INHIBITION OF REVERSE TRANSCRIPTASE ACTIVITY BY BENZOPHENANTHRIDINE ALKALOIDS[1]

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ABSTRACT.—Benzophenanthridine alkaloids, fagaronine **4**, *O*-methylfagaronine **5**, nitidine **1**, allonitidine **3** and methoxydihydronitidine **2** have been shown to possess inhibitory activity against reverse transcriptase of RNA tumor viruses. The enzyme inhibition (50%) by these alkaloids was found in the range of 6–60 μg per milliliter of the reaction mixture when polynucleotide-oligodeoxynucleotide complexes were used as template primers. The results suggested that the benzophenanthridine alkaloids interacted with the template primers (particularly of the A:T base pairs) and not with the enzyme proteins. Kinetics reaction of the reverse transciptase inhibition showed that the alkaloids stopped the DNA polymerase synthesis instantly, probably by interacting with the template primer.

Reverse transcriptase is RNA-directed DNA polymerase present (1–4) in RNA tumor viruses. Such viruses are recognized as causative agents of malignant diseases, especially in avian and murine systems. Reverse transcriptase is required in the formation of viral DNA (provirus) which on integration with the host genome leads to the viral infection and transformation of susceptible cells (5–8). Recently, presence or isolation of virus-related components, reverse transcriptase, and type-C viral related nucleic acids have been reported in normal human cells (9–10), woolly monkey sarcoma (11), the gibbon ape lymphosarcoma (12), human leukemic cells (11, 13, 14), human breast cancer cells (15), human brain tumor cells (16) and human milk (17, 18). The presence of reverse transcriptase in the normal tissues as well as in oncogenic RNA viruses indicates that this enzyme may play an important role in the regulation of cell function, expression of genetic information, gene modification or mutation and cell transformation. The direct involvement of RNA tumor viruses in human cancer is a matter of controversy. However, there is convincing evidence that RNA tumor viruses at some stage of viral replication are linked to some types of human cancer, at least in some cases of leukemic cells (19–21).

The animal and tissue culture studies reveal that reverse transcriptase is essential for provirus synthesis but is not required for maintenance and transcription of the proviral DNA (6). The inhibitors of reverse transcriptase do not produce any effect after integration of the proviral genome into the host DNA. Therefore, potential value of the inhibitors is limited to its prophylactic use. There are different classes of inhibitors which can specifically inhibit the viral replication. The inhibitors may be substrate analogues, template-primer analogues, enzyme binding, and divalent cation-binding agents. The exact mechanism of antiviral actions of inhibitors is sometimes difficult to demonstrate. But any inhibitor which can block the viral replication at any step of viral oncogenesis should be considered useful.

Benzophenanthridine alkaloids possess interesting biological and pharmacological properties. A number of these alkaloids (fig. 1) showed antileukemic activity and inhibited lung carcinoma. Benzophenanthridine alkaloids, namely

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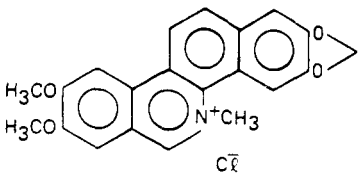
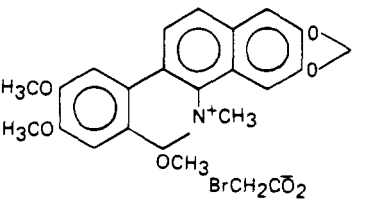
nitidine chloride (NSC 146397) and 6-methoxy-5,6-dihydronitidine (NSC 146396) isolated from *Fagara macrophyla* were found to be highly cytotoxic, exhibited antileukemic activity in both leukemia L1210 and P388 systems in mice and inhibited Lewis lung carcinoma (22–24). Synthetic 6-methoxy-5,6-dihydronitidine and allonitidine (NSC 171554) also showed antileukemic activity (25, 26).



