Specific inhibitors of eukaryotic DNA synthesis and DNA polymerase α, 3-deoxyaphidicolin and aphidicolin-17-monoacetate

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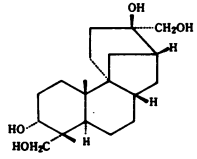
ABSTRACT

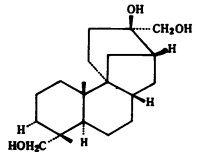
Of several phytotoxins isolated from culture filtrates of Phoma betae Frank PS-13, an incitant of leaf spot disease of sugar beet, three have been identified as aphidicolin, 3-deoxyaphidicolin and aphidicolin-17-monoacetate. Aphidicolin is a selective inhibitor of eukaryotic DNA polymerase α (Ikegami et al. (1978) Nature 275, 458-460). Consequently, we studied the action mechanism of 3-deoxyaphidicolin and aphidicolin-17-monoacetate. These aphidicolin analogues markedly inhibited the in vivo DNA synthesis of sea urchin embryos and HeLa cells but not RNA and protein syntheses. Only DNA polymerase α, not DNA polymerase β and γ, was inhibited by these drugs. The mode of action of these analogues on DNA polymerase α from the sea urchin was competitive inhibition with respect to dCTP with K_i values of 0.44 μg/ml for deoxyaphidicolin and 0.89 μg/ml for aphidicolin monoacetate, respectively. None of the other three dNTPs competed with these drugs. A similar inhibitory mode was observed using the enzyme from HeLa cells and toad oocytes. These drugs at a concentration of 2 μg/ml caused a delay in the cleavage of fertilized eggs of the sea urchin and decomposition before blastulation, indicating the possibility of achromosomal cleavage because of the absence of DNA synthesis. Based on the above, it is concluded that these analogues can be used as other inhibitors of eukaryotic DNA synthesis and DNA polymerase α.

INTRODUCTION

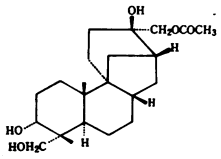
Aphidicolin, a tetracyclic diterpene-tetraol, is a well known specific inhibitor of eukaryotic DNA polymerase α (3,4) and is widely used in the study of eukaryotic DNA synthesis, DNA repair and so on. This drug, first discovered as an inhibitor of herpes virus growth, is an antibiotic isolated from the culture filtrates of the mold, Cephalosporium aphidicola Petch (1,2). When other specific inhibitors having different inhibitory mechanisms are available,



Aphidicolin



3-Deoxyaphidicolin



Aphidicolin-17-monoacetate

the study of the mechanism of DNA synthesis is facilitated.

Recently, from the culture filtrates of Phoma betae Frank PS-13, an incitant of leaf spot disease in sugar beets, several bioactive compounds were isolated based on the leaf test on sugar beets and lettuce germination inhibition. Three of these compounds have been identified as aphidicolin, 3-deoxy-aphidicolin and aphidicolin-17-monoacetate (Ichihara et al., to be published). In this paper, we determined the inhibitory mechanism of these aphidicolin analogues and obtained results showing that aphidicolin and its analogues have similar inhibitory effects on eukaryotic DNA synthesis and DNA polymerases.

MATERIALS AND METHODS

[^{3}H]dTTP (43.3 Ci/mmole), [^{3}H]dCTP (18.4 Ci/mmole), [^{3}H]dGTP (12 Ci/mmole), [^{3}H]dATP (19.2 Ci/mmole), [^{3}H]thymidine (21 Ci/mmole), [^{3}H]uridine (28.5 Ci/mmole) and [^{3}H]leucine (136 Ci/mmole) were purchased from Amersham International Ltd. dTTP, dCTP, dGTP and dATP were obtained from Yamasa. Calf thymus DNA was from Miles. Deoxyaphidicolin and aphidicolin monoacetate were purified from culture filtrates of the fungus, Phoma betae as will be described elsewhere (Ichihara et al., to be published). These drugs were dissolved in dimethylsulfoxide and added to the assay mixture to give a final concentration of 2 % dimethylsulfoxide(v/v).

