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STIMULATION OF POLLEN TUBE GROWTH IN VITRO BY THIOURACIL AND OTHER ANTIMETABOLITES OF NUCLEIC ACID BASES

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ABSTRACT

25 Purines and pyrimidines were tested for the effect on pollen tube growth and germination. 2-Thiouracil most strongly stimulates tube growth. However, 2-thiouracil decreases or does not affect metabolism of sugars. Some derivatives of thiouracil also stimulate tube growth, whereas others inhibit growth. Pollen growth response is markedly affected by the concentration of the compound tested.

I. INTRODUCTION

Thiouracil and certain analogues of purine and pyrimidine bases generally inhibit cell growth. These unnatural compounds substitute for uracil and other moieties in the cells' nucleic acids; this substitution modifies the template on which proteins are synthesized (HAMERS, 1956). The decreased growth of bacteria and plant viruses in the presence of such analogues has been related to a net decrease in some enzymes and a net increase in others (CHANTRENNE and DEVREUX, 1958). Treatment of plants with thiouracil can inhibit leaf and bud formation and seed growth (HESLOP-HARRISON, 1962; SHIMENO and KINOSHITA, 1963). Similarly thiouracil have toxic effect on *Lemna minor* in concentration as low as 1 p.p.m. (NICKELL, 1962). In contrast, ŠORMOVÁ *et al.* (1960) reported that at 100 p.p.m. thiouracil stimulated pea seedling root growth and inhibited hypocotyl elongation (HAMERS, 1956). Thiouracil saw also reported to stimulate root cell elongation and to inhibit cell division in root tissue culture (WOODSTOCK, 1963).

II. METHODS

Solutions of reagent grade chemicals in glass distilled water were used in all in vitro growth experiments. Purine and pyrimidine derivatives purchased from Mann Research Laboratories, New York, or synthesized in laboratories of the Czechoslovak Academy of Sciences, Prague, were tested in 0.3 M sucrose containing 10 p.p.m. boric acid. Equal aliquots of pollen of *Nicotiana alata* Link and Otto

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TABLE 1

Effect of substituted pyrimidines and purines on pollen germination (*Nicotiana alata*)

	Compound added to control solution	10 ppm			100 ppm			1000 ppm		
		Germ. %	Tube length μ		Germ. %	Tube length μ		Germ. %	Tube length μ	
			Mean	S.E. ±		Mean	S.E. ±		Mean	S.E. ±
	(Control = 0.3M sucrose + 10 ppm H_3BO_3 . .	70	274	10.0)						
1	Uracil	70	270	9.9	71	266	9.7	56	270	11.3
2	4–Aminouracil	71	291	10.0	66	295	9.6	55	315[b]	11.0
3	4–Amino–3–methyluracil	64	295	9.0	65	270	8.9	56	224[c]	9.3
4	4–Amino–2–thiouracil	65	274	9.6	69	315[b]	11.4	62	315[b]	12.8
5	5–Aminouracil	68	270	8.6	70	270	9.1	70	282	8.6
6	6–Azauracil	75	320[b]	11.9	69	315[b]	12.1	71	278	11.3
7	5–Bromuracil	69	295	10.5	67	257	8.9	54	216[c]	8.4
8	4, 5–Diaminouracil-sulfate	73	286	9.6	63	253	8.9	0	—	—
9	5, 6–Dihydrouracil	64	262	8.6	68	270	9.7	72	374[c]	12.4
10	1, 4–Dimethyl–2–methylmercaptouracil	64	291	10.8	68	286	9.9	0	—	—
11	5–Nitrouracil	73	291	9.3	65	332[c]	12.8	44	< 60	—
12	4–Propylthiouracil	64	274	10.7	18	174[c]	8.3	0	—	—
13	2–Thiouracil	72	324[b]	12.2	75	523[c]	22.6	48	324[a]	17.0
14	Thymine	61	249	11.0	61	203[c]	7.9	60	224[c]	8.2
15	6–Azathymine	66	278	11.0	63	266	10. 1	68	232[b]	9.5
16	2–Thiocytosine	65	295	9.6	70	282	7.9	73	328[c]	11.2
17	2–Amino–4,6–dimethylpyrimidine	66	286	10.7	65	278	10.7	72	407[c]	15.1
18	4,6–Diamino–2–thiopyrimidine	62	270	9.1	70	278	9.8	74	469[c]	16.5
19	Adenine	67	262	9.3	37	253	10.5	0	—	—
20	8–Azaadenine	70	324[b]	13.0	69	357[c]	15.1	72	332[b]	15.1
21	2–Thioadenine	66	270	9.8	65	278	10.0	0	—	—
22	Guanine	64	241[a]	8.9	57	220[c]	7.1	—	—	—
23	8–Azaguanine	76	357[c]	13.6	66	332[c]	13.3	26	158[c]	6.8
24	6–Thioguanine	68	315[b]	10.8	65	307[a]	9.6	—	—	—
25	2–Thioxanthine	68	299	10.6	57	262	8.4	—	—	—

