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Deamination of 5-azacytidine by a human leukemia cell cytidine deaminase

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CYTOSINE ARABINOSIDE has become an important agent in the treatment of human leukemia, but its effectiveness in some cases appears to be limited by the induction of elevated levels of the catabolic enzyme cytidine deaminase.[1] Recently, a new cytidine antimetabolite, 5-azacytidine, has demonstrated activity in acute myelocytic leukemia.[2] The anti-tumor activity of 5-azacytidine has been attributed to its metabolism to the nucleotide form and incorporation into RNA.[3] Studies of the pharmacology of 5-azacytidine in mice have disclosed excretion of unaltered drug, as well as 5-azauracil and various ring-cleavage metabolites.[4] However, the enzymatic deamination of 5-azacytidine by human leukemic cells has not been demonstrated previously and is the subject of this report.

Unlabeled 5-azacytidine (NSC No. 1028165), tetrahydrouridine (NSC No. 112907) and cytosine arabinoside (NSC No. 63878) were obtained from the Division of Drug Research and Development, National Cancer Institute. Cytidine was obtained from Schwartz–Mann, Orangeburg, N.Y., and cytidine-2-^{14}C from ICN Isotope & Nuclear Division, Cleveland, Ohio. Cytosine arabinoside-2-^{3}H was purchased from New England Nuclear Corp., Boston, Mass. Glutamic dehydrogenase from bovine liver and in ammonia-free solution was obtained from CalBiochem.

Human leukemic granulocytes were obtained from untreated patients with chronic granulocytic leukemia in the chronic phase of the disease. Heparinized peripheral blood was allowed to sediment after addition of dextran,[5] and the leukocyte-rich plasma removed after 30–120 min. Cells were concentrated by centrifugation at 500 *g* for 15 min, and the cell pack was taken up in 5 ml of 0·15 M NaCl. Red blood cells were lysed by the addition of 3 vol. of cold distilled water, and isotonicity was restored after 30 sec by addition of 1 vol. of 0·6 M NaCl. Cells were recentrifuged and resuspended in 0·05 M Tris, pH 7·5. Granulocytes were lysed by three cycles of freeze–thawing, followed by 10 light strokes with a Dounce homogenizer. The supernatant, containing cytidine deaminase activity of 5–10 $\times$ 10^3 units*/ml and sp. act. 0·5 units/mg of protein, was stored at 4° until use.

Deamination of cytidine and of cytosine arabinoside was assayed as previously described,[1] separating product from substrate by ion-exchange chromatography on Dowex-50-H$^+$ resin. Deamination of 5-azacytidine was assayed by measuring the rate of ammonia production accompanying the conversion of 5-azacytidine to 5-azauridine. In the assay, 100–200 μg of supernatant protein was incubated with substrate in 0·05 M Tris, pH 7·5, in a total volume of 0·4 ml at 37°. NH_4^+ production was determined by addition of the incubation solution to a cuvette containing glutamic dehydrogenase, 24 units; alpha-keto-glutarate, 17·0 μmole; EDTA, 1·0 μmole; NADH, 0·3 μmole and sodium phosphate buffer, pH 7·5, 50 μmole, in a total volume of 1 ml. The initial velocity of glutamic dehydrogenase activity, as measured by the decrease in absorbance at 340 nm, was directly proportional to NH_4^+ generated in the deaminase reaction. By this method deamination of 5-azacytidine was shown to be linear with supernatant protein concentrations of 100–500 μg/0·4 ml reaction volume and, in the presence of excess 5-azacytidine, to be linear with time for 60 min.

