90. Structural Requirements for the Enzymatic Deamination of Cytosine Nucleosides

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Dedicated to Professor Dr. *Tadeus Reichstein* on the occasion of his eightieth birthday

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Summary

Thirty-three 1-β-D-pentofuranosylcytosine nucleosides were examined as substrates of crude cytidine deaminase from mouse kidney. In addition to previous observations of structural features in substrates required for enzymatic deamination [*e.g.*, a free 3′-hydroxyl in the 'down' ribo(arabino)configuration] we find that: modification of the aglycon by substitution of a fluorine atom at C(5) results in a several-fold increase in the deamination velocity relative to cytidine whereas insertion of a methyl group at C(5) decreases the deamination velocity. This decrease is even more pronounced when a methyl group is substituted at C(6).

Though xylosylcytosine and 3′-deoxy-3′-fluoro-xylo-C are not substrates for this deaminase, those xylofuranosylcytosines bearing good leaving groups (*e.g.*, bromo, mesyloxy, or tosyloxy) at C(3′) are deaminated with substantial deamination velocities. This is probably due to a prior chemical reaction leading to arabino nucleosides bearing a free 'down' 3′-hydroxyl.

A different situation is obtained with arabino nucleosides. Though ara-C and 2′-deoxy-2′-fluoro-ara-C are substrates for this deaminase, substitution of bulky groups (*e.g.*, chloro, bromo, or mesyloxy) at C(2′) substantially decreases the susceptibility to deamination. An hypothesis is offered to explain these differences between xylo- and arabino-cytosines.

The presence of a free hydroxyl group at position 5′ is not essential for enzymatic deamination.

In the last decade, 1-β-D-arabinofuranosylcytosine (ara-C) has emerged as one of the most useful agents for the chemotherapy of human acute leukemia [1–4]. It is now used mostly in combination with other drugs, such as thioguanine [5], cyclophosphamide-vincristine-prednisone [6] and other combinations. In man, monkeys, dogs and mice [7–10], ara-C is rapidly converted by cytidine deaminase [E.C. 3.5.4.5] to 1-β-D-arabinofuranosyluracil (ara-U), a biologically inactive

product. Efforts to inhibit or circumvent this enzymatic inactivation of ara-C have resulted in the synthesis of a number of deaminase inhibitors to prevent such deamination or of analogs of 2′-deoxycytidine or ara-C which would not serve as subtrates for this enzyme [9–20]. It was demonstrated long ago [21] that *E. coli* cytidine deaminase showed a high degree of structural specificity for the sugar moiety of cytosine nucleosides. Thus 5-methyl- or 5-fluoro-cytidines or 2′-deoxy-cytidines were substrates for *E. coli* cytidine deaminase whereas 1-β-D-xylo-furanosylcytosine (xylo-C) or its 5-methyl analog were not. Reports by *Camiener* [11] with human liver deaminase, by *Dollinger et al.* [10] with human liver and mouse kidney deaminases, and by *Wright et al.* with pig kidney deaminase [22] suggested the following structural requirements in substrates for deamination by these enzymes: the presence of a 2-carbonyl function in the aglycon, an un-substituted N(3) in the ring, and a free 3′-OH in the 'down' (ribo) configuration. Substitution of the 4-amino group with an exocyclic alkylamino, hydrazino, oxo, thio, or hydroxylamino group resulted in compounds which were increasingly refractory to deamination. Introduction of certain halogens or a methyl group into position 5 did not alter the deamination rates markedly. In general, these reports suggested that the sugar moiety of the 1-β-D-pentofuranosyl type (probably of the ribo, 2′-deoxyribo, or arabino configuration) is required for enzymatic deamination.

The present study was undertaken to further investigate structural requirements for the enzymatic deamination by comparing the deamination of certain cytosine nucleosides in relation to cytidine. For this purpose, a crude kidney homogenate from BDF_1 mice was used.