Determination of Activities of DNA Polymerases

The activities of DNA polymerase α, β and γ from the sea urchin were assayed using a reaction mixture (50 µl) containing 50 mM Tris-maleate buffer, pH 7.4, 0.4 mg/ml bovine serum albumin, 40 mM NaCl, 7 mM $MgCl_2$, 0.1 mg/ml activated DNA, 20 µM each of dCTP, dGTP, dATP and dTTP and 1 µCi [^{3}H]dTTP. After incubation at 26° C for 15 - 60 min, the radioactivity incorporated into the acid-insoluble fraction was determined as described previously (4).

Preparation of DNA Polymerases

DNA polymerase α and β were prepared from 40 g of the unfertilized eggs of the sea urchin, Hemicentrotus pulcherrimus. The crude extract, after phase separation, was applied to a hydroxyapatite column (2.7 x 5.0 cm) as described previously (9). Two peaks were obtained. The former peak, eluted at 120 mM phosphate, was resistant to N-ethylmaleimide (MalNEt) and the another, eluted at phosphate concentrations from 200 mM to 280 mM, was sensitive to MalNEt. Two peaks were separately pooled, dialyzed against solution A (20 % glycerol, 2 mM EDTA and 10 mM 2-mercaptoethanol) containing 100 mM potassium phosphate, pH 7.4 and applied to a phosphocellulose column (1.1 x 6.5 cm) equilibrated with the same buffer solution. Elution was performed with a linear gradient of

phosphate buffer in solution A. DNA polymerase α activity was observed at approximately 180 mM phosphate and DNA polymerase β activity was eluted at 290 - 340 mM phosphate.

DNA polymerase γ was obtained from 12.5 g of the sperm of the sea urchin. The sperm were washed with 1 M glucose and homogenized with 100 ml of 30 mM phosphate buffer, pH 7.4 containing 20 % glycerol, 0.1 M 2-mercaptoethanol, 10 mM EDTA and 1 M NaCl. After sonication for 5 min, the homogenate was centrifuged at 30,000 rpm 60 min using an Hitachi RP 30 rotor. The supernatant was extensively dialyzed against solution A containing 50 mM potassium phosphate buffer, pH 7.4 and applied to a phosphocellulose column (1 x 8 cm). Elution was from 50 mM to 0.5 M phosphate buffer, pH 7.4 in solution A. The fractions containing DNA polymerase γ activity (aphidicolin resistant and MalNEt sensitive) were collected.

DNA polymerase α from HeLa cells was partially purified as reported previously (10). The enzyme from the toad, Bufo bufo japonicus was obtained by column chromatography as previously described (11).

Determination of DNA, RNA and Protein

Aphidicolin analogues were dissolved in dimethylsulfoxide and dispersed in embryo suspensions to give a final concentration of 0.4 % dimethylsulfoxide (v/v). 0.5 μCi of tritiated precursor was added to gastrulae (1.2×10^3 embryos in 0.5 ml of sea water) and incubated for 30 min at 20° C. After incubation, 100 % trichloroacetic acid was added to the suspension at a final concentration of 10 %. Radioactivity incorporated into the acid-insoluble fraction was determined as described previously (3,12).

HeLa cells (5×10^5 cells/ml) were incubated for 30 min at 37° C with 1 μCi of each tritiated precursor and the indicated concentration of aphidicolin analogues. After incubation, the cells were collected by tripsinization and the acid-insoluble radioactivity was determined by the method of Horwitz et al (13).

RESULTS

Effects of Aphidicolin Analogues on in vivo DNA, RNA and Protein Synthesis

The in vivo effects of the analogues on macromolecular synthesis were investigated using sea urchin embryos and HeLa cells. Fifty percent of the inhibition of DNA synthesis in sea urchin embryos was observed at a concentration of 0.6 μg/ml deoxyaphidicolin and 0.4 μg/ml aphidicolin monoacetate (Fig. 1). At least 20 % inhibition of RNA synthesis was observed at a concentration of 20 μg/ml. This decrease may have been the secondary effect of the arrest of