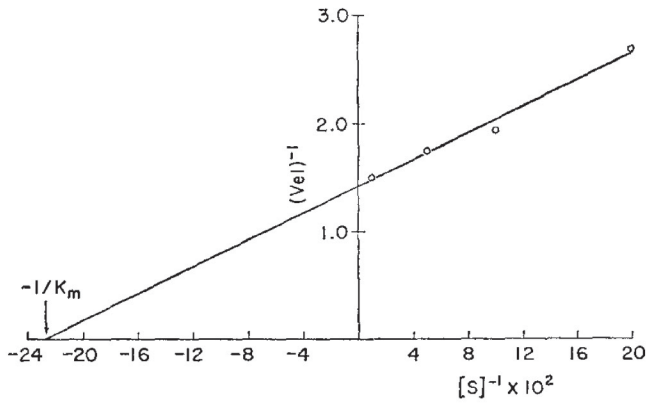

FIG. 1. Double reciprocal plot of 5-azacytidine concentration vs reaction velocity. A K_m value of $4·3 \times 10^{-4}$ M was determined from this plot.

* Units of cytidine deaminase = nmoles of substrate consumed per hr.

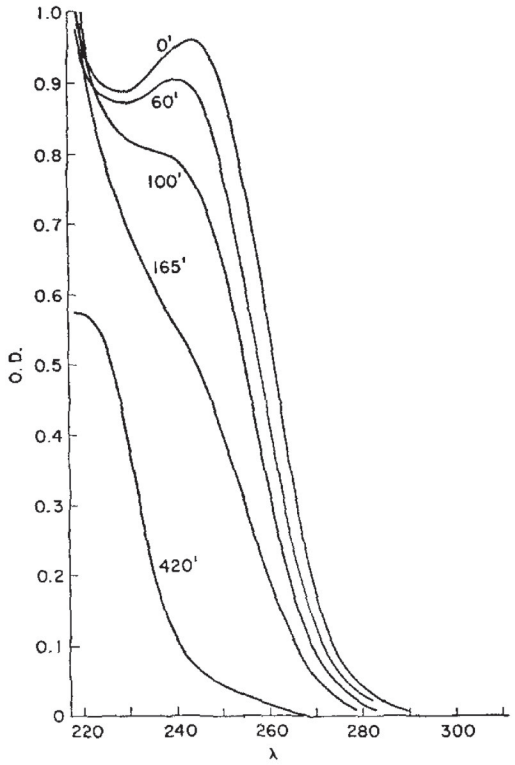

FIG. 2. Sequential u.v. absorption spectra of deamination of 5-azacytidine. See text for experimental conditions.

The deamination of 5-azacytidine was also observed by u.v. spectrophotometric scanning using a Cary, model 15. The reaction solution consisted of 5-azacytidine, 0·4 μmole; Tris, pH 7·5, 0·15 m-mole and 420 μg of supernatant protein in a total volume of 3 ml.

Deamination of 5-azacytidine by the extract from human leukemic leukocytes was linear with time and protein concentration. A double reciprocal plot of substrate concentration versus NH_4^+ production (Fig. 1) yielded a K_m value of $4{\cdot}3 \times 10^{-4}$ M which was 20-fold higher than the corresponding K_m value for cytidine, $2{\cdot}2 \times 10^{-5}$ M, and 4-fold higher than the value for cytosine arabinoside, 1·1 ×

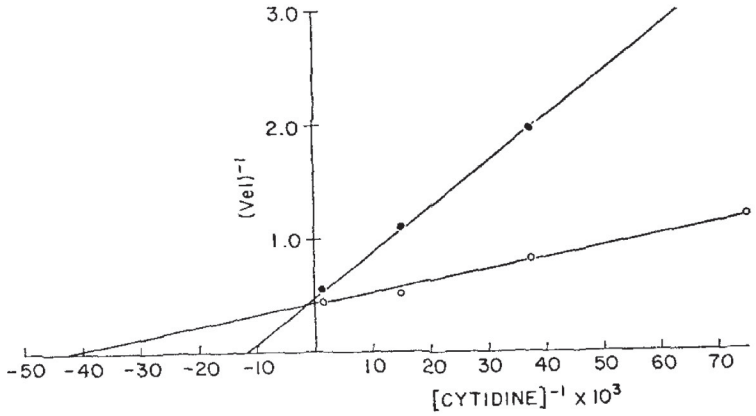

FIG. 3. Competitive inhibition of the deamination of cytidine by 5-azacytidine, $6{\cdot}7 \times 10^{-4}$ M. Cytidine-2-^{14}C was incubated with enzyme in the presence of unlabeled 5-azacytidine, and uridine-2-^{14}C was isolated as described in text. (○——○) = cytidine-2-^{14}C; (●——●) = cytidine-2-^{14}C with 5-azacytidine.

