the data on the growth of the organism in the presence of 0.01 μmole per ml. of thymidine or 5-methyldeoxycytidine and the loss of viability in the absence of these compounds or in the presence of 5-methylcytosine. Thus the free pyrimidine can be neither deaminated nor coupled to form a nucleoside by a nucleoside phosphorylase or by a transglycosidase.

Washed cells, strain 15T−, were suspended at about 2 × 10⁹ per ml. in mineral medium containing deoxycytidine or 5-methyldeoxycytidine at 0.02 μmole per ml. At intervals, 0.1 ml. of 60 per cent perchloric acid was added to 2 ml. aliquots. The chilled tubes were centrifuged and the ultra-
violet absorption spectra were determined. Within a minute about 60 per cent of the 5-methyldeoxycytidine had been converted to thymidine; the deoxycytidine was deaminated more slowly.

Inhibition in Vivo—The possible inhibition of utilization of 5-methyldeoxycytidine by a number of pyrimidine derivatives was determined by following the turbidity and viability of strain 15− in the presence of 5-methyldeoxycytidine at 0.017 μmole per ml. and a 10-fold molar ratio

![Graph](http://www.jbc.org/)

Fig. 4. The growth response of *E. coli* strain 15− to thymidine and to 5-methyldeoxycytidine. The pyrimidines were present at 0.016 μmole per ml. ●, no pyrimidine; ○, 5-methylcytosine; X, thymidine; △, 5-methyldeoxycytidine.

(0.17 μmole per ml.) of test inhibitor. 5-Methylcytosine, 5-methylcytosine glucoside, and cytosamine were without significant effect.

Inhibitory compounds were of three types. Inhibitions were observed with cytosine derivatives which were not deaminated. Thus cytosine glucoside produced a lag in increase of viable count and turbidity and about a 10 per cent decrease in the rate of growth obtained.

Inhibitions were also obtained with cytosine derivatives which could be deaminated and with their deamination products as well. Thus at a molar ratio of 10:1, deoxycytidine and cytidine produced 50 to 75 per cent inhibitions of increase in viable count and in turbidity in a 2 hour interval (Fig. 5). Deoxyuridine at this concentration was about as inhibitory as
3 times the concentration of deoxycytidine. Uridine was far less inhibitory in this test.

**Fig. 5.** The inhibitory activity of cytosine derivatives toward *E. coli* strain 157 growing in the presence of 5-methyldeoxycytidine at 0.017 μmole per ml. •, no addition; X, deoxycytidine; O, 5-methylcytosine; △, cytidine; □, cytosine glucoside. These compounds were present at 0.17 μmole per ml.

**DISCUSSION**

The function of the pyrimidine nucleoside deaminase of *E. coli* is unknown at present. It may be suggested as a working hypothesis that the enzyme does indeed operate in one of the possible routes of thymidine biosynthesis, that involving the formation of a methyl group on a cytosine derivative, e.g. deoxycytidine.

Although most workers consider it unlikely that pyrimidines are first formed as 6-amino compounds, it has been observed that, in mammalian systems in which deoxyuridine is converted to thymidine, the ring structure of the former nucleoside does not enter deoxycytidine (19, 20). It has been suggested then that the cytosine series of compounds has an independent origin. Nevertheless, in bacterial mutants requiring pyrimidines for growth, uracil and cytosine requirements have been interchangeable. In *E. coli* strains, Wc− and B4−, tested in our laboratory by isotopic techniques, uracil fills the complete uracil and cytosine requirement of the organisms (21, 22). Furthermore, when strain B4− was grown in uracil-2-C14 plus unlabeled deoxycytidine, the uracil and cytosine of DNA and RNA possessed equivalent amounts of label per mole of pyrimidine formed.
Thus there is no metabolic evidence that the cytosine series does have an independent origin in *E. coli*.

At first glance, the existence of the active pyrimidine nucleoside deaminase appears wasteful to the economy of *E. coli*. Thus, if the main pathway of pyrimidine nucleoside metabolism begins with deoxyuridine and leads via amination to deoxycytidine, the existence of the deaminase would merely effect the regeneration of the deoxyuridine. On the other hand, it is possible that the main pathway of pyrimidine transformation in *E. coli* occurs at the nucleotide level via the condensation of orotic acid or uracil with pyrophosphoryl ribose phosphate to form the ribose nucleotide. In this event the deaminase may act as a scavenger mechanism for the recovery of useful pyrimidine derivatives after dephosphorylation of the key nucleotide.

If the conversion of 5-methyldeoxycytidine to thymidine is a normal pathway for the generation of the DNA thymidine, the control of the deaminase in *vivo* would become a mechanism of some interest. The fact that a variety of enzyme substrates and products inhibits the conversion to thymidine, among them natural substances such as deoxycytidine, cytidine, and deoxyuridine, suggests the possibility that the formation of these compounds may govern the availability of thymidine for DNA synthesis. In this light it may be noted that uridine is a more potent inhibitor than deoxyuridine when thymine or thymidine is used to support growth of a thymine-requiring bacterium (22), whereas deoxyuridine is much more active as an inhibitor than uridine in the presence of 5-methyldeoxycytidine as the source of thymidine.

The DNA of plants is particularly rich in 5-methylcytosine. Pyrimidine nucleosides, particularly thymidine, are readily utilized in plants, which also appear capable of utilizing free pyrimidines to a significant extent (17). The nucleosides may presumably be phosphorylated by appropriate kinases to the nucleotides. It may be imagined that the conversion of 5-methyldeoxycytidine to thymidine is an important pathway in DNA synthesis in those organisms. However, if plants were cells in which the pyrimidine nucleoside deaminase was limiting, two results might be obtained: (a) 5-Methyldeoxycytidine would accumulate and perhaps be phosphorylated. The nucleotide might then substitute for deoxycytidylic acid; (b) thymidine would be in short supply for DNA synthesis, producing phenomena of cytoplasmic elongation and unbalanced growth, even as is commonly observed during the differentiation of plant cells.

**SUMMARY**

The deoxyribose of 5-methylcytosine has been isolated. The compound is deaminated *in vitro* by a partially purified pyrimidine nucleoside
deaminase from *Escherichia coli*. The product, thymidine, was isolated and characterized. The rates of deamination of deoxycytidine and the 5-methyl nucleoside were compared.

A thymine-requiring strain of *E. coli* can utilize 5-methyldeoxycytidine to fill its thymine requirement in permitting a normal rate of growth. The conversion *in vivo* of 5-methyldeoxycytidine to thymidine is inhibited by a number of natural nucleosides. The significance of these results is discussed.

**BIBLIOGRAPHY**

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