THE EFFECTS OF 8-AZAGUANINE ON THE MITOTIC CYCLE AND CELL GROWTH IN *VICIA FABA* ROOTS

by

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ABSTRACT

An investigation was made of the effects of the purine analogs, 8-azaguanine, 8-azaxanthine, and 6-mercaptopurine, and the purines adenine and guanine, on cell growth, mitosis, and desoxyribonucleic acid (DNA) synthesis in primary roots of Vicia faba seedlings grown in aerated solutions. Root elongation was used as a measure of cell elongation; mitotic frequency was determined in free cell suspensions prepared from 1 mm-long root tips; the relative content of DNA was determined microspectrophotometrically by the two-wavelength method.

It was shown that the balance between root elongation and mitosis in the root tip could be influenced by the amount of aeration and by adenine. Increased aeration stimulated root elongation and depressed mitotic frequency; adenine stimulated mitosis, inhibiting, at the same time, root elongation.

8-azaguanine, in concentrations of 10 p.p.m. and higher, stopped mitosis within 24 hours and greatly reduced root elongation and the fresh and dry weights of roots within 72 hours. This inhibitory effect on both root elongation and mitosis was positively correlated with aeration. 8-azaguanine in a concentration of 1 p.p.m. significantly reduced mitotic frequency but slightly stimulated root elongation. The inhibition of root elongation could be best, though incompletely, reversed by 40 p.p.m. adenine or guanine. The mitotic inhibition could be partially reversed by 40 p.p.m. guanine; adenine, at a concentration of 80 p.p.m., not only completely relieved mitotic inhibition, but

increased the mitotic frequency to a level higher than that of the water controls. Concentrations of 6-mercaptopurine and 8-azaxanthine comparable with those of 8-azaguanine had no inhibitory effects.

Roots treated with 20 p.p.m. 8-azaguanine for 24 hours and then transferred into 80 p.p.m. adenine showed a higher mitotic frequency than the control within 24 hours after transfer. Roots transferred from 8-azaguanine into water showed some mitosis 48 hours after transfer; in this case mitosis was restricted to the partially differentiated and elongated cells of the provascular bundles.

DNA content of interphase nuclei in the controls showed this distribution: a sharp 2C peak (about 65 per cent nuclei), a much lower 4C peak (about 20 per cent nuclei), and intermediates (about fifteen per cent). There were no polyploid nuclei in the apical meristem of the root. The DNA content of chromosomal groups in different mitotic stages demonstrated the accuracy of the two-wavelength method which was used. The DNA content of nuclei in roots treated with 20 p.p.m. 8-azaguanine was distributed in a 2C peak (about 80 per cent nuclei) and a 4C peak (about 20 per cent nuclei). There were no intermediates in treated roots and no nuclei contained a higher amount of DNA than 4C. The percentage of 4C nuclei did not increase with time.

From the evidence that the mitotic inhibition induced by 8-azaguanine could be completely reversed within 24 hours by subsequent treatment with adenine, and from the findings concerning the distribution of DNA in inhibited nuclei, it may be concluded that 8-azaguanine was not

incorporated into DNA. The possibility that 8-azaguanine exerts its inhibitory effects through interference with ATP metabolism is discussed.

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The University of British Columbia

Faculty of Graduate Studies

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DOCTOR OF PHILOSOPHY

of

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B.S.A. University of British Columbia
M.A. University of British Columbia

IN ROOM 33, BIOLOGY BUILDING

MONDAY, DECEMBER 30th, 1957, at 10:00 a.m.

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Plant Physiology I.	D. J. Wort
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INTRODUCTION

A number of analogs of the natural purines, adenine and guanine, have been shown to suppress growth and cell multiplication in a variety of species. The hope that such compounds might provide a basis for practical cancer chemotherapy has led to extensive study of their action as antimetabolites.

Included in this group is the guanine analog, 8-azaguanine. Roblin et al. (1945) reported that this compound acted as a guanine antimetabolite in Escherichia coli. Kidder et al. (1949) obtained growth inhibition in Tetrahymena and an almost complete cessation of growth of adenocarcinoma E 0771 in mice, though the growth of tumor resumed shortly after the treatment was discontinued. During these experiments positive results were obtained also with spontaneous mammary cancer and lymphoid leukemia in mice. However, two years later the same authors (Kidder et al., 1951) reported that 8-azaguanine did not inhibit some other tumors (sarcoma 37 and melanoma S 91). Partial reversal of inhibition in susceptible tumors was produced by guanine. In a series of papers, Matthews (1951, 1952, 1953) reported inhibition of mosaic virus on Nicotiana leaves, the inhibition being reversible by both guanine and adenine. The results of the studies, only a small part of which is mentioned above, were summarized by Gellhorn and Hirschberg (1955) as follows: 8-azaguanine was found to be active against 10 experimental tumors out of 15 tested, and interfered with 23 out of 59 non-tumorous systems (such as viruses, bacteria, Drosophila, and biosynthesis of several organic compounds). Since their review does not include

research which had been done with higher plants, this work will be described here in more detail.

Using very low concentrations of 8-azaguanine (0.1 - 10.0 gamma per liter) de Ropp (1950, 1951) obtained complete or partial growth inhibition in a variety or normal and crown-gall tissues (garden chrysanthemum stem infected with Agrobacterium tumefaciens; bacteria-free crown-gall tissue of sunflower; excised tomato roots; and excised sunflower embryos). The inhibition was not reversed with guanine. On the other hand, the inhibition of virus tumor tissue from Rumex acetosa by concentrations of 8-azaguanine as low as 1 p.p.m. was completely reversed by guanine (Nickell et al., 1950). Fries (1954) noted considerable inhibition of growth of decotylised pea seedlings with 0.1-0.3 micromolar 8-azaguanine; the inhibition could be partially prevented by guanine, adenine and, to a lesser extent, by high concentrations of yeast nucleic acid. The author also observed a decrease of combined nucleic acids with increasing concentrations of the analog. Finally, Pryzina (1956) reported a complete inhibition in root tips of Vicia faba within 24 hours by concentrations of 10 p.p.m. of 8-azaguanine; the mitotic inhibition was not accompanied by an inhibition of root elongation. The mitotic inhibition was best reversed by adenine and guanine, adenine being superior.