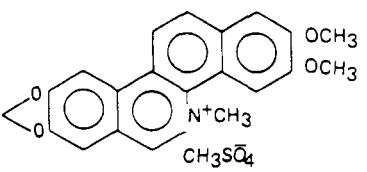
NITIDINE CHLORIDE

1



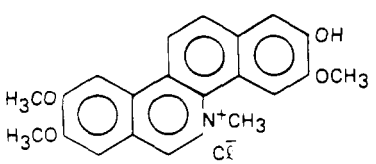
6–METHOXY–5,6–DIHYDRONITIDINE BROMOACETATE

2



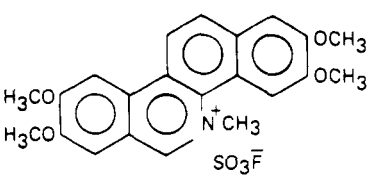
ALLONITIDINE METHYLSULFATE

3



FAGARONINE CHLORIDE

4



O–METHYLFAGARONINE FLUOROSULFONATE

5

FIG. 1. *Structure of Benzophenanthridine Alkaloids.*

Fagaronine isolated (27, 28) from *Fagara zanthoxyloides* Lam. and *O*-methylfagaronine (NSC 179207) possessed (23) good activity against leukemia P388. Nitidine, 6-methoxy-5,6-dihydronitidine and *O*-methylfagaronine also demonstrated inhibition of catechol *O*-methyltransferase and transfer RNA methyltransferase (29).

In the previous work (30–32) on the specific inhibitors of reverse transcriptase, fagaronine was found to be a potent inhibitor of this enzyme since low molar concentration caused 50% inhibition. This activity compares well with other known inhibitors. In order to explore further inhibitory activity of alkaloids related to fagaronine, this paper reports an inhibition of reverse transcriptase activity of RNA tumor viruses by some other members of the benzophenanthridines. The alkaloids reported here also showed good inhibition of reverse transcriptase activity as compared with fagaronine.