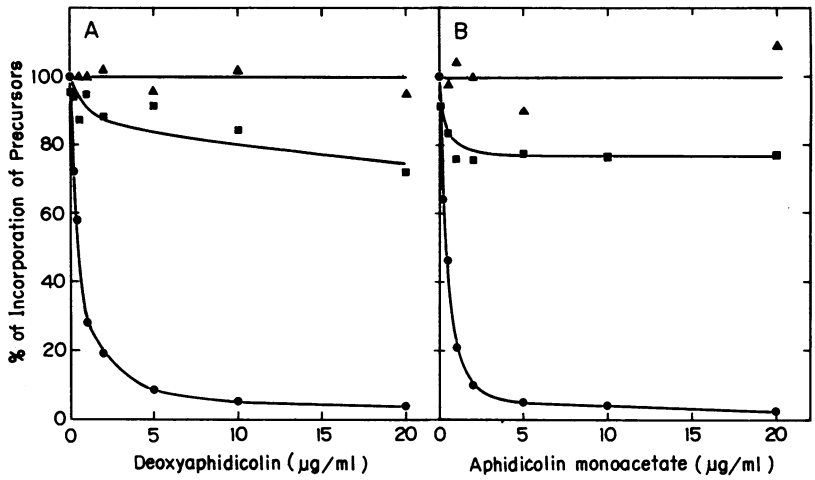


Fig. 1. Effects of aphidicolin analogues on DNA, RNA and protein synthesis in sea urchin embryos. The results are expressed as % of the incorporation observed in the absence of the drugs. 100 % incorporated [^{3}H]thymidine (●), [^{3}H]uridine (■) and [^{3}H]leucine (▲) represent 11,000, 820 and 11,300 cpm, respectively. A, deoxyaphidicolin; B, aphidicolin monoacetate.

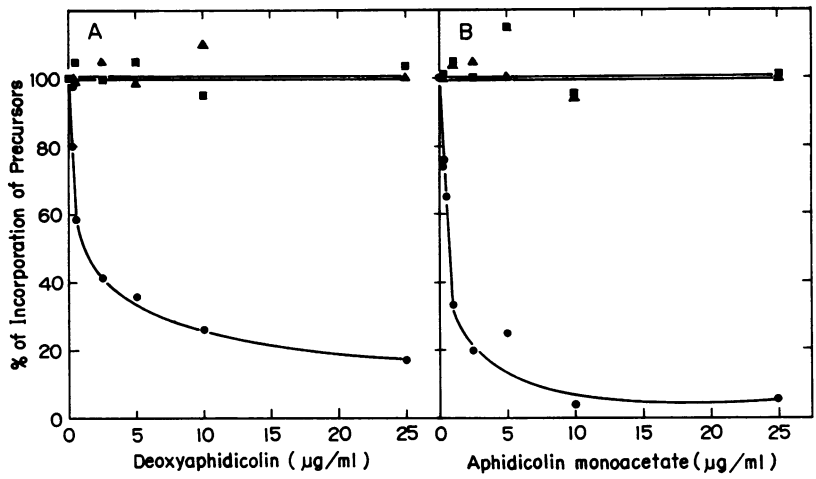


Fig. 2. Effects of aphidicolin analogues on DNA, RNA and protein synthesis in HeLa cells. The ordinate numbers are expressed as % of the incorporation observed in the absence of the analogues. The incorporation of [^{3}H]thymidine (●), [^{3}H]uridine (■) and [^{3}H]leucine (▲) was 15,245, 2,694 and 465 at 0 μg/ml of the drugs, respectively. A, deoxyaphidicolin; B, aphidicolin monoacetate.

