Differentiation in Fern Gametophytes Treated with Purine and Pyrimidine Analogs

Abstract. *Analogs of the purines, adenine and guanine, and of the pyrimidines, cytosine and uracil, completely inhibited two-dimensional differentiation in the gametophytes of the fern,* Asplenium nidus *L. Analogs of thymine caused a nonspecific growth inhibition without suppressing two-dimensional growth. The effects of the analogs were reversed by the respective bases or their derivatives.*

The transition of the filamentous one-dimensional protonema to the characteristic two-dimensional gametophyte in polypodiaceous ferns has been shown to be causally connected with RNA synthesis. By the use of 8-azaguanine, which is an effective inhibitor of RNA synthesis, a reversal of growth of the gametophytes of *Dryopteris erythrosora* and *D. borreri* from two-dimensional to one-dimensional forms, accompanied by a decreased synthesis of RNA or proteins, has been demonstrated (*1*). This evidence was not considered conclusive to prove a role for RNA in the differentiation of fern gametophytes, in that it was based on the use of a single inhibitor, and did not eliminate the probable effects of the analog on DNA synthesis. This report describes the contrasting effects of a number of specific inhibitors of RNA and DNA synthesis, respectively, on the differentiation in the gametophytes of *Asplenium nidus* L. That the purine and pyrimidine bases of RNA are required in regulating two-dimensional differentiation in the gametophytes is indicated.

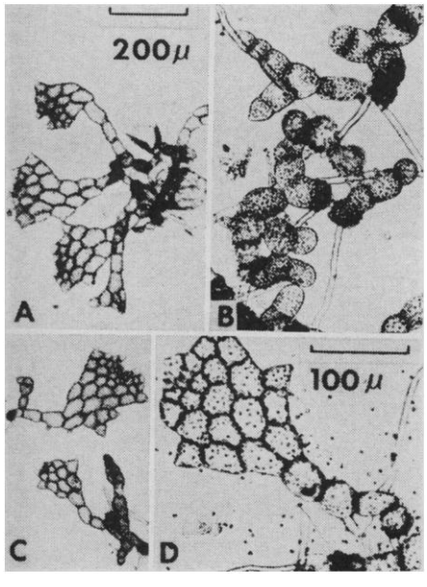

Fig. 1. Gametophytes of *Asplenium nidus* growing in (*A*) modified Miller medium; (*B*) modified Miller medium plus 2-thiouracil (1 mg/liter); (*C*) modified Miller medium plus 2-thiouracil (1 mg/liter) and uracil (40 mg/liter); and (*D*) modified Miller medium plus 5-fluorodeoxyuridine (0.1 mg/liter). All photographs were taken after a growth period of 4 weeks. Magnification for *A* and *C* is given in *A*, and that for *B* and *D* is given in *D*.

Spores of *Asplenium* were sown in 9-cm petri dishes containing 25 ml of Miller and Miller's (*2*) medium, modified by substituting ferric citrate for ferric tartrate. The analogs tested were added as supplements to the modified Miller medium. The cultures were grown at 23° to 25°C and subjected to a daily photoperiod of 5½ hours of mixed fluorescent and incandescent light of about 2000 lux intensity at the level of the petri dishes. The maximum length attained by the gametophytes before differentiation into two-dimensional forms was considered as the extent of one-dimensional growth. Criteria adopted to measure two-dimensional growth included width, cell number, and surface area of the gametophytes after a growth period of 4 weeks (*3*). The data presented are averages of at least 20 gametophytes from each treatment.

Table 1 shows the effects of several purine and pyrimidine analogs on the two phases of growth of the gametophytes (*4*). From the low values for width, cell number, and surface area, it is clear that the purine analogs, 8-azaadenine and 8-azaguanine, and the pyrimidine analogs, 2-thiocytosine, 5-fluorouracil, 2-thiouracil, and 6-azauracil, which are known to interfere with RNA synthesis (*5, 6*), completely inhibited two-dimensional differentiation in the gametophytes (Fig. 1*b*). An equally clear inhibition of one-dimensional growth also occurred in these inhibitors; however, during further periods in culture the filaments elongated and exceeded the controls in length without showing two-dimensional growth. In contrast to analogs

Table 1. Effects of purine and pyrimidine analogs on the one-dimensional and two-dimensional phases of growth of the gametophytes of *Asplenium nidus*.