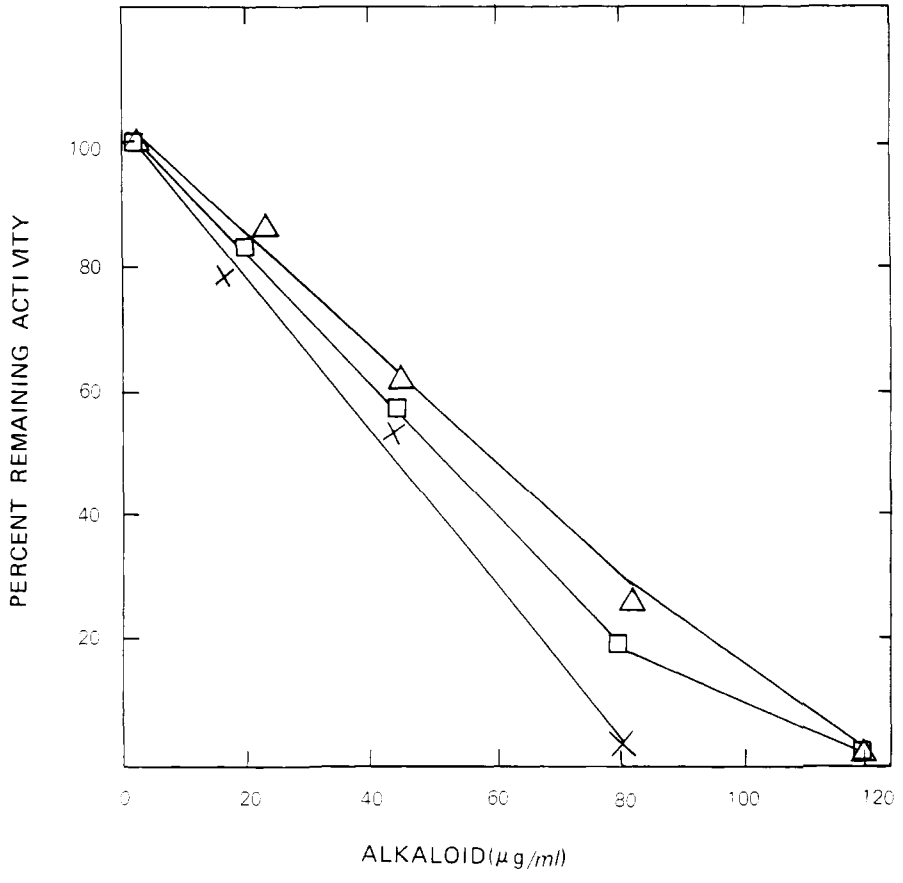


FIG. 2. *Effect of Benzophenanthridine Alkaloids on AMV Reverse Transcriptase.* In a standard assay mixture of 0.01 ml (Material and Methods) containing 5 μl of reverse transcriptase, different concentrations of nitidine (-×——×-), methoxydihydronitidine (-△——△-) or allonitidine (-□——□-) were added for enzyme inhibition.

MATERIAL AND METHODS

REVERSE TRANSCRIPTASE ENZYME.—The purified enzyme[2] from avian myeloblastosis virus (AMV) had a specific activity of 56,663 units/mg and a protein content of 0.217 mg/ml. The

[2]Life Science Research Labs., St. Petersburg, Fla., obtained through the courtesy of Dr. J. Beard. The enzyme preparation contained 12,296 units of reverse transcriptase/ml (1 unit of enzyme activity is expressed as the incorporation of 1 nmole of deoxythymidine monophosphate into an acid-insoluble product in 10 min. at 37°). The enzyme was assayed by the established method of the laboratory.

enzyme was purified, detected and assayed by a published method (33). Reverse transcriptase[3] from Rauscher murine virus (MuLV) propagated in JLS-V9 cell line had an activity of 4.00 nmoles of thymidine monophosphate incorporation/30 minutes/ml. 70S RNA[4] from MuLV was purified according to the published method (34).

THE TEMPLATES AND OTHER CHEMICALS.—The templates[5], poly rA.oligo dT, poly dA.oligo dT, poly rC.oligo dG, substrates, [^{3}H]-thymidine triphosphate[6] (^{3}H-TTP, Sp. act. 14 Ci/nmole), [^{3}H]-deoxyguanosine triphosphate[6] (^{3}H-dGTP, Sp. act. 14 Ci/nmole) and unlabelled nucleotide[5] were obtained commercially. All other reagents were of analytical grade.