While the literature is voluminous it does not provide a clear picture of the mechanism of action of 8-azaguanine. These mechanisms have been suggested: incorporation of the analog into nucleic acids

(Bennett et al., 1950, Parks, 1955, Mandel, 1955, and many others), inhibition of different enzyme systems through interference with ribonucleic acid (Creaser, 1955 a, b), interference with the synthesis of the structural components of nucleic acids (rev. in Skipper, 1953). The susceptibility of animal tumor tissues probably depends on the concentration of guanase (8-azaguanine deaminase) which converts inhibitory 8-azaguanine into inactive 8-azaxanthine. Thus Hirschberg et al. (1952) found very low concentration of guanase in 8-azaguanine-susceptible tumors. Similarly, Hirschberg et al.(1953) found that the analog severely damaged a human brain neoplasm, glioblastoma multiforme, which is devoid of guanase, while the effect on fetal brain tissue containing some guanase was much less severe. Dietrich and Shapiro (1953) could potentiate the carcinostatic action of 8-azaguanine with a riboflavin analog, flavotin; the result was interpreted as an indirect inhibition of guanase through product inhibition (xanthine). Finally, Mandel (1955) demonstrated the enhancement of 8-azaguanine toxicity through the inhibition of guanase by 4-amino-5-imidazolecarboxamide.

The history of studies with 6-mercaptopurine is similar. After a demonstration of inhibition of growth in Lactobacillus casei and of embryogenesis in Rana pipiens, the compound was shown to be inhibitory to mouse sarcoma 180 (Clarke et al., 1952); the authors expressed the opinion that the mechanism of action of this analog "may be related to the mercapto group rather than to the purine moiety, i.e., become involved in sulfhydryl metabolism in some as yet unknown way". The

inhibition of mouse sarcoma 180 was confirmed by Biesele (1955); this author has also demonstrated the prevention of inhibition by very small concentrations of coenzyme A and concluded that the compound might act as an antimetabolite of coenzyme A rather than interfere with nucleic acid metabolism. The review of Gellhorn and Hirschberg (1955) shows that 6-mercaptopurine had been active against 13 tumors out of 15 tested, and interfered with 29 out of 59 non-tumor systems. Very interesting results with this compound have been obtained by Nickell (1955): while 6-mercaptopurine slightly stimulated the growth of Lemna minor in aseptic culture, it strongly inhibited the growth of virus tumor tissue from Rumex acetosa; the results indicated the possibility of different types of metabolism in these two materials.

The present study was undertaken in order to provide further detailed information on the action of these purine antimetabolites at the cell level, specifically the effects of these compounds on cell growth and the mitotic cycle. With respect to the latter it was hoped that the use of cytochemical methods would show whether or not the expected inhibition of cell reproduction by 8-azaguanine and 6-mercaptopurine was dependent only on the suppression of mitosis per se, or whether this effect involves the suppression of interphase processes such as DNA reproduction. Furthermore, it was considered that accurate microspectrophotometric DNA determinations in single nuclei, in conjunction with metabolite-antimetabolite studies would provide the technical means of investigating whether these compounds were incorporated into DNA.

MATERIALS AND METHODS

In this investigation the cytological effects of purines and several purine analogs were studied in roots of Vicia faba seedlings which had been grown in aerated aqueous solutions of these substances. This method of seedling culture had been previously adapted by Setterfield and Duncan (1955) from the similar technique outlined by Brown and Broadbent (1950) and Brown (1951).

Normal, large seeds were selected and soaked in tap water in shallow trays for 48 hours, after which the seed coats were peeled off and the naked seeds held at 20±1°C for four days in flats filled to a depth of 6" with vermiculite. To diminish bacterial and fungal contamination fresh vermiculite was used for each run and flats were steam-sterilized before use. Seedlings with healthy, straight primary roots 5-10 cm long were transferred directly from vermiculite into trays filled with warm tap water at 20°C for washing and further selection. The seedlings were then separated into groups (usually three) according to the length of the root, and the groups subdivided into replicates (in most cases four replicates of six roots each per group), as uniformly as possible, according to the thickness of the roots. In most cases an experiment consisted of six different treatments of twelve roots per treatment. To obtain uniform replicates, it was necessary to germinate 300-400 seeds prior to each run. Each root was marked at a point 30 mm from

the tip with thick India ink, applied with a fine glass rod. This mark served as a reference point in measuring root elongation during subsequent treatment.

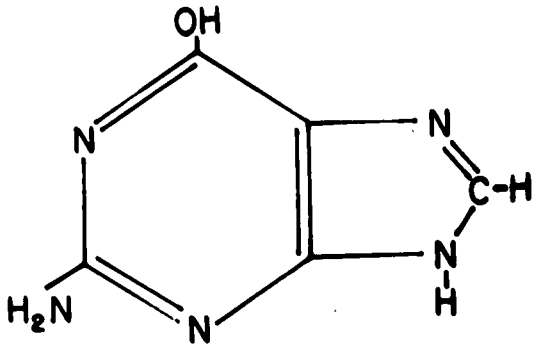
Seedlings with plumule removed were then placed on plastic plates over 1000 ml Pyrex beakers containing the treatment solutions, with roots extending down into the solutions through equally spaced holes in the plates. Aeration of the solutions was provided by a small diaphragm pump which delivered air through rubber tubing to a sintered glass diffusion tube in each beaker. Air flow to various beakers was balanced by means of clamps fitted to the connecting rubber tubing. The experiments were carried out at 20±1°C in diffuse light. Relative humidity was close to 100 per cent.