[a] significant at $P < 0.02$ level [b] significant at $P < 0.01$ level [c] significant at $P < 0.001$ level

Growth conditions are as listed in Table 2.

or *Petunia hybrida* clone W 166 K were added to drops of germination media and growth measurements made after 6 hours. Values of percent germination and mean tube length represent the mean of counts from two replicate samples of 100 grains and 50 tubes respectively. The significance of differences between mean tube lengths in the controls and individual chemical treatments were tested by Student's "T" test.

III. Results

In experiments with *N. alata* pollen, 2-thiouracil was the most stimulating of the compounds tested (Table 1). Of other thiopyrimidines: 4-amino-2-thiouracil, 4, 6-diamino-2-thiopyrimidine and 2-thiocytosine were stimulatory at higher concentrations; 4-propyl-thiouracil strongly inhibited germination and tube growth at 100 and 1000 p.p.m. The ring substituted aza-derivatives of uracil, adenin and guanin also stimulated tube growth at low concentrations as did 5-nitrouracil and 6-thioguanine. The nitrogen analogue of guanine, 8-azaguanine stimulated pollen growth at low concentrations but inhibited at 1000 p.p.m. Thioderivatives of adenine, and xanthine did not stimulate pollen tube growth. Of the nucleic acid bases: uracil did not inhibit tube growth at 1000 p.p.m., the highest concentration tested; adenine inhibited only at high concentrations and thymine and guanine were inhibitory at low concentrations.

The halide derivative of uracil, 5-bromouracil, is a commonly used and generally more effective inhibitor of cell growth and protein synthesis than thiouracil. It had no effect at 10 or 100 p.p.m. but inhibited pollen growth at 1000 p.p.m. Although the concentration ranges differs, this agrees with observations of the effect of 5-bromouracil on leaf growth in *Lemna minor* (Nickell, 1962). 5-Bromouracil is the only uracil analogue studied in detail in diverse living systems. Although rapid substitution is reported in bacterial RNA, 5-bromouracil is not incorporated into plant nucleotides and nucleic acids (Šebesta, Bauerová, Šorm and Šormová, 1960). Since 5-bromouracil apparently does not directly modify protein synthesis, plant growth inhibition by this compound would be expected only at relatively higher concentrations, as was indeed observed in this present study.

Table 2 lists the six longest pollen tubes observed in germinating *N. alata* and the compound and concentrations that produced the growth. As might be expected, the compounds which resulted in greatest mean tube lengths (Table 1), also produced the longest single tubes. The length of these tubes indicates that it is possible to establish in vitro conditions which result in pollen tube lengths and growth rates approximating those observed in vivo. Pollen can probably be made to grow as normally in vitro as in vivo when the proper environment provided (Brewbaker and Kwack, 1964).