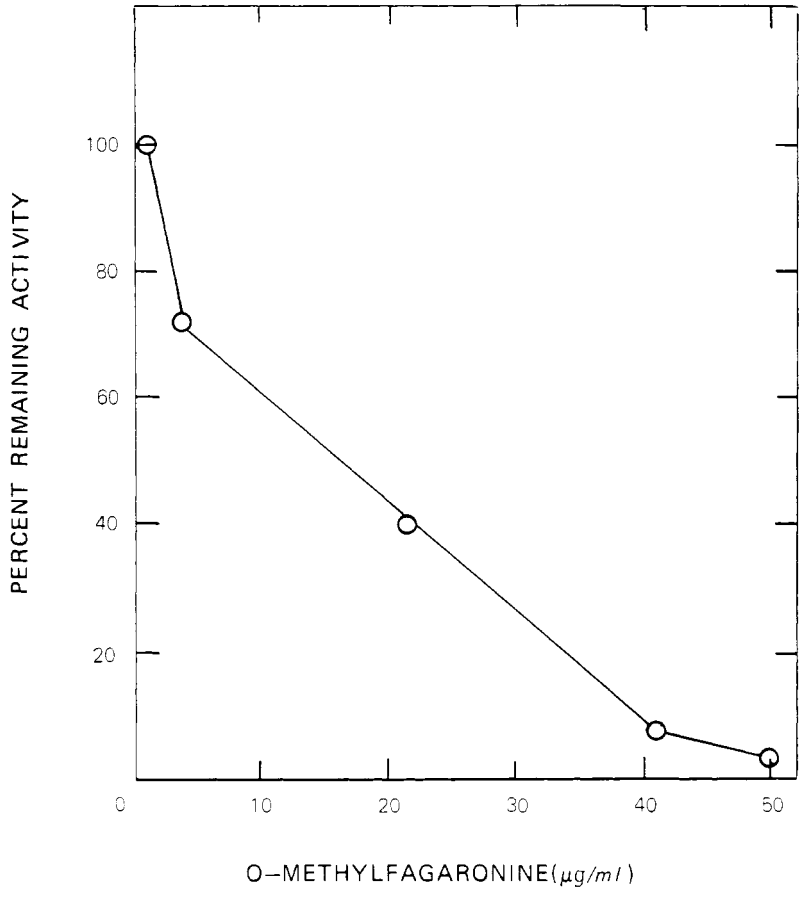


FIG. 3. *Effect of O-Methylfagaromine on AMV Reverse Transcriptase.* In a standard assay mixture of 0.01 ml (Material and Methods) containing 5 μl of reverse transcriptase, different concentrations of O-Methylfagaronine (-○——○-) were added for enzyme inhibition.

REVERSE TRANSCRIPTASE ASSAY AND ENZYME INHIBITION.—All templates were dissolved in buffer containing 0.01 M Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, pH 7.0), 0.01 mM ethylenediaminetetraacetic acid tetrasodium salt (EDTA) and 0.10 M NaCl.

[3]Bionetic Laboratory Products, Kensington, Md.

[4]Gift from Dr. M. Reitz, Litton Bionetics Inc., Bethesda, Md.

[5]P.L. Biochemicals Inc., Milwaukee, Wis. Templates abbreviations are: Poly rA.oligo dT, polyriboadenylic-oligodeoxythymidylic acid, Poly dA.oligo dT, polydeoxyadenylic-oligodeoxythymidylic acid, Poly rC.oligo dG, polyribocytidylic-oligodeoxyguanylic acid.

[6]Schwarz-Mann.

Activated calf thymus DNA was prepared by incubating 4.50 mg of DNA[7], 5 mg of bovine serum albumin (BSA) in 10 ml of buffer containing Tris HCl, pH 7.0 (0.05 M) and 5.00 mM $MgCl_2$ with 0.01 mg of crystalline pancreatic deoxyribonuclease[8] for 15 min at 37°. It was cooled at 4°, further incubated at 70° for 5 min, and stored at 4°. Appropriate concentrations of benzophenanthridine alkaloids[9] solutions were made in dimethylsulfoxide (DMSO).

Reverse transciptase assays were performed by preparing 100 μl enzyme assay mixture containing 5.00 umoles Tris HCl, pH 7.3, 8.00 umoles KCl, 0.10 umole $MnCl_2$, 0.50 umole dithiothreitol (DTT), 2.00 umole [^{3}H]-TTP (200 cpm/pmole), 20.00 μg BSA, 2.00 μg poly rA.oligo dT and 5.00 μl purified enzyme fraction. The assay mixture was incubated at 37° for 30 minutes. The enzyme reaction was stopped by cooling at 4° and by the addition of 25 μl of 0.10 M EDTA. One hundred μl of each assay mixture was spotted uniformly onto a 2.50 cm. circular filter paper.[10] The filter papers were washed batchwise six times by swirling in 10 ml of 5% $Na_2HPO_4 \cdot 7H_2O$ per filter paper, followed by two washings each of water and 95% ethanol. The filter papers were dried and the amount of radioactivity determined[11] in a toluene-based scintillation fluid.