Broad beans of the variety "Windsor" (crops 1955 and 1956) supplied by the Brackman-Kerr Milling Co., New Westminster, B.C., were used in all experiments. The chemicals, 8-azaguanine, 6-mercaptopurine hydrate, 8-azaxanthine, guanine hydrochloride, and adenine, were obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio. The chemicals were used as supplied, without any further attempt at purification. The structural formulae of the chemicals are indicated in Fig. 1. Distilled and subsequently "demineralized" water of less than 1 p.p.m. ion concentration, obtained by letting distilled water run slowly through Illco-Way Research Model De-ionizer, was used throughout the experiments for preparing of solutions and for rinsing of glassware after washing.

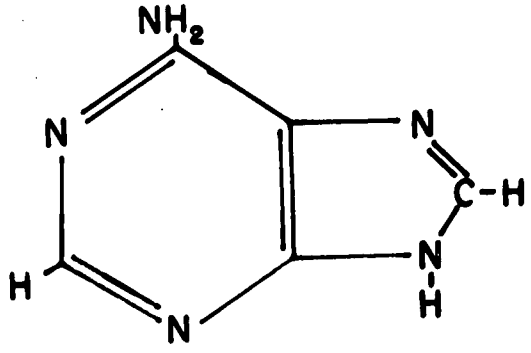
PLATE I

Figure 1. Structural formulae of purines and purine analogs used in this investigation.

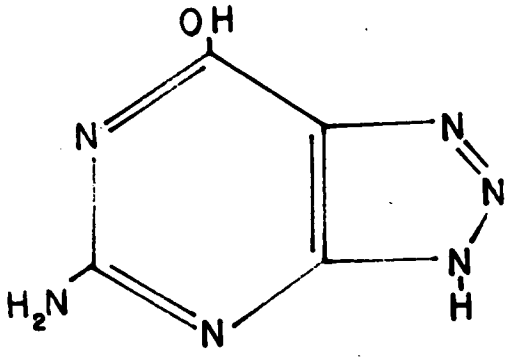
PLATE I



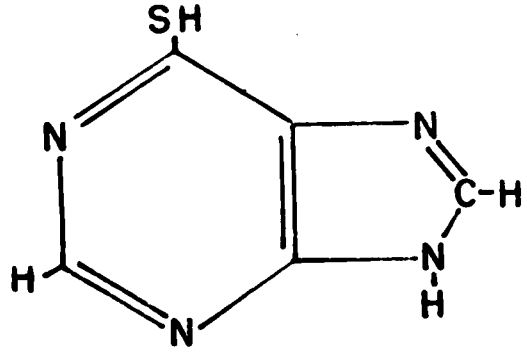
GUANINE



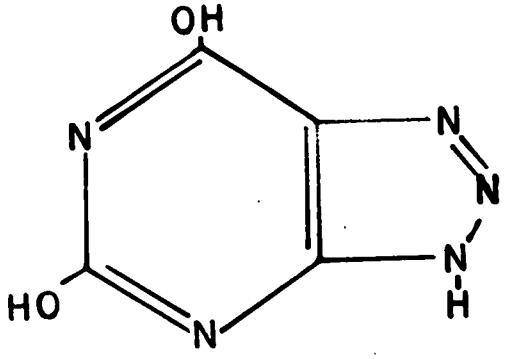
ADENINE



8-AZAGUANINE



6-MERCAPTOPURINE



8-AZAXANTHINE

Chemicals were dissolved in water by heating to less than boiling point directly in beakers; where necessary, compounds were first pulverized in a small porcelain mortar. After cooling the volume of solutions was made up to 1000 ml and the pH adjusted to 6.0±0.1 with dilute HCl and KOH. The pH was measured again at the end of each run. In all experiments, concentrations of chemicals are expressed in parts per million (p.p.m.). Since the molecular weights of adenine, guanine hydrochloride, and 8-azaguanine differ slightly, a correction was necessary in order to provide equivalence in molarity. For this reason concentrations of adenine and guanine hydrochloride were adjusted to those of 8-azaguanine, i.e. where 20 p.p.m. of guanine hydrochloride is indicated, actually 24.66 p.p.m. were administered, and for adenine, 17.76 p.p.m.

All glassware, except volumetric items, was cleaned by the Calgon-metasilicate method of Scherer (1955). Pipettes and graduate cylinders were cleaned in the usual sulphuric acid-potassium dichromate solution, followed by rinsing in running, distilled, and finally "de-ionized" water.

The roots were measured to the nearest millimeter at 24, 48, and 72 hours. Where no roots were collected until the end of the run the growth rate curves were constructed on the basis of the root length (i.e. the distance between the root tip and the mark less 30 mm) at each time. If some roots were collected during the treatment the growth rate curves were calculated from the average growth increments for each 24

hour period. Most of the runs were started with 12 roots per treatment, from which three roots were collected at each time; the average increment was then calculated from 12 roots at 24 hours, from 9 roots at 48 hours, and from 6 roots at 72 hours. If a root had not grown at all it was discarded and not included in calculations; this happened occasionally when the root meristem was broken in measuring.

Where the fresh weights were taken, the roots were rinsed in distilled water and the sections between the mark and the root tip removed. These portions of the roots were then quickly surface dried with filter paper, collected in cooled, stoppered vials, and weighed immediately in the vials. The dry weights were taken after drying to constant weight in an oven at 95°C on previously dried and weighed filter papers.