Materials and Methods. – All compounds except cytidine, deoxycytidine (*Calbiochem*, La Jolla, Calif.), ara-C (kindly provided by Dr. *Harry B. Wood* of Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, *National Cancer Institute*) and 5′-deoxyribo-5-fluoro-C (*Hoffmann-La Roche*, Nutley, N.J.) were synthesized in our laboratories and references for their synthesis are given in *Tables 1–3*. All nucleosides listed herein are 1-β-D-pentofuranosyl derivatives.

Kidney homogenate was prepared from BDF_1 mice by homogenizing 4–8 kidneys in 0.25M sucrose; the crude homogenate (20%, *w/v*) was centrifuged in an *International Model* PR-2 centrifuge at 1000× g and diluted 1:1 with 0.25M sucrose (= homogenate). Each assay tube contained *Krebs-Ringer* buffer pH 7.4, homogenate, 4 μmoles of cytidine (CR) or ara-C or the substances to be tested in a volume of up to 0.3 ml. After incubation of 30 min to 1 h, 200 μl of cold 10% trichloroacetic acid (TCA) was added, the precipitated protein removed by centrifugation, the supernatant added to a *Conway* microdiffusion dish (*Fisher Scientific*, Philadelphia, Pa.), and the formed ammonia trapped and assayed spectrophotometrically according to the procedure of *Roth et al.* [38] as applied to cytidine deaminase studies [39]; limit of detection of ammonia is 5 nmol/mg/h. Protein was assayed according to *Lowry et al.* [40]. The values indicated are relative ones, as evaluated from 2–3 experiments (individual values do not deviate from the mean by more than ±15%, except in those ones where the relative velocities are equal to or less than 0.05), except the one marked with 'a' *(Table 1)* where only 1 experiment could be performed due to lack of sufficient material. The deamination of cytidine was always registered as being 1.0. In some instances, high blanks were registered in the photometric assay (O.D. 500 nm > 0.50) indicating partial decomposition of the compound and non-enzymatic release of ammonia. This chemical reaction probably takes place in the *Conway* dishes and makes relative velocity rates for such compounds less accurate. Normally, the blanks were below 0.1.

Results and Discussion. – The introduction of a fluoro function at position 5 of the cytosine moiety strongly enhances the substrate susceptibility of the nucleoside to enzymatic deamination. This effect is strongest in the ribosyl- and 2′-deoxy-

ribosyl-cytosine nucleoside series *(Table 1)*. Thus the relative deamination velocity of 5-fluorocytidine (ribo-5-fluoro-C) is more than 8-fold that of cytidine, and that for 2′-deoxyribo-5-fluoro-C is $\sim 10\times$ greater than that of 2′-deoxycytidine. This increase in substrate susceptibility to deaminase is also noted in the arabino nucleoside series *(Table 2)* wherein ara-5-fluoro-C is also a better substrate than the parent nucleoside, ara-C, or cytidine. The introduction of a methyl substituent on C(5) of ribo-, 2′-deoxyribo-, or arabino-cytosines markedly reduces the deamination velocity relative to the corresponding parent nucleoside unsubstituted at C(5). The order of deamination is $F \gg H > CH_3$. These data are generally in accord with those of *Creasey* [29] with mammalian deaminases. Introduction of methyl substituent on C(6) significantly reduces the susceptibility of the ribo- or arabino-cytosine nucleoside to deamination by the enzyme.

Table 1. *Relative Deamination Velocity of Cytidine and 2′-Deoxycytidine and Related Ribonucleosides*

Nucleoside	Relative Deamination	Nucleoside	Relative Deamination
Cytidine (ribo-C)	1.00	2′-Deoxy-ribo-5-methyl-C [23]	0.15
Ribo-5-fluoro-C [21]	8.20	2′-Chloro-ribo-C [24]	0.23
Ribo-5-methyl-C [23]	0.32	2′-Chloro-ribo-5-fluoro-C [25]	6.42
2′-Deoxy-ribo-C	0.72	5′-Deoxy-ribo-5-fluoro-C[b])	3.23
2′-Deoxy-ribo-5-fluoro-C [21]	7.24[a])	Ribo-6-methyl-C [26]	0.18

[a]) One determination only.
[b]) The authors are grateful to Dr. *A. R. Cook*, Hoffmann-La Roche, Nutley, N.J. for this sample.