Although 2-thiouracil is facilitates near normal tube elongation in vitro, we do not want to infer that this compound occurs endogenously the stylar tissues. It did not affect in vivo pollen tube growth in experiments with *Petunia hybrida*. In vitro *P. hybrida* pollen responded well to added 2-thiouracil (Table 3). Movement of solutions up the

TABLE 2

Maximum pollen tube lengths in purine and pyrimidine analogues

Compound	Concentration p.p.m.	Maximum length observed microns
None	—	560
2-Thiouracil	100	1205
4,6-Diamino-2-thiopyrimidine	1000	830
5,6-Dihydrouracil	1000	790
2-Amino-4,6-dimethylpyrimidine	1000	745
8-Azaguanine	10	745
8-Azaadenine	10	720

Large populations of *Nicotiana alata* pollen were cultivated in 0,3 M solution of sucrose in glass-distilled water containing 10 p.p.m. boric acid 6 hrs. at $\sim$ 26°C.

TABLE 3

Effect of 2-Thiouracil on sugar metabolism by germinating pollen (*Petunia hybrida*)

Radioactive substrate	Experiment	Counts per minute $C^{14}O_2$ recovered 4 hours	
		Control	2-Thiouracil
Glucose–U–C^{14}	1 –	6440	4750
	2 –	5500	4800
Sucrose–U–C^{14}	1 –	2200	2070
	2 –	2950	2400
Growth observed	% Germination	64	58
	Mean length μ	10.9 $\pm$ 0.74	13.2 $\pm$ 0.77

Experiments were run in shaking Warburg flasks, 26°C. Initial counts of glucose –U–C^{14} added = 1.44×10^5 c.p.m.; sucrose–U–C^{14} = 1.9×10^5 c.p.m.; 10 mgs pollen contained in a total volume of 1.0 ml germinating media of 0.2 M sucrose and 20 p.p.m. B as H_3BO_3; 750 p.p.m. 2-thiouracil was added. Growth was stopped by addition of 4 percent H_2SO_4 from the side arm. Center wells contained 0.15 ml 5 M KOH. Radioactive substrates were supplied by Atomic Energy Center, Amersham, England. Since addition of acid to pollen distorts the tubes growth measurements were made on separate samples of pollen from identical sources. Measurements were computed on counts of 2 samples of 100 grains each, germinated 3 hours, 26° C $\pm$ 500 p.p.m. 2-thiouracil.

Petunia style can be readily demonstrated by placing the cut style base into a dye solution. But, when cross- or self-pollinated styles of *P. hybrida* were placed in solutions of 2-thiouracil the rate of tube growth down the style was unaffected.

Pollen of several other angiosperm genera were also tested for response to 2-thiouracil. 2-Thiouracil stimulated pollen tube growth of most species tested, however the degree of growth stimulation varied with the species and age of the pollen. In view of this pollen growth response to 2-thiouracil we next evaluated the ability of this compound to stimulate a ubiquitous metabolic pathway.

We investigated mono- and disaccharide metabolism by germinating *Petunia hybrida* pollen in a medium containing C^{14}-labeled glucose or sucrose. Germination was halted and CO_2 released by adding sulfuric acid. Carbon dioxide was trapped in KOH solution and its radioactivity measured.

2-Thiouracil did not increase the rate of conversion of glucose or sucrose to CO_2 (Table 3) despite its ability to stimulate tube elongation. In fact, in the presence of this compound the rate of conversion of sucrose to CO_2 decreased slightly and glucose metabolism appeared to decrease markedly.

The decrease in $C^{14}O_2$ recovered may merely indicate that 2-thiouracil increases the amount of CO_2 incorporated into cell constituents via such mechanisms as carboxylation of phosphoenol pyruvate. This pathway for CO_2 fixation has been demonstrated in germinating pine pollen (STANLEY, 1958). However, studies which compared sucrose and glucose metabolism in germinating apple pollen (HRABĚTOVÁ and TUPÝ, 1961; TUPÝ, 1962) indicate that the observed decrease in recovered $C^{14}O_2$ is related to the quantity of glucose metabolized and not the CO_2 incorporation.

Presumably 2-thiouracil does not significantly increase the rate of glucose release from the sucrose and thus does not dilute the glucose-U-C^{14}. Germinating apple pollen preferentially uses the fructofuranose component of sucrose (TUPÝ, 1962). In our present studies nonradioactive sucrose was present in high concentrations (0.2 M) along with the radioactive glucose ($\sim 10^{-7}$ M). Thus, if 2-thiouracil increased the rate of metabolism of sucrose, more fructofuranose would be available to decrease utilization of glucose-U-C^{14}. However, 2-thiouracil also decreased the quantity of $C^{14}O_2$ released from sucrose-U-C^{14} in a sucrose medium (Table 3). Therefore, we must conclude that the observed pollen growth stimulation of 2-thiouracil is not related to an increased rate of catabolism of available carbohydrates to CO_2.