Mitotic frequency determinations were carried out using the method described by Setterfield et al.(1954). Root tips were fixed in freshly prepared 1:3 acetic alcohol for 15 min., washed in running water for 15 min., hydrolyzed 10 min. in 1 N HCl at 60°C, washed in two changes of distilled water, and stained for 1 hour in leucobasic fuchsin. The material was then washed in three changes of SO_2 water of 10 min. each, and rinsed in distilled water. In a few experiments roots were fixed in 10 per cent neutral formalin; in this case the fixing time was extended to six hours and washing to twelve hours.

Root caps were removed by gently wiping the roots with filter paper and one millimeter-long apical segments were removed from the stained roots using a device specially designed to minimuze errors in cutting.

This consisted of a plastic plate provided with a longitudinal groove with a stop at one end and narrow slits transecting the groove at measured intervals. Roots were placed in the groove, and 1 mm tips excised by slicing with a razor blade in the appropriate slit. The root tips were then held in 1 ml aliquots of filtered 5 per cent pectinase solution (Nutritional Biochemicals Corporation) at pH 4.0 for 6-24 hours, washed in two changes of distilled water of 5 min. each, and shaken vigorously in 1 ml of water in corked vials. To obtain free cell suspensions from root tips fixed in formalin it was necessary to force the tissue fragments several times through the needle of a hypodermic syringe. However, acetic-alcohol fixed root tips disintegrated readily on shaking. The free cells were centrifuged down for 5 min. at 1200 r.p.m. and mounted on slides in a drop of neutral Karo syrup-phosphate buffer mixture. The three roots collected at each time for each treatment were processed together and two slides were prepared as replicates from each sample of cells.

Samples of 1700 - 1900 meristematic cells were scored in random transects of the slide, under high power magnification, in order to determine the proportions of cells in interphase and in different stages of mitosis. In this scoring prometaphase was included in prophase, and very early prophases were considered as interphases. Four samples were counted for each treatment at each time (one in the upper and one in the lower half of each slide), and arithmetic means of the cells in mitosis were expressed as mitotic frequency. All cell types that have been seen to divide commonly in controls have been considered as meristematic. These included, besides isodiametric cells, also partially elongated cells of

the central pith. In some treatments the ratio of prophases: telophases was calculated.

The DNA content was measured in Feulgen stained nuclei. In preparing material, the staining procedure was identical with that described above except that the staining time was extended to 90 min. and the temperatures and time of fixing, washing, hydrolyzing, staining, and washing in $S\theta_2$ water were carefully standardized. Single lots of 1 N HCl, leucobasic fuchsin, and of the stock solution of potassium metabisulfite were used for preparation of all the material used for measurements. The control and treated root tips in each experiment were carried through the various steps in the procedure in the same vial. Each root tip was then placed on a slide in a drop of 45 per cent glacial acetic acid, the root cap removed and the epidermis peeled off under a dissecting microscope. An apical portion of the root 0.2 mm in length was then cut off, squashed, and the coverslip floated off in a Petri plate filled with 45 per cent acetic acid. The time was controlled so that the cells were treated with the acetic acid for a total time of 90 min. (this included dissecting, squashing, and floating off the coverglasses). The slides were then passed through two changes of five min. each, of 70 per cent, 95 per cent, and absolute ethanol, xylene, and toluene, and were finally mounted in Shillaber's immersion oil of $N_D = 1.5150$. Prepared slides were stored in darkness in a refrigerator.

The Feulgen dye content of individual nuclei was measured by the two-wavelength method of Patau (Patau, 1952, Patau and Swift, 1953)

using a microspectrophotometer of the type described by Swift (1950 a, b). The instrument included the following main components: a ribbon-filament (tungsten) light source, a Beckman DU spectrophotometer which isolated essentially monochromatic light (slit width 0.1 mm), a Leitz binocular microscope with Aristophot stand, a 90 X apochromatic oil immersion objective (NA 1.32) and 10 x periplanatic ocular, an I P 21 electron multiplier phototube, a Farrand Type B control unit, and a Rubicon galvanometer. Details of the assembly and alignment of the optical components, as well as a thorough discussion of procedures used in operating and testing the apparatus are given by Swift and Rasch (1956).

The choice of the two wavelengths used in these measurements was made in the usual way, by first obtaining the absorption curve of the central portion of a homogeneous nucleus, and from this selecting two wavelengths (λ_1 and λ_2), such that $E^{*}\lambda_1 = 1/2\ E\lambda_2$. On this basis wavelengths 490 millimicrons (λ_1) and 511 millimicrons (λ_2) were chosen and used throughout these experiments.

The following procedure was followed in measuring individual nuclei: the image of the nucleus was centered in the aperture of an iris diaphragm located immediately beneath the photocell and the diaphragm stopped down to a diameter slightly greater than that of the nuclear image. At each wavelength the intensity of light passing through this aperture to the photocell was measured both with the nucleus in position (I_n) and through a blank area on the slide (I_o). Transmission (T) is

* E = extinction.

given by the ratio I_n/I_o. This ratio was determined twice by replicate measurements, yielding an average value of T at each wavelength (T λ_1 and T λ_2).

The relative dye content within the measured area A is given by $M = L_1 D c^2$, where $L_1 = 1 - T\,\lambda_1$, c is the diameter of A, and $D = \frac{1}{2 - L_2/L_1} I_n \frac{1}{L_2/L_1 - 1}$. The value D is a correction factor for inhomogeneity of dye distribution within A. This correction is based on the fact that inhomogeneity increases T to a proportionally greater extent at λ_2 than at λ_1. The ratio $L_2/L_1 = \frac{1 - T\,\lambda_2}{1 - T\,\lambda_1}$, which is the variable in the calculation of D, therefore decreases with decreasing homogeneity. Convenient tables of D prepared by Swift and Rasch (1956) were used in calculations.