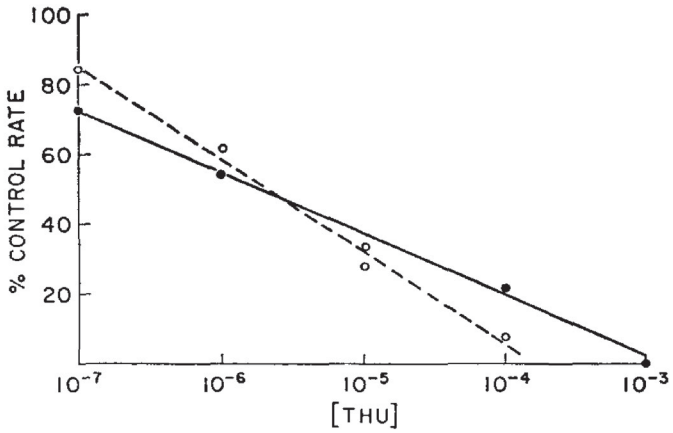

FIG. 4. Inhibition of the deamination of 5-azacytidine, 2 mM (○– – –○) and cytidine-2-^{14}C, 0·7 mM (●——●) by tetrahydrouridine. Assays were performed as described in text, except that tetrahydrouridine was added to reaction solution in concentration indicated 5 min prior to addition of substrate.

10^{-4} M. A marked change in the u.v. absorption of the reaction solution was observed as the reaction proceeded. Sequential tracings, as shown in Fig. 2, revealed disappearance of the pyrimidine ring peak at 242 nm. The decline in absorption at 242 nm was directly proportional to the NH_4^+ generated in the reaction, and was not accompanied by the appearance of a new absorption maximum, as seen with the conversion of other cytidine derivatives to their uridine analogs. This was likely due to the instability of the deamination product, 5-azauridine, which has been shown to rapidly break down to form ribosyl-*N*-formyl biuret and ribosyl-*N*-biuret.[6]

Further experiments were directed at determining whether the deamination of 5-azacytidine was carried out by the same enzyme catalyzing deamination of cytosine arabinoside. Thus, 5-azacytidine was found to be a competitive inhibitor of the deamination of both cytidine and cytosine arabinoside (Fig. 3). The K_i values for 5-azacytidine were $3{\cdot}2 \times 10^{-4}$ M with cytosine arabinoside as substrate, and $2{\cdot}55 \times 10^{-4}$ M with cytidine as substrate, both of which values agreed well with the previously determined K_m value for 5-azacytidine of $4{\cdot}3 \times 10^{-4}$ M. In addition, tetrahydrouridine, a potent inhibitor of the deamination of cytidine and of cytosine arabinoside,[7] also inhibited deamination of 5-azacytidine (Fig. 4). At substrate concentrations 35–40 times the K_m values for each substrate, the I_{50} for tetrahydrouridine inhibition of the deamination of both 5-azacytidine and cytosine arabinoside was 5×10^{-5} M. These results are compatible with the hypothesis that both cytidine analogs are deaminated by the same enzyme as has been suggested by Camiener[8] in his study of human liver cytidine deaminase.

It is thus apparent that 5-azacytidine is subject to enzymatic deamination by peripheral leukemic leukocytes, and that increased levels of the enzyme cytidine deaminase may compromise the effectiveness of 5-azacytidine in treating human leukemia. It is possible that inhibitors of pyrimidine deamination such as tetrahydrouridine may be helpful in preventing catabolism of this antineoplastic agent.

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