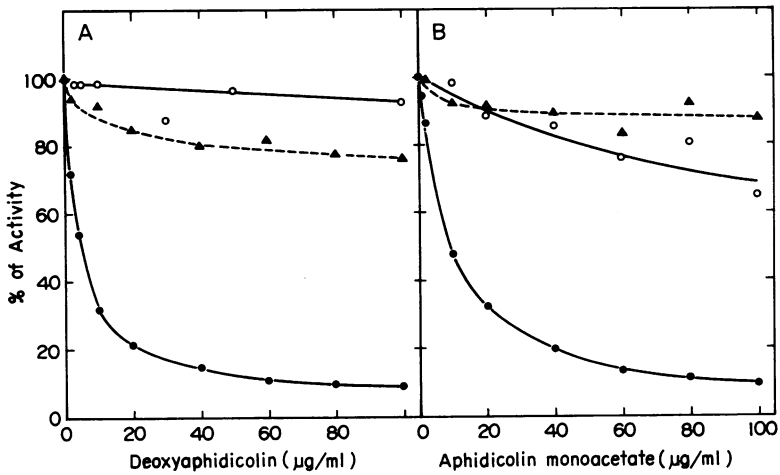


Fig. 3. Effects of aphidicolin analogues on the activities of DNA polymerases from the sea urchin. The source of DNA polymerases and assay conditions are described in Materials and Methods. The analogues were dissolved in dimethylsulfoxide and added to the assay mixture to give a final concentration of 2 % dimethylsulfoxide(v/v). The values on the ordinate are expressed as % of incorporation of [^{3}H]dTTP in the absence of drugs. A, deoxyaphidicolin, 100 % activity of DNA polymerase α (●), β (o) and γ (▲) represents 60, 19 and 0.35 pmoles/60 min, respectively. B, aphidicolin monoacetate, 100 % of DNA polymerase α (●), β (o) and γ (▲) represents 160, 19 and 0.42 pmoles/60 min, respectively.

DNA synthesis. DNA synthesis in HeLa cells was reduced by 50 % in the presence of 1 μg/ml of deoxyaphidicolin or 0.5 μg/ml of aphidicolin monoacetate (Fig. 2). RNA and protein synthesis was not inhibited at all up to a concentration of 25 μg/ml. Based on these findings, it is concluded that deoxyaphidicolin and aphidicolin monoacetate inhibit in vivo DNA synthesis but not RNA and protein synthesis.

Effects of Aphidicolin Analogues on Activities of DNA Polymerases

The activity of sea urchin DNA polymerase α was selectively inhibited by these agents (Fig. 3). Deoxyaphidicolin was a more effective inhibitor than aphidicolin monoacetate under the same assay conditions. Fifty percent inhibition by deoxyaphidicolin and aphidicolin monoacetate was observed at concentrations of 5 and 9 μg/ml, respectively, in the presence of 20 μM dCTP.

A slight inhibition of the activity of sea urchin DNA polymerase β and γ by the drugs was observed at higher concentrations tested. More than 80 % of the activity of DNA polymerase β and nearly 100 % of the activity of DNA polymerase γ were detected even in the presence of 100 μg/ml deoxyaphidicolin. In

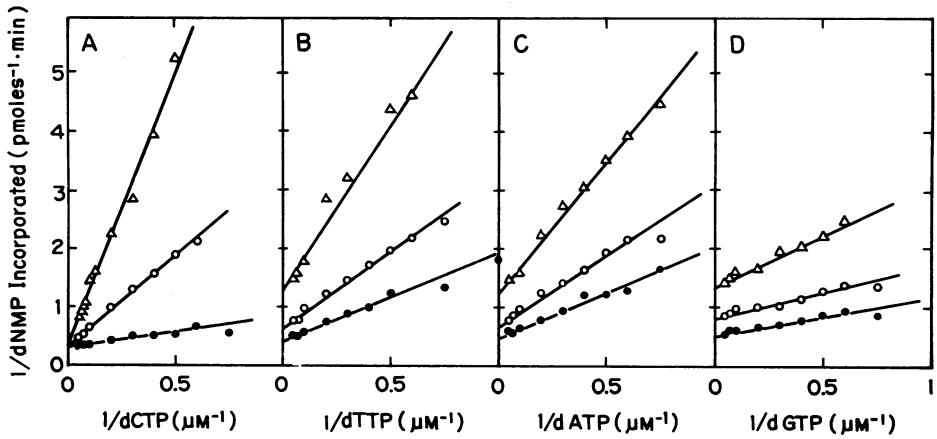


Fig. 4. Lineweaver-Burk's plots showing the effects of dNTP concentration on the inhibition of DNA polymerase α by deoxyaphidicolin. The assay was performed as described in Materials and Methods except that the concentrations of dCTP (A), dTTP (B), dATP (C) and dGTP (D) were varied in the presence of fixed concentrations of deoxyaphidicolin. (●) 0 μg/ml; (○) 2 μg/ml; (▲) 6 μg/ml.