All measurements were carried out with microscope condenser stopped down to a numerical aperture of approximately 0.4, and with the monochromater slit width of 0.1 mm. Where frequency distributions of DNA per nucleus were sought, nuclei were measured at random in several fields on the slide. These areas were mapped and the nuclei numbered so that unintentional duplications of measurements of individual nuclei could be avoided and, where necessary, nuclei could be remeasured.

RESULTS

About one quarter of the results reported here have been obtained in runs under high aeration. After it had been realized that high aeration, besides stimulating root elongation (Fig. 14), considerably depresses mitosis (Fig. 15) and thus renders data on mitotic frequencies less suitable for expressing differences due to treatments, the rest of the runs have been done under medium aeration. The average air flow has been measured with a "flowrator" tube (Fischer and Porter) and gave these values: high aeration - cca 0.0070 CFM (cubic feet per minute) per beaker; medium aeration - cca 0.0035 CFM per beaker. Results presented without a note have been obtained under medium aeration. A note about pH has been made only where final value deviated from the original pH 6.0 ± 0.1 by more than 0.2.

a. Preliminary experiments:

In preliminary experiments an investigation was made of the effects on root elongation of seed size, presence of the plumule, and degree of aeration of water (used later as control). An examination was also made of the effect of fixatives on mitotic scoring, and of the effects of washing during preparation of roots on mitotic frequency and the prophase: telophase ratio.

Concerning the first three preliminary experiments, only a short summary is reported here. A small but noticeably greater rate of root elongation was observed in the roots of seedlings grown from relatively small seeds. However, these roots were also much thinner, provided smaller amounts of tissue per unit root length for the determination of fresh and dry weight and for mitotic counts, and thus were less satisfactory. Increased air flow through the culture beakers stimulated root elongation, though when the rate of aeration reached the point where gas bubbles settled on the roots growth declined sharply. The presence of the plumule slightly stimulated root elongation but the difference was very small.

TABLE I

Effect of fixative on scoring of mitotic frequency in root tips.

Roots were collected directly from vermiculite. Three root tips from each treatment were processed together and from the mixture of cells two slides per treatment were prepared.

		Number of cells							Per cent Mitosis (±S.E.)
Treatment	Slide	Pro	Meta	Ana	Telo	Total mitosis	Inter-phases	Grand total	
Acetic-alcohol fixation	0a/1a	106	34	17	37	194	1,621	1,815	10.68
	0a/2a	103	29	22	35	189	1,632	1,821	10.37
	0a/1b	101	21	14	40	176	1,647	1,823	9.65
	0a/2b	115	20	13	33	181	1,648	1,829	9.89
		425	104	66	145	740	6,548	7,288	10.15 ± .23
formalin fixation	0b/1a	125	24	5	52	206	1,624	1,830	11.25
	0b/2a	106	19	6	56	187	1,626	1,813	10.31
	0b/1b	111	19	6	48	184	1,664	1,848	9.95
	0b/2b	132	21	6	38	197	1,680	1,877	10.49
		474	83	23	194	774	6,594	7,368	10.50 ± .27

Table I is included to illustrate in detail the technique of mitotic frequency scoring. While the difference between mitotic frequencies of the two samples is not statistically significant (difference - .35 per cent, t calc. - .97, t at.05 - 2.45), the difference between the average number of anaphases per count is statistically highly significant (fixed in acetic alcohol - 16.50±2.02, fixed in formalin - 5.75±.25, difference - 10.75, t calc. - 5.29, t at .01 - 3.71).* The nuclei and chromosomes treated with acetic-alcohol appeared larger, swollen, while those from formalin were more compact and more densely stained. The difference in average number of anaphases per count was obviously due to the fact that a large number of anaphases with contracted and shrunken arms due to formalin fixation were counted as telophases. Since it was much more difficult to prepare cell suspensions from formalin-fixed root tips it was decided to use acetic alcohol fixative in all subsequent runs. The prophase:telophase ratio for root tips fixed in acetic alcohol was 2.93.

TABLE II

Effect of preparatory washing and handling of roots on mitotic frequency and the prophase:telophase ratio.

Unwashed roots collected directly from vermiculite; washing and handling lasted for approximately two hours. The t test was used for estimation of significance. Mitotic frequencies are means of four counts as shown in Table I.

Treatment Run	Mitotic frequency (%) ± S.E. Unwashed	Washed	Difference (%)
18/57	11.17± .16	9.21± .45	1.96**
20/57	12.59± .27	11.18± .12	1.41*
Prophase:Telophase ratio	2.82	2.23	

* Statistical methods and values of t from Goulden (1953)

During the preparatory washing and handling of roots preceding fixation the mitotic frequency in the root tips decreased. The decrease was highly significant in the first run and significant in the second run. The decrease in mitotic frequency was accompanied by a decrease in the prophase:telophase ratio. The significance of the changes in prophase: telophase ratio is considered in the discussion.

b. Effects of purines and purine analogs on root elongation, mitosis, and fresh and dry weight of roots.

Responses of root elongation to various concentrations of 8-azaguanine are represented graphically in Figs. 2 and 4. Low concentrations (up to 1.0 p.p.m.) consistently gave a small stimulation which increased with concentration (Fig. 4). Even the stimulation with 1.0 p.p.m. was not statistically significant: the analysis of variance gave these results: F value for treatment = 1.98, F at .05 = 4.60. Within the range (5-40 p.p.m.) the effect was reversed - inhibition was increased with concentration (Fig. 2). The inhibition by 80 p.p.m. of 8-azaguanine (not shown in Fig. 2) was slightly greater than that by 40 p.p.m. However, this result was unreliable since the chemical tended in some runs to precipitate in this highest concentration. The growth curves of roots inhibited by 8-azaguanine showed a typical levelling off with time: for example the total elongation of roots treated with 20 p.p.m. 8-azaguanine was 77.6, 56.2, and 44.7 per cent of that of control at 24, 48, and 72 hours respectively, or, expressed in another way, the average consecutive daily increments of roots treated with