Table 2. *Relative Deamination Velocity of Certain Arabinofuranosyl-Cytosines*

Nucleoside	Relative Deamination	Nucleoside	Relative Deamination
Ara-C	0.70	Cytidine (ribo-C)	1.00
Ara-5-fluoro-C [27]	1.11[a])	2′-Deoxy-ribo-C	0.72
Ara-5-methyl-C [28]	0.10		
Ara-6-methyl-C[b])	0.10		
5′-Deoxy-ara-C [30]	0.59		
3′-Deoxy-ara-C[c])	0.09		
3′-Amino-ara-C[c])	0.05		
2′-Fluoro-ara-C [36]	0.40		
2′-Fluoro-ara-5-fluoro-C[d])	2.45		
2′-Chloro-ara-C [37]	0.05		
2′-Bromo-ara-C[e])	< 0.05		
2′-Mesyloxy-ara-C[e])	< 0.05		

[a]) High blank.
[b]) Kindly provided by Dr. *B. A. Otter* of this Institute.
[c]) *D. H. Hollenberg, K. A. Watanabe & J. J. Fox*, unpublished.
[d]) *U. Reichman, K. Hirota, K. A. Watanabe & J. J. Fox*, unpublished.
[e]) *R. S. Klein, D. H. Hollenberg & J. J. Fox*, unpublished.

Of the parent 1-β-D-pentofuranosylcytosines, cytidine is the best substrate for the mouse kidney deaminase followed in decreasing order by 2′-deoxycytidine, ara-C, xylo-C and lyxo-C. The last two substrates are hardly deaminated at all.

Substitution at the 5′-position does not remove the susceptibility of the resulting nucleoside to deamination as shown previously [11] [30]. Thus, 5′-deoxy-ara-C is deaminated at a velocity comparable to that of ara-C. In the ribo series, 5′-deoxy-ribo-5-fluoro-C is deaminated about 3 times faster than cytidine; however, this increase is due probably in great measure to the influence of the 5-fluoro substituent. *Wempen et al.* [31] have also demonstrated the susceptibility of 5′-deoxy-5′-fluoro-ribo-C to deamination by cytidine deaminase derived from *E. coli B.* With 5′-deoxy-5′-methoxy derivatives, *Krajewska & Shugar* [32] showed that such cytosine nucleosides were not deaminated by deaminase from *Salmonella typhimurium* whereas they were deaminated to some extent by deaminases from human white blood cells.

The 3′-hydroxyl function is essential for deamination by this enzyme. Thus neither 3′-deoxy- or 3′-amino-ara-C *(Table 2)* are deaminated. These data and the fact that neither xylo-C, 3′-fluoro (or chloro)-xylo-C nor lyxo-C are deaminated *(Table 3)* warrants the conclusion, as suggested by *Camiener* [11], that not only must the nucleoside contain a 3′-hydroxyl function but this functional group must also be in the 'down' (ribo or arabino) configuration. It is highly likely that the 3′-'down' (ribo) hydroxyl is a binding site of the enzyme to substrate. Yet to be explained is the susceptibility to enzymatic deamination of those xylo-C derivatives in *Table 3* which bear a bromo, mesyloxy, or tosyloxy group at 3′. These latter derivatives [unlike 3′-fluoro-(or chloro)-xylo-C] bear excellent leaving groups on C(3′) which are readily susceptible to nucleophilic displacement by the neighbouring *trans*-2′-hydroxyl function leading to 'down' oxides which are converted to 2,2′-anhydro-ara-C's and finally to ara-C's (see *Scheme*). Such transformations of these 3′-substituted xylo nucleosides occur readily at slightly alkaline pH[1]). Indeed,