Concn. (mg/liter)	One-dimensional length (μ)	Two-dimensional*		
		Width (μ)	Cell No.	Surface area (relative units)
No analog added (basal medium)				
	280.6†	182.9	20.5	10.6
8-azaadenine				
5.0	211.4‡	35.7	4.9	2.8
8-azaguanine				
30.0	187.2‡	42.6	3.9	2.4
2-thiocytosine				
20.0	160.0‡	32.0	3.5	2.5
5-fluorouracil				
1.0	175.0‡	42.0	3.7	2.8
2-thiouracil				
1.0	150.0‡	35.0	3.2	2.6
6-azauracil				
50.0	125.3‡	33.8	2.9	2.1
5-fluorodeoxyuridine				
0.1	262.0†	122.7	15.7	8.8
5-bromouracil				
500.0	248.0†	153.3	16.9	9.0
6-azathymine				
400.0	251.7†	168.5	17.2	8.9

* In all cases, width, cell number, and surface area were determined in 4-week-old cultures. † Measured 20 days after sowing. ‡ Measured 4 weeks after sowing.

Table 2. Reversal of growth inhibition in the gametophytes of *Asplenium nidus* by nucleic acid bases and their derivatives. Data are based on 4-week-old cultures. The figures in parentheses indicate the ratios of the optimum reverser to the analog for the effects indicated. The concentrations of the analogs used are the same as given in Table 1. +++, complete reversal; increasingly lesser effects are shown by ++ and +; −, no reversal.

Reversal of non-specific growth inhibition	Reversal of two-dimensional growth inhibition			
5-fluoro-deoxy-uridine	8-aza-adenine	8-aza-guanine	2-thio-cytosine	5-fluoro-uracil
		Adenine		
−	+++ (10)	++ (1)	−	−
		Adenylic acid		
−	+++ (10)	−	−	−
		Adenosine		
−	+++ (10)	++ (1)	−	++ (25)
		Guanine		
−	+++ (1)	+++ (1)	−	+ (50)
		Guanylic acid		
−	+++ (10)	+ (10)	−	−
		Cytosine		
−	−	−	+++ (50)	+ (50)
		Cytidylic acid		
+++ (50)	−	−	++ (10)	+++ (50)
		Cytidine		
+++ (200)	++ (10)	−	++ (1)	+++ (25)
		Uracil		
−	++ (1)	−	−	+++ (10)
		Uridylic acid		
−	+ (1)	−	−	++ (25)
		Uridine		
−	+ (10)	−	−	+++ (25)
		Thymine		
−	−	−	−	−
		Thymidylic acid		
+++ (25)	++ (50)	−	−	−
		Thymidine		
+++ (25)	++ (25)	−	−	−

of RNA bases, thymine analogs like 5-fluorodeoxyuridine, 5-bromouracil, and 6-azathymine which interfere with DNA synthesis (*6*, *7*) did not appreciably inhibit induction of two-dimensional growth in the gametophytes (Fig. 1*d*). A slight decrease observed in the length of the protonema and in the width, cell number, and surface area of the gametophytes grown in thymine analogs may be due to a nonspecific growth inhibition by the added compounds. These results imply that inhibition of RNA synthesis leads to an inhibition of two-dimensional growth of the gametophytes, while effects of interfering with DNA synthesis are exerted as a general retardation of growth. Indeed, when grown in a medium containing ribonuclease (0.8 to 1.0 mg/ml), two-dimensional differentiation in the gametophytes of this same species was completely blocked (*8*).

Further work showed that the nonspecific growth inhibition due to 5-fluorodeoxyuridine was completely reversed by thymidine, thymidylic acid, cytidine, and cytidylic acid; thymine, cytosine, and the purines were ineffective in reversing this inhibition (Table 2). The inhibition of two-dimensional growth induced by analogs of adenine and guanine was annulled by the corresponding purine bases and their derivatives; pyrimidines were ineffective in completely reversing this inhibition, although in some cases partial reversals did occur. Similarly, inhibition caused by antagonists of cytosine and uracil was reversed by the corresponding pyrimidine bases or their derivatives, but not by the purines (Fig. 1, *a* and *c*). Thus there is a requirement for the purine and pyrimidine bases of RNA in the induction of a two-dimensional growth pattern in the gametophytes of *Asplenium*; the requirement for the purines, adenine and guanine, was not met by the pyrimidines, cytosine, uracil, or thymine, and a requirement for cytosine and uracil was not satisfied by the purines or by thymine.