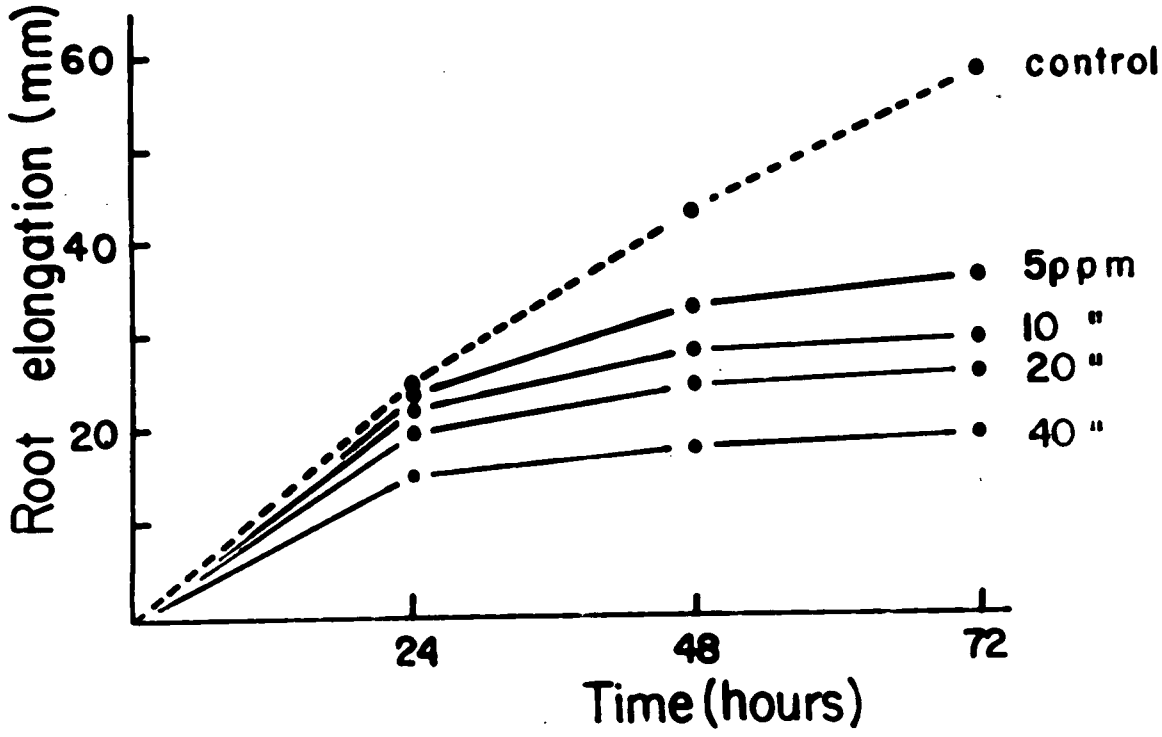
PLATE II

Figure 2. Inhibition of elongation of Vicia faba roots by "high" concentrations of 8-azaguanine. The concentrations are expressed as parts per million (p.p.m.). Values in the figure represent means of following number of runs of 12 roots each: control - 8, 5 p.p.m. - 4, 10 p.p.m. - 7, 20 p.p.m. - 6, 40 p.p.m. - 3. Growth of treated roots was calculated as per cent of control in each run, and the individual controls adjusted to the mean of all the controls.*

Figure 3. Effects of "high" concentrations of 8-azaguanine on mitosis in root tips. In each run four separate scorings of 1,700 - 1,900 meristematic cells were carried out for each concentration at each time. Presented values are means of following number of runs: control - 4, 5, 10, and 40 p.p.m. 8-azaguanine - 1, 20 p.p.m. 8-azaguanine - 4. Mitotic rates in treated roots were calculated as per cent of control and the means adjusted to the average control.*

* Methods described for Figs. 2 and 3 were used in all subsequent figures showing root elongation and mitotic frequencies. All experiments were caried out on primary roots of Vicia faba.

PLATE II


Root elongation (mm)
60
40
20
control
5ppm
10 "
20 "
40 "
24
48
72
Time (hours)