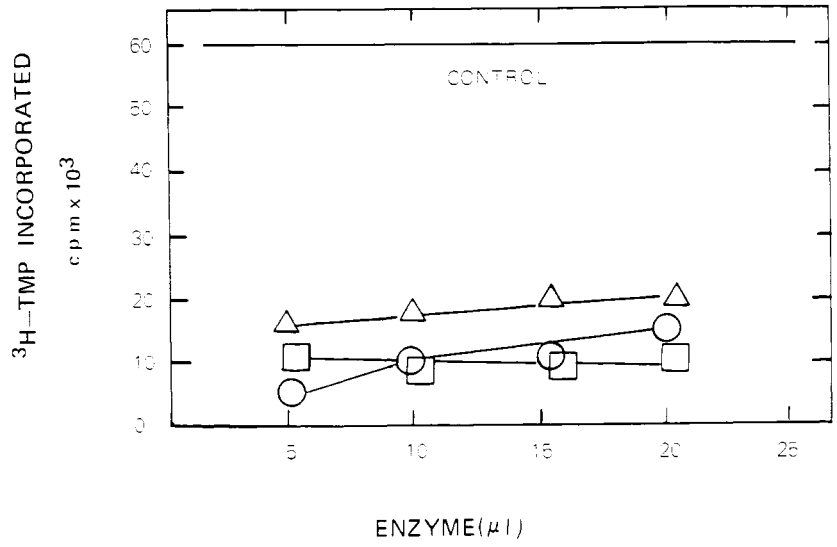


FIG. 4. *Effect of Increasing Concentrations of AMV Reverse Transcriptase on Benzophenanthridine Alkaloids-inhibited Reaction Mixture.* In a standard assay mixture of 0.01 ml (Material and Methods) containing 5 μg of poly rA.oligo dT and 5 μg of methoxydihydronitidine (-△——△-) or allonitidine (-□——□-) or O-methylfagaronine (-○——○-) increasing concentrations of enzyme were added. The control value was 60,000 cpm (average) of [^{3}H]-TMP incorporation.

For testing enzyme inhibition, two reaction mixtures were prepared, one mixture contained enzyme, buffer, and BSA; and the other mixture consisted of template, substrate, DTT, and salts. Appropriate concentrations of the benzophenanthridine alkaloids in DMSO were mixed with the mixture containing enzyme. The final concentration of the ingredients and the assay procedure was the same as mentioned above. The control assay mixture contained an equivalent volume of DMSO without alkaloid which represented 100% enzyme activity. The inhibitory results were calculated by determining the remaining radioactivity and expressed as percent control activity.

RESULTS AND DISCUSSION

INHIBITION OF AMV AND MULV REVERSE TRANSCRIPTASE ACTIVITY.—The in-

[7]Sigma Chemical Co., St. Louis, Mo.
[8]Worthington Biochemical Corporation.
[9]Gift samples obtained from Drug Synthesis and Chemical Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.
[10]DE-81 Whatman.
[11]Beckman Model LS 150.

hibitory activity of benzophenanthridine alkaloids (fig. 1) on reverse transcriptase from AMV and MuLV at increasing concentration of the alkaloids was studied. The inhibitory curves of nitidine chloride (**1**), 6-methoxy-5,6-dihydronitidine bromoacetate (**2**) and allonitidine methylsulfate (**3**) based on the effects of the alkaloids of AMV reverse transcriptase are shown in figure 2. The 50% enzyme inhibitions of alkaloids **1**, **2** and **3** were in the ranges of 40–50 μg/ml, 50–60 μg/ml and 45–50 μg/ml of the reaction mixture respectively. The inhibition range was

TABLE 1. *Effect of different template primers on inhibition of AMV reverse transcriptase activity by benzophenanthridine alkaloids.*[a]

Template primer	Radioactive substrate	% Inhibition
O-METHYLFAGARONINE		
Poly rA.oligo dT[b]	TTP	98
Poly dA.oligo dT[b]	TTP	97
Poly rC.oligo dG[c]	dGTP	3
Activated DNA[d]	TTP	96
70 S RNA[e]	TTP	95
ALLONITIDINE		
Poly rA.oligo dT[b]	TTP	95
Poly dA.oligo dT[b]	TTP	94
Poly rC.oligo dG[c]	dGTP	2
Activated DNA[d]	TTP	93
70 S RA[e]	TTP	92
METHOXYDIHYDRONITIDINE		
Poly rA.oligo dT[b]	TTP	94
Poly dA.oligo dT[b]	TTP	93
Poly rC.oligo dG[c]	dGTP	2
Activated DNA[d]	TTP	92
70 S RNA[e]	TTP	91