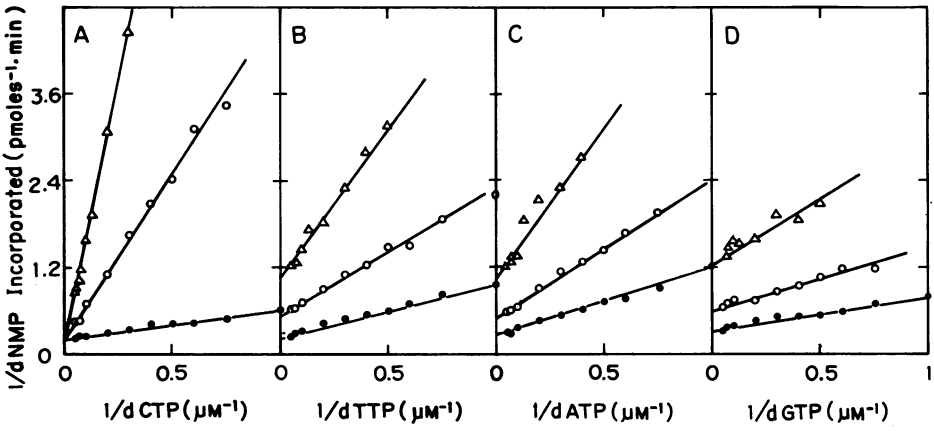


Fig. 5. Lineweaver-Burk's plots showing the effects of dNTP concentration on the inhibition of DNA polymerase α by aphidicolin monoacetate. The assay was performed as described in Materials and Methods except that the concentrations of dCTP (A), dTTP (B), dATP (C) and dGTP (D) were varied in the presence of fixed concentrations of aphidicolin monoacetate. (●) 0 μg/ml; (○) 10 μg/ml; (▲) 30 μg/ml.

Table I Kinetic parameters of aphidicolin analogues and DNA polymerase α from unfertilized eggs of the sea urchin.

K_m and K_i values were calculated from the results of Figs. 3 and 4. K_i values for aphidicolin and its analogues were determined in the presence of 20 μM dCTP when dTTP, dATP or dGTP was a variable substrate.

Substrate	dCTP	dTTP	dATP	dGTP
Inhibitory mode	competitive	noncompetitive	noncompetitive	noncompetitive
K_m value (μM)	1.8	4.0	4.0	4.0
K_i value (μg/ml)				
Deoxyaphidicolin	0.44	2.5	3.6	3.7
Aphidicolin monoacetate	0.89	7.5	9.0	9.1
Aphidicolin	0.17	0.7		