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9. I thank Mrs. L. Raghavan for technical help and Hoffman-La Roche, Basle, Switzerland, for the 5-fluorouracil and 5-fluorodeoxyuridine.

5 October 1964

Ethylation: Biological Formation of an S-Ethyl Homolog of Lincomycin

Abstract. Streptomyces umbrinus *var.* cyaneoniger *produces the antibiotic lincomycin. When grown in the presence of ethionine an additional antibiotic is produced. Physical and chemical analyses and incorporation of radioisotopes indicate that the new antibiotic is the* S-*ethyl homolog of lincomycin.*

Ethionine has been reported to be a naturally occurring compound (*1*), and several examples of biological ethylation with it as an ethyl donor are known (*2*). The reactions occurring represent transethylation in place of normal transmethylation, and the ethyl group occurs attached to either an oxygen or a nitrogen atom in the final product. We now report an example of biological ethylation in which a new antibiotic, a homolog of the antibiotic lincomycin (*3*), was formed when *Streptomyces umbrinus* var. *cyaneoniger* (var. nov.) (*4*) was grown in the presence of ethionine. In this case the ethyl group is attached to a sulfur atom in the final product.

Streptomyces umbrinus var. *cyaneoniger,* isolated from soil, was grown under conditions of aeration and agitation at 28°C for 4 days on a medium consisting of soybean meal, 2 percent; glucose, 2 percent; corn steep liquor, 1 percent; sodium chloride, 0.3 percent; and calcium carbonate, 0.2 percent (*5*). An antibiotic, found in the culture broth, was isolated as the crystalline hydrochloride salt. It had the following properties: m.p. 152°–156°C; $[\alpha]_D$ +131 (*c*, 1.01, H_2O); *pK* 7.4 (neutralization equivalent 434); and essentially no absorption in ultraviolet and visibile light. Analysis (percentage): C, 48.25; H, 7.98; N, 6.24; S, 7.10; Cl, 8.13; O, 21.56; *O*—CH_3, 0.45; *N*—CH_3, 2.6; *C*—CH_3, 4.8; acetyl, 0.83. Calculated (percentage) for $C_{18}H_{34}N_2O_6S \cdot HCl$: C, 48.78; H, 7.97; N, 6.32; S, 7.24; Cl, 8.01; O, 21.68. These properties, together with the mobility on paper chromatography and the infrared absorption spectrum (*6*) indicate that the antibiotic is identical with lincomycin (*3*).

In other similar fermentations of *Streptomyces umbrinus* var. *cyaneoniger,* 0.006 percent DL-ethionine was added after 48 hours of growth. At harvest time a second antibiotic was detected in the culture broth by paper bioautography against *Corynebacterium xerosis* on *p*H 7.9 nutrient agar medium. By developing on No. 1 Whatman paper in the system, *n*-butyl alcohol, water, isoamyl alcohol, and dichloroacetic acid (100 : 75 : 50 : 1), the R_F values of lincomycin and the new compound were 0.50 and 0.65, respectively. A concentrate containing the two antibiotics was prepared by the procedure used for the isolation of lincomycin as follows. The antibiotics were adsorbed from the culture filtrate by activated charcoal (1.5 percent weight/volume) and eluted with a mixture of acetone and water (9 : 1). The eluate was concentrated to an aqueous phase and adjusted to *p*H 10.5. This was extracted with two equal volumes of *n*-butyl alcohol. The combined *n*-butyl alcohol extracts were extracted twice with one-fifth volume of water adjusted to *p*H 2.5 with hydrochloric acid. The aqueous phase was lyophilized. The residual solid was about 90 percent pure antibiotic, containing lincomycin and the new compound in the ratio of about 10 : 1. The antibiotics were present, as shown by paper chromatography, in essentially the same ratio in the culture broth. Crystallization from acetone and methanol (5 : 1) resulted in no resolution because the antibiotics cocrystallized.

Lincomycin and the new compound were resolved by partition chromatography on diatomaceous earth, in the system composed of ethyl acetate and 0.5*M* phosphate, *p*H 6.2. The distribution coefficients were 0.05 and 0.09 for lincomycin and the new compound, respectively. Most of the new compound, which contained less than 2 percent lincomycin as judged by paper chromatography, was eluted with an amount of developing fluid equal to 3 to 5.5 times the void volume of the column. This portion of eluate was extracted with water adjusted to *p*H 2.5 with hydrochloric acid. The extract was