[a]For each inhibition alkaloid, 5 μg/assay was used.
[b]See standard assay conditions under "Material and Methods".
[c]Standard assay mixture contained 2 μg of this template primer and 2.50 nmoles of [^{3}H]-dGTP.
[d]Standard assay mixture contained 2.2 μg activated DNA, 10 nmoles each of dATP, dCTP, dGTP and 2.5 nmoles of [^{3}H]-TTP.
[e]70 S RNA (MuLV) concentration in the standard assay mixture was 0.05 OD units (260 nm).

very close perhaps due to the close relationship of the alkaloids in chemical structure (fig. 1). Similar results were obtained (data not shown) on MuLV reverse transcriptase activity. Inhibition of AMV reverse transcriptase by fagaronine chloride (**4**) and *O*-methylfagaronine fluorosulfonate (**5**) was stronger than alkaloids **1**, **2** and **3**. Fifty percent inhibitions by alkaloids **4**(30) and **5** (fig. 3) were obtained with 6–12 μg/ml and 12–18 μg/ml of the reaction mixture respectively. Similar results were obtained on MuLV reverse transcriptase activity

for alkaloids **4** (30) and **5** (data not shown). It was also noted that AMV reverse transcriptase was more sensitive to benzophenanthridine alkaloids than MuLV reverse transcriptase since the latter enzyme required higher doses of alkaloids (5–10 μg/ml) to cause an equal percent of inhibition. DNA and RNA polymerases of *E. coli* were less senstiive to inhibition by these alkaloids than viral polymerases (unpublished work).

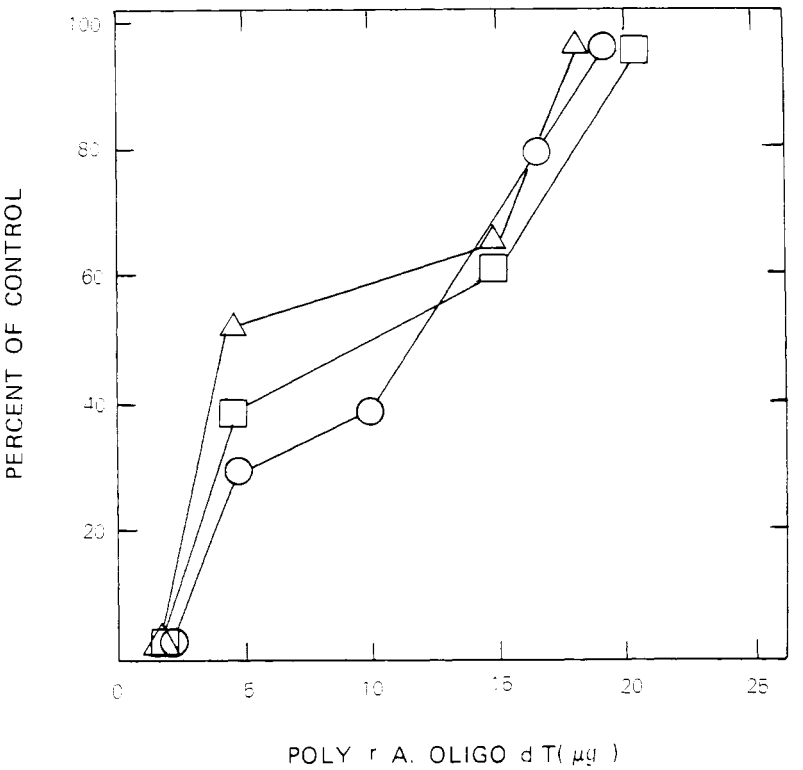


FIG. 5. *Effect of Increasing Concentrations of Template Primer on Benzophenanthridine Alkaloids-inhibited Reaction Mixture.* The standard assay mixture (0.01 ml) contained 5 μl of AMV reverse transcriptase and 5 μg of methoxydihydronitidine (-△——△-) or allonitidine (-□——□-) or O-Methylfagaronine (-○——○-).