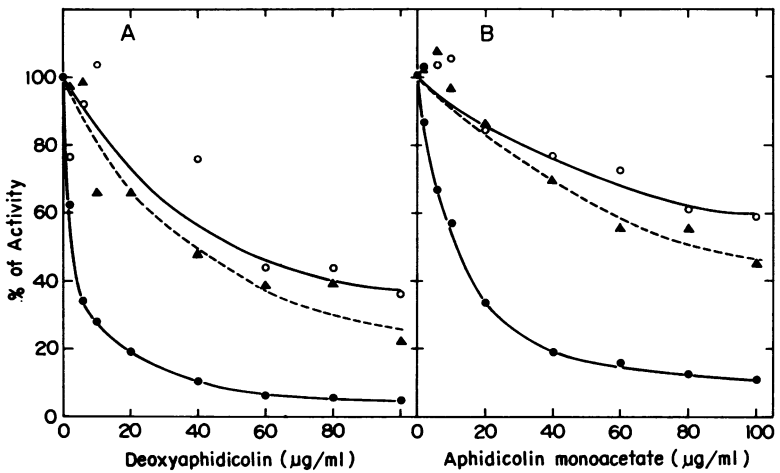


Fig. 6. Effects of aphidicolin analogues on the activity of DNA polymerase α from HeLa cells under truncated conditions. The reaction mixture contained 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 0.2 mg/ml activated DNA, 50 mM KCl, 0.4 mg/ml bovine serum albumin, 2 mM 2-mercaptoethanol, 10 μM dGTP and 0.2 μCi [^{3}H]dGTP. The other dNTPs included are 100 μM each of dATP, dTTP and dCTP (o), 100 μM each of dCTP and dATP in the absence of dTTP (▲), 100 μM each of dTTP and dATP in the absence of dCTP (●). Incubation was carried out for 1 hr at 37° C. The ordinate numbers are expressed as % of incorporation of [^{3}H]dGTP in the absence of the drugs. A, deoxyaphidicolin, 100 % activity of (o), (▲) and (●) represents 35.5, 21.3 and 29.5 pmoles, respectively. B, aphidicolin monoacetate, 100 % activity of (o), (▲) and (●) represents 29.7, 20.8 and 25.2 pmoles, respectively.

Table II Effects of aphidicolin analogues on truncated DNA synthesis using DNA polymerase α from mature oocytes of Bufo bufo japonicus

The reaction mixture (50 µl) contained 50 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 40 mM NaCl, 0.4 mg/ml bovine serum albumin, 0.1 mg/ml activated DNA, 20 µM each of 4 dNTPs (one of these was removed from the mixture as indicated and 0.4 µCi of the indicated [^{3}H]dNTP was used in its place) and the indicated concentrations of aphidicolin analogues. Incubation was carried out for 1 hr at 37° C.

Experimental system	[^{3}H]dNTP	Deoxyaphidicolin (µg/ml)		Remaining activity	Aphidicolin monoacetate (µg/ml)		Remaining activity
		0	15		0	15	
		pmoles dNMP		%	pmoles dNMP		%
Complete system	[^{3}H]dTTP	7.72	4.69	60.8	13.11	8.69	66.3
-dATP	[^{3}H]dTTP	2.16	1.16	53.7	7.49	3.50	46.7
-dGTP	[^{3}H]dTTP	5.66	2.47	43.6	8.23	5.23	63.5
-dCTP	[^{3}H]dTTP	3.63	0.46	12.7	8.10	1.51	18.6
Complete system	[^{3}H]dGTP	8.78	5.69	64.8	8.89	6.08	68.4
-dATP	[^{3}H]dGTP	4.41	2.27	51.5	4.48	2.87	64.1
-dTTP	[^{3}H]dGTP	5.71	2.40	42.0	6.18	4.02	65.0
-dCTP	[^{3}H]dGTP	4.84	0.77	15.9	6.26	1.14	18.2

the case of aphidicolin monoacetate which has no inhibitory effects on DNA polymerase β activity, DNA polymerase γ activity was slightly inhibited at higher drug concentrations.

Mode of Inhibitory Action on DNA Polymerase α

The inhibitory mode of aphidicolin analogues was studied using the DNA polymerase α of sea urchin embryos. As shown in Fig. 4 and 5, both drugs competed only with dCTP but not the other three dNTPs. The K_I values for deoxyaphidicolin and aphidicolin monoacetate were approximately 0.44 and 0.89 µg/ml, respectively, when calculated from the results (Figs. 4A and 5A) of the double reciprocal plots of DNA polymerase α activity as a function of dCTP concentration. From the kinetic parameters summarized in Table I, it is concluded that the inhibitory effects of deoxyaphidicolin and aphidicolin monoacetate are three times and six times less effective than that of aphidicolin, respectively. Higher Ki values were obtained from double reciprocal plots as functions of dATP, dGTP or dTTP. These higher values are due to the existence of dCTP in the reaction mixture.

Similarly, DNA polymerase α from HeLa cells was inhibited by these analogues as shown in Fig. 6. That is, high sensitivity was observed only in the case of the reaction mixture containing no dCTP. The absence of one of the other three dNTPs did not change the extent of inhibitory action of the analogues. The results indicate that the extent of inhibition was dependent upon