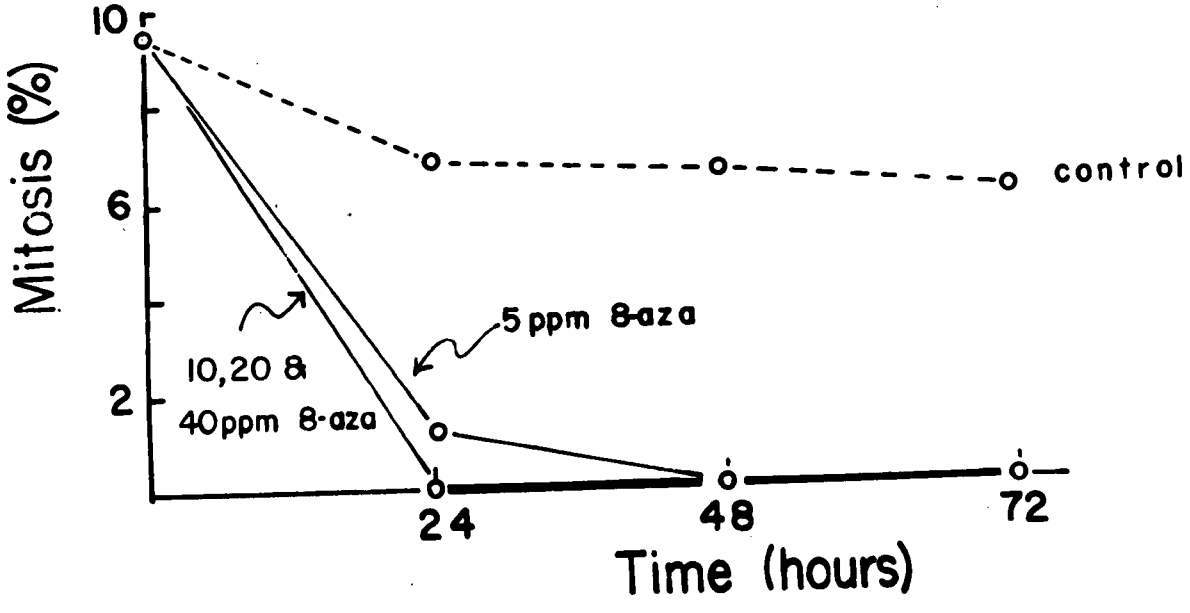
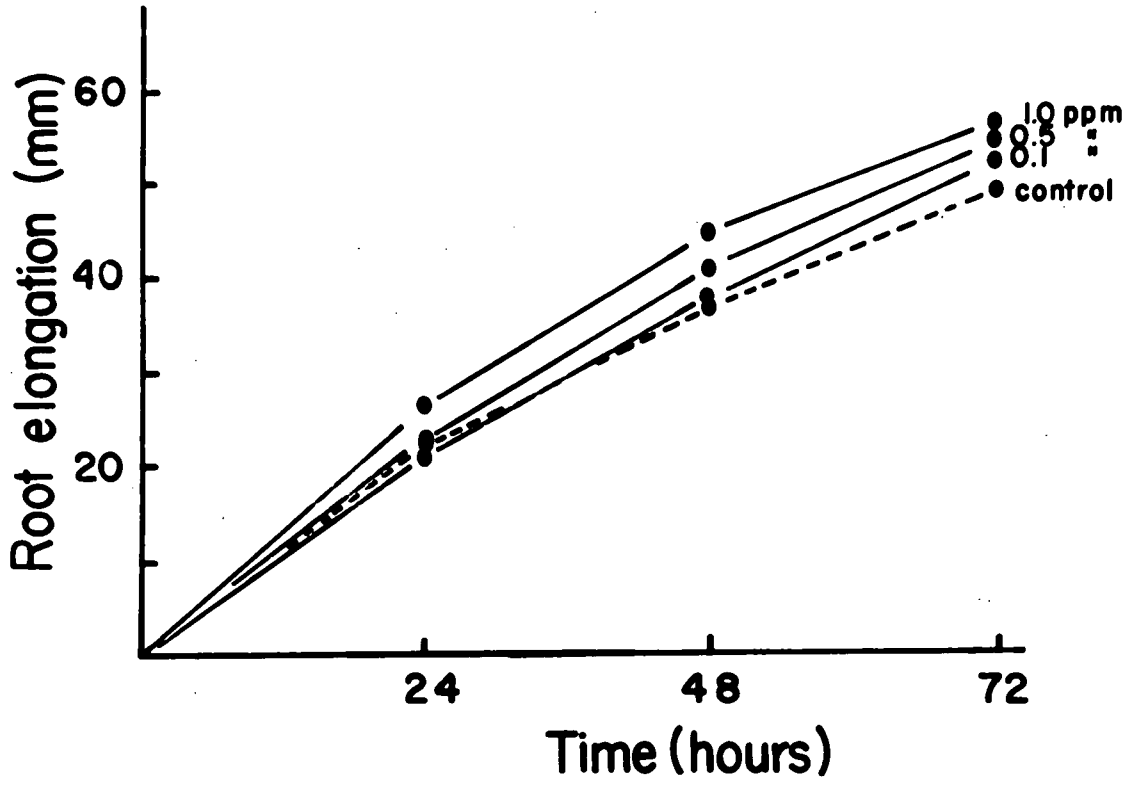

Mitosis (%)
10
6
2
control
5 ppm 8-aza
10,20 &
40ppm 8-aza
24
48
72
Time (hours)