MECHANISM OF INHIBITION OF REVERSE TRANSCRIPTASE BY BENZOPHENANTHRIDINE ALKALOIDS.—To determine whether direct binding of the alkaloids **2, 3** and **5** with AMV or MuLV reverse transcriptase enzyme was responsible for the inhibition, the effect of increasing concentrations of enzymes was studied on alkaloid-inhibited reaction mixture (fig. 4). When the amount of AMV or MuLV reverse transcriptase was increased from 5 to 20 μl in the assay mixture, the original percent of inhibition was not significantly changed. Similar results were obtained in the case of alkaloid **1**. The results indicated that the enzyme did not interact with the alkaloids and the mode of enzyme inhibition was other than the direct binding of the enzyme with the alkaloids. However, the increase of enzyme concentration in the fagaronine-inhibited reaction mixture completely restored the enzyme activity (30). The reversal of enzyme inhibition could be explained

by assuming the formation of an enzyme-template-alkaloid ternary complex in the reaction mixture which was fully dissociated by the addition of excess enzyme.

To explain further the mode of enzyme inhibition, different template primers such as poly rA.oligo dT, poly dA.oligo dT, poly rC.oligo dG, activated DNA, and 70S RNA were used as presented in table 1. The percent inhibitions of alkaloids **5** and **3** with different template primers occurred between 95–98 and 92–95

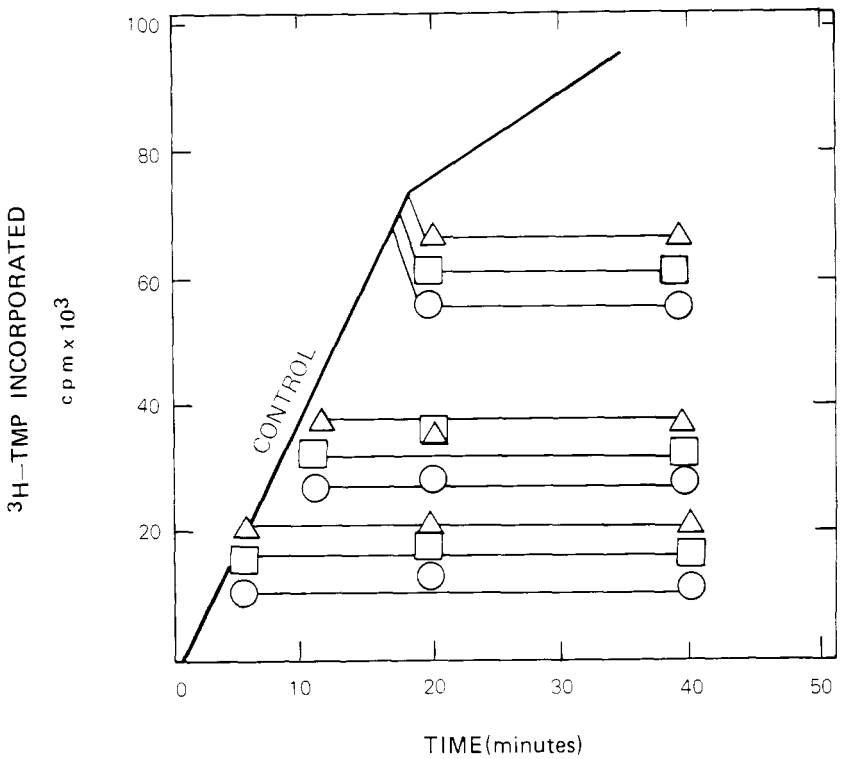


FIG. 6. *Effect of Addition of Benzophenanthridine Alkaloids during AMV Reverse Transcriptase Kinetic Reaction.* For each assay, 2 ml of standard mixture containing 100 μl of enzyme was divided into four parts: A(0.60 ml), B(0.50 ml), C(0.50 ml), D(0.40 ml). From A, 0.10 ml sample was withdrawn at 0 minute and the remaining quantity was incubated at 37°. One tenth ml samples from A were withdrawn at 5, 10, 20, 30 and 40 minutes after incubation which represented control. B, C, and D parts were also incubated at 37° and 50 μg/ml solutions of methoxydihydronitidine (-△——△-) or allonitidine (-□——□-) or O-Methylfagaronine (-○——○-) were added 5, 10, and 20 minutes after incubation. One minute after addition of alkaloid, 0.10 ml samples were withdrawn from B, C, and D at 5 minutes intervals. Radioactivity of each sample was determined.