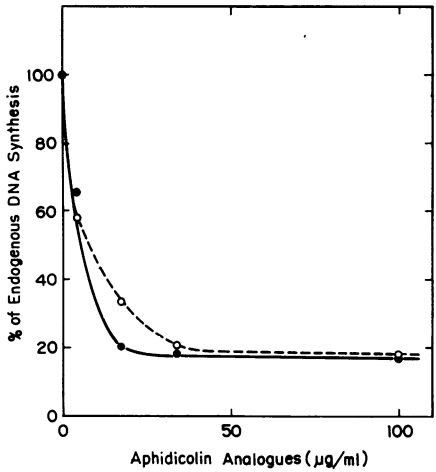


Fig. 7. Effects of aphidicolin analogues on the DNA synthesis of isolated nuclei from HeLa cells. The reaction mixture contained 40 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM 2-mercaptoethanol, 4 mM ATP, 100 μM each of dATP, dCTP and dGTP, 10 μM dTTP, 0.2 μCi [^{3}H]dTTP and 20 μl of isolated nuclei suspension in a total volume of 75 μl. Incubation was carried out for 30 min at 37° C. The ordinate is expressed as % of incorporation of radioactivity into the acid-insoluble fraction in the absence of the drugs. 100 % activity was 40.8 pmoles. (•) deoxyaphidicolin, (o) aphidicolin monoacetate.

the concentration of dCTP, suggesting that the inhibition of DNA polymerase α from HeLa cells by aphidicolin analogues is due to competition with dCTP. Actually, from the results of double reciprocal plots of the activity as a function of dCTP concentration (data not shown), deoxyaphidicolin and aphidicolin monoacetate inhibited the enzyme competitively with respect to dCTP with Ki values of 4.3 and 8.6 μg/ml, respectively. DNA polymerase β from HeLa cells was not inhibited by the analogues.

Effects of the analogues on the activity of DNA polymerase α obtained from the toad, Bufo bufo japonicus, were also studied in the presence of only three dNTPs (Table II). Greater inhibition was observed only in the system containing no dCTP. These results suggest that each analogues inhibits the DNA polymerase α of the toad competitively with respect to dCTP.

From the above, it is concluded that these analogues competitively inhibit α-type DNA polymerase from various species with respect to dCTP as shown in the case of aphidicolin.

Effects of Aphidicolin Analogues on The Endogenous DNA Synthesis of Isolated Nuclei

Endogenous DNA synthesis using the isolated nuclei of sea urchin embryos

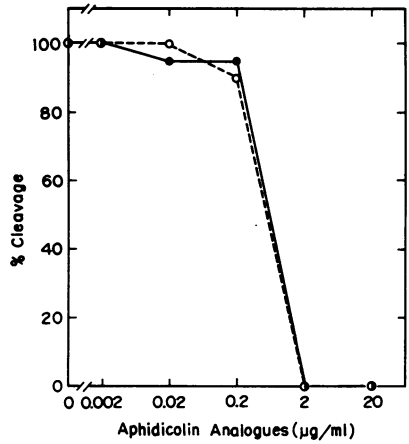


Fig. 8. Inhibition by aphidicolin analogues of mitotic division of sea urchin embryos. Embryos of the sea urchin were treated with various concentrations of aphidicolin analogues immediately after insemination. Morphological changes were observed for 98 min with a microscope. Aphidicolin analogues were dissolved in dimethylsulfoxide and then dispersed in sea water to give a final concentration of 0.4 % dimethylsulfoxide, which did not affect cleavage. (●) deoxyaphidicolin, (○) aphidicolin monoacetate.

was resistant to relatively high concentrations of each aphidicolin analogue. Approximately 90 % of the activity achieved in the absence of the drug remained in the dosage at 100 μg/ml. In the same experimental system, 67 % of the activity was detected in the presence of 100 μg/ml aphidicolin. On the other hand, significant inhibition by the analogues was observed in the DNA synthesis of HeLa cells (Fig. 7). Fifty percent inhibition was observed at 5 and 8 μg/ml of deoxyaphidicolin and aphidicolin monoacetate, respectively. A higher sensitivity of endogenous nuclear DNA synthesis in HeLa cells than that in sea urchin embryos toward these drugs was also observed in the case of aphidicolin (5).

Effects of Aphidicolin Analogues on The Development of The Fertilized Eggs of The Sea Urchin

The fertilized eggs of the sea urchin were exposed to these analogues so as to investigate their effects on mitosis during the early cleavage stage. In the control experiments, half of the fertilized eggs cleaved 80 min after insemination and all embryos attained the two-cell stage within 85 min. When treated with deoxyaphidicolin or aphidicolin monoacetate at a final concentration of 2 μg/ml, the fertilized eggs remained uncleaved even after 100 min.