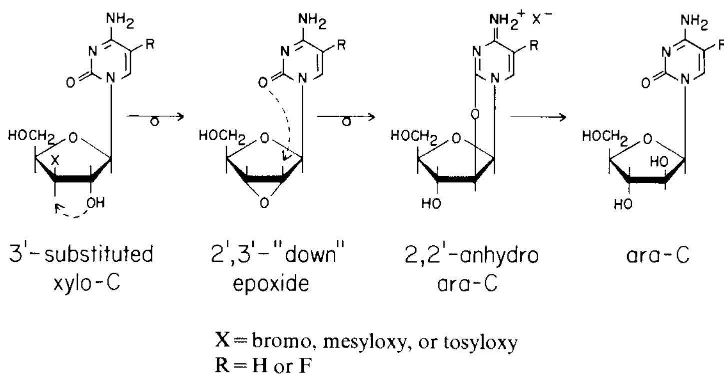
Table 3. *Relative Deamination Velocities of Certain Xylo- und Lyxo-furanosylcytosines*

Nucleoside	Relative Deamination	Nucleoside	Relative Deamination
Xylo-C [33]	0.07	Cytidine	1.0
Xylo-5-methyl-C [33]	0.06	Ara-C	0.70
3′-Fluoro-xylo-C [22]	< 0.05		
3′-Chloro-xylo-C[a])	0.06		
3′-Bromo-xylo-C[a])	0.57		
3′-Mesyloxy-xylo-C[a])	0.76		
3′-Mesyloxy-xylo-FC[a])	0.30[b])		
3′-Tosyloxy-xylo-C[a])	0.61		
3′-Tosyloxy-xylo-FC[a])	1.09[b])		
Lyxo-C [33]	0.05		

[a]) *D.H. Hollenberg, U. Reichman, K.A. Watanabe & J.J. Fox*, unpublished.
[b]) High blank.

[1]) *D.H. Hollenberg* and *U. Reichman*, unpublished data.

Scheme. *Reaction sequence for conversion of 3′-substituted-xylo-C's to ara-C type nucleosides*



X = bromo, mesyloxy, or tosyloxy
R = H or F

one of us (*JJF*) has hypothesized [35] that the growth inhibitory activity of 3′-bromo-xylo-C against leukemic cells in culture may be due to its chemical conversion *in vitro* to ara-C. Such a conversion is also plausible under conditions of the enzymatic deamination reaction (pH 7.4, 30 min, 37°).

A totally different picture is obtained with the 2′-substituted-ara-C derivatives *(Table 2).* 2′-Fluoro-ara-C is almost as good a substrate for the deaminase as 2′-deoxycytidine due probably to the closeness of size of this halogen to the hydrogen atom. It is noteworthy that substitution of a fluoro atom in place of hydrogen or hydroxyl in the 2′-'up' (arabino) configuration (unlike a fluoro substitution at C(5) of the aglycon) does not increase the relative deamination velocity. The general order for 2′-'up' substituents is OH ≅ H > F > Cl, Br, mesyloxy. The latter 3 arabino derivatives containing good leaving groups at position 2′ are not deaminated to any significant extent – unlike the situation in the corresponding xylo series *(Table 3).* This difference may be a result of the enzyme binding to the 3′-'down'-OH groups in these arabinocytosines thus preventing anchimeric assistance of the 3′-OH for displacement of the 2′-leaving group for formation of the 'down' epoxide *(Scheme).* The failure of those arabinocytosines to undergo deamination may be a consequence of bulkiness of the 2′-'up'-substituent or of conformational alterations *(viz. syn* versus *anti)* resulting therefrom, which prevent the active site of the enzyme from reacting with the site of deamination on the substrate.

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