It is conceivable that 2-thiouracil stimulates pollen growth by acting as an enzyme sulfhydryl reductant. However, certain sulfhydryl containing analogues failed to stimulate pollen growth, while other nonsulfhydryl analogues increased pollen growth (Table 1 & 2). Furthermore, in vitro tests of the effect of reduced glutathione, 2, 3-dimercaptopropanol (BAL) and thiourea over a range of concentrations showed no significant pollen growth stimulation. However, recent studies with isolated mitochondria showed that aminothiols are effective sulfhydryl activators while the usual $\sim$SH containing compounds, such as were tested here on pollen, are not active (BLUM and SANADI, 1964). Assuming that the pyrimidine and purine analogues are all equally absorbed by the pollen, our studies do not exclude the possibility of an indirect and differential activation similar to that observed in isolated mitochondria.

IV. DISCUSSION

Experiments in which plant tissues are grown in the presence of

pyrimidine and purine analogues suggest that their effect on cell elongation is related to their participation in nucleic acid synthesis and/or cell wall extension (ŠEBESTA, BAUEROVÁ, ŠORM and ŠORMOVÁ, 1960; HEYES, 1963). When 8-azaguanine is supplied to growing isolated pea roots the purine analogue is incorporated only into cellular RNA (HEYES, 1963); cell division is inhibited, cell expansion is increased, and protein, nucleic acids and enzyme synthesis decreased. This is similar to the growth stimulating effect of 2-thiouracil on isolated roots (WOODSTOCK and BROWN, 1963). Pollen tube cells do not divide; but both 2-thiouracil and 8-azaguanine stimulate pollen cell elongation (Table 1). The percent increase in walls is much greater in pollen tubes than in root cells. The difference in magnitude of the response in pollen is probably related to the mechanism of cell wall extension in the different plant tissues, rather than a difference in molecular sites of incorporation.

An evaluation of pollen protein changes during germination may suggest other hypotheses to explain why pollen elongation is so markedly affected by certain analogues of nucleotide bases. Studies of gross protein nitrogen changes in pollen have been interpreted as indicating that pollen does not synthesize proteins during growth (POZSAR, 1960). Tube extension accordingly would result from water absorption and mobilization of already existing protein. If this suggestion is valid it would explain why RNA modification by these analogues does not inhibit pollen growth. Since proteins are not being synthesized their functional level would not be affected. However, the decreased glucose oxidation observed in the presence of 2-thiouracil (Table 3) indicates that some protein synthesis occurs during pollen germination, as do results of radioisotope studies with pollen (LINSKENS, 1953; STANLEY, YOUNG and GRAHAM, 1958; TUPÝ, 1964).

Although an explanation of pollen growth response in terms of cell protein synthesis is attractive, we cannot rule out the possibility that 2-thiouracil affects cell growth indirectly. For example, 2-thiouracil might merely free more substrate, or a moiety essential to pollen tube formation. Also, growth responses to the different compounds (Table 1) may be related to their dissociation in the germinating solutions (ŠKODA, 1963). A reciprocal interaction may exist between cell constituents and antimetabolites added. The buffering systems and organic acids of the pollen may affect antimetabolites activity and the added antimetabolite may affect the pH of the germinating media. No attempt was made to evaluate these effects, or to relate the pK's and the effectiveness of the various antimetabolites tested.

Studies utilizing C^{14} labeled thiouracil are now in progress. A future report will include details relating to the site of incorporation of thiouracil in germinating pollen and the effect of thiouracil on pollen tube wall structural patterns. Through studies of the mechanism of pollen tube growth stimulation by 2-thiouracil it is hoped to further understanding of the relationships between nucleic acids, protein, and the formation of plant cell walls.

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