respectively except with poly rC.oligo dG in which 2–3% inhibition was observed. Alkaloid **2** showed 91–94% inhibition with all template primers except poly rC.oligo dG which showed 2% enzyme inhibition. The data similar to that in table 1 were obtained for alkaloids **4** (30) and **1** (data not shown). The enzyme inhibition by the alkaloids presented in table 1 was due to the interaction of the alkaloids with the template primers used. If there was interaction between the enzyme and the alkaloids, the same percent of enzyme inhibition would have been ob-

served regardless of the different template primers employed. A high degree of enzyme inhibition by poly rA.oligo dT, poly dA.oligo dT, 70S RNA and activated DNA template primers as compared to poly rC.oligo dG was due to the strong binding affinity of the alkaloids with adenine-thymine (A:T) template primers.

The interaction of template primers and alkaloids was further confirmed by examination of the effects of increasing concentrations of template primers on the alkaloid-enzyme inhibited reaction mixture. Figure 5 shows that the enzyme inhibitions of alkaloids **5**, **3** and **2** were reversed completely when poly rA.oligo dT concentration in the assay mixture was increased from 2–20 μg. This could be explained by the fact that the initial binding of template-alkaloid-enzyme was overcome by the excess template primer resulting in complete dissociation of the complex product. Since the increase of template primer in the alkaloid-enzyme-template inhibited reaction mixture restored the enzyme inhibition linearly, it was clear that the enzyme molecule did not interact with the alkaloid. Similar interaction was also demonstrated by the alkaloids **4** (30) and **1** (35), thus conforming to the earlier reports. The interaction or competition of KCl or substrate, [^{3}H]-TTP or [^{3}H]-dGTTP was ruled out since these were present in the reaction mixture in 8- or 25-fold, respectively, relative to the alkaloids. The possibility of interaction of metal ions with the alkaloid was not considered since the enzyme activity was not changed when the concentrations of $MnCl_2$ in the reaction mixture were increased from 0.10 umole to 0.50 umole (data not shown). All the experimental results discussed above demonstrate that the alkaloids have specific interaction with A:T base pairs of the templates rather than direct binding with the enzyme.

The effect of alkaloids on the time course of the reaction of AMV reverse transcriptase is shown in figure 6. The control experiment consisted of reaction mixture without the alkaloid, which means that the DNA synthesis by reverse transcriptase was linear with respect to incubation time. During the time course of the reaction, alkaloids were added at different time intervals of incubation. When the alkaloids were added at 10, 20, 30 and 40 minutes after initiation of the polymerization reaction, the enzyme activity was abruptly changed. Further incubation did not change the kinetics of polymerization or degrade the product. The results indicated that the alkaloids stopped [^{3}H]-TMP incorporation instantly, probably by interaction with the template primer. Similar results were observed for alkaloids **1** and **4** (30).

In conclusion, the data presented here revealed that the benzophenanthridine alkaloids mentioned above inhibited the reverse transcriptase activity of RNA tumor viruses by interaction with the adenine-thymine (A:T) template primers and stopped DNA synthesis at the initiation of the polymerization processes. Alkaloids **4** and **5** were found to be potent inhibitors of reverse transcriptase since 30–40 μg/ml of alkaloids inhibited more than 90% of the enzyme activity. Alkaloids **1**, **2** and **3**, which were closely related to each other in chemical structure, also displayed good inhibition against AMV and MuLV reverse transcriptase as compared with fagaronine. Since these alkaloids interfered with the viral DNA polymerase synthesis *in vitro*, they could be promising prophylactic agents in cancer chemotherapy. Synthesis of more alkaloids related to fagaronine and nitidine would be useful with a view to decrease the cytotoxic action but increase the inhibitory activity of these alkaloids against reverse transcriptase. It would also be worthwhile to study the effects of benzophenanthridine alkaloids on viral transformation and replication in cell culture.

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