The timing of the first cleavage in the presence of 0.2 μg/ml analogues was essentially the same as that of control (Fig. 8). These inhibitory effects on the first cleavage are nearly the same as reported using aphidicolin. Thereafter, the embryos in the presence of 0.2 μg/ml analogues (70 % *in vivo* DNA synthesis was observed under the same conditions (Fig. 1)) undergo nearly normal cell cycle progression and normal blastulation and gastrulation occur although a delay in development was observed (at 4 hr after insemination, control and treated embryos attained the 16-cell and the 8-cell stages, respectively). In the presence of 2 μg/ml or 20 μg/ml analogues, the fertilized eggs cleaved but with a prominent delay. At 6 hr after fertilization, the control had attained the early blastula stage. However, the embryos in the presence of 2 μg/ml or 20 μg/ml deoxyaphidicolin showed the abnormal 16-cell stage or 1- to 8-cell stages (a mixture of 1-cell, 2-cell, 4-cell and 8-cell and also irregular cleavages was observed). Aphidicolin monoacetate was more effective than deoxyaphidicolin and the embryos showed the abnormal 4-cell or mainly 1-cell stage at the same doses of aphidicolin monoacetate, respectively. Analogues-treated embryos continued to cleave and then decomposed. These embryos never hatched or formed blastulae. Microscopic observation showed no nuclei in blastomeres of embryos treated with the analogues. From the above, the morphological results were found to be quite similar to those obtained with starfish embryos treated with aphidicolin (7). Thus, it is concluded that cleavage in the presence of high doses of these analogues is achromosomal.

DISCUSSION

In this paper, it has been demonstrated that 3-deoxyaphidicolin and aphidicolin-17-monoacetate, which are natural products of *Phoma betae*, are specific inhibitors of eukaryotic DNA synthesis and DNA polymerase α. The inhibition by these drugs of the enzyme was due to competition with dCTP. These inhibitory effects of aphidicolin analogues are essentially similar to those of aphidicolin. These observations, however, are important in elucidating the relationship between the structure of the inhibitors and inhibitory activity toward DNA polymerase α. Although aphidicolin is the most effective (Ki value of 0.17 μg/ml) as an inhibitor of DNA polymerase α, deoxyaphidicolin (Ki value of 0.44 μg/ml) and aphidicolin monoacetate (Ki value of 0.89 μg/ml) are also useful for investigating eukaryotic DNA synthesis. At the beginning of our study on the inhibitory mode of these drugs, we expected a different mode of action from aphidicolin but failed to find any significant difference in the

biochemical and morphological effects of aphidicolin or those of its analogues.

Higher Ki values were calculated when the concentrations of noncompetitive substrates varied in the double reciprocal plots (Table I). The inhibitory level of DNA polymerase α by these inhibitors was dependent on the dCTP concentration in the reaction mixture. In the other words, the apparent Ki value becomes larger when the dCTP concentration increases. A true Ki value is obtained when a competitive substrate (dCTP) is varied in Lineweaver-Burk's plots or when a noncompetitive substrate is varied in the same plots in the absence of dCTP. If the apparent Ki value is assumed to be Ki(1 + [dCTP]/Km for dCTP), the Ki value calculated from apparent Ki values and the formula fit each other well.

Deoxyaphidicolin was more effective than aphidicolin monoacetate in inhibiting the activity of DNA polymerase α. On the other hand, in vivo DNA synthesis was more strongly inhibited by aphidicolin monoacetate than by deoxyaphidicolin (Figs. 1 and 2). This discrepancy can be explained if it is assumed that aphidicolin monoacetate is partially hydrolyzed to aphidicolin in vivo. This assumption is supported by the fact that the effects of aphidicolin monoacetate on the morphological changes of embryos of the sea urchin are more prominent than those of deoxyaphidicolin.

Endogenous DNA synthesis in isolated nuclei from the sea urchin was somewhat resistant to aphidicolin analogues whereas DNA synthesis in isolated nuclei from HeLa cells was quite sensitive to these analogues. These observations are reasonably consistent with the findings on aphidicolin (5,6,8). The reason for the differences remains to be clarified.

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