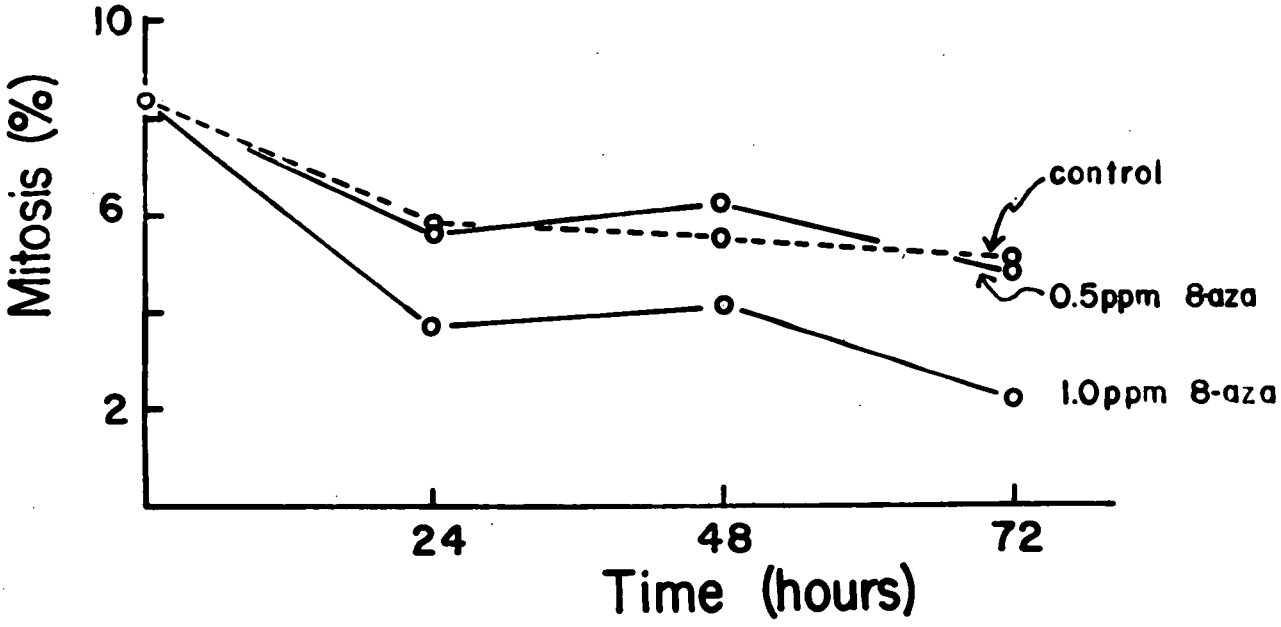
PLATE III

Figure 4. Effect of "low" concentrations of 8-azaguanine on root elongation. Presented values are means of following number of runs: control - 4, 0.1 p.p.m. - 1, 0.5 p.p.m. - 3, 1.0 p.p.m. - 2.

Figure 5. Effect of "low" concentrations of 8-azaguanine on mitosis in root tips. Presented values are means of following number of runs: control - 3, 0.5 p.p.m. - 2, 1.0 p.p.m. - 1.

PLATE III


Root elongation (mm)
60
40
20
1.0 ppm
0.5 "
0.1 "
control
24
48
72
Time (hours)


Mitosis (%)
10
6
2
control
0.5ppm 8aza
1.0ppm 8-aza
24
48
72
Time (hours)

20 p.p.m. 8-azaguanine were 19.2, 4.9, and 1.5 mm while those of control were 24.8, 18.2, and 14.3 mm.

The effects of 8-azaguanine on mitotic frequency in the root meristem are shown in Figs. 3 and 5. Concentrations of 10-80 p.p.m. caused a complete cessation of mitosis within 24 hours while with the concentration of 5 p.p.m. the mitotic rate declined to about 1 per cent within 24 hours and ceased within 48 hours (Fig. 3). In 8-azaguanine-inhibited root tips, about 5 - 10 per cent of nuclei had the appearance of very early prophases. These "pseudo-prophases" (term used by Firket, 1957) are larger than interphase nuclei, show some condensation of chromosomes and very large nucleoli with diffused borders as compared with smaller, sharply delineated nucleoli in normal interphasic nuclei. A concentration of 0.5 p.p.m. had practically no effect on mitotic rate; a concentration of 1.0 p.p.m. lowered mitotic frequency to about one half of that of control (Fig. 5).

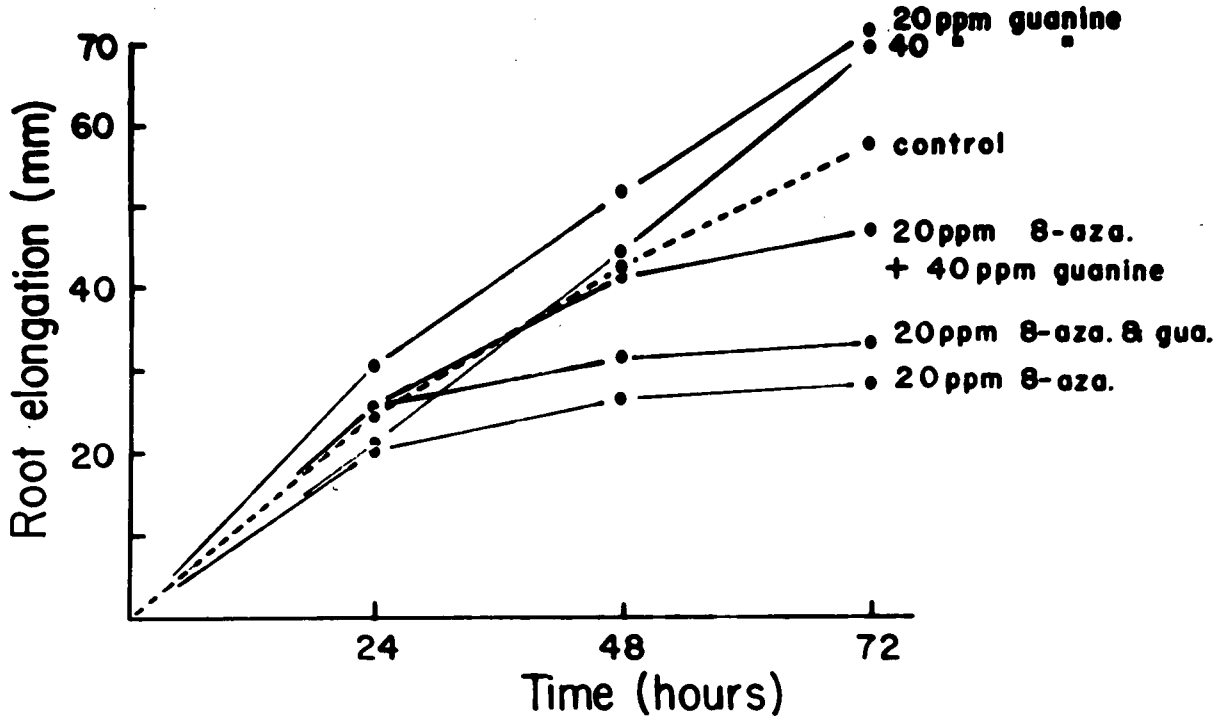
The observed inhibitory effects of 8-azaguanine at rather low concentrations might be interpreted on the basis of the concept that this compound interfered with purine metabolism. If this was the case, it might be possible to reverse this inhibition by supplying the natural purine metabolites - guanine and adenine - to the roots. The effects of guanine alone and guanine in combination with 20 p.p.m. of 8-azaguanine on root elongation and mitosis are represented in Figs. 6 and 7. Guanine alone slightly stimulated both root elongation and mitosis. Best but not complete reversal of the inhibition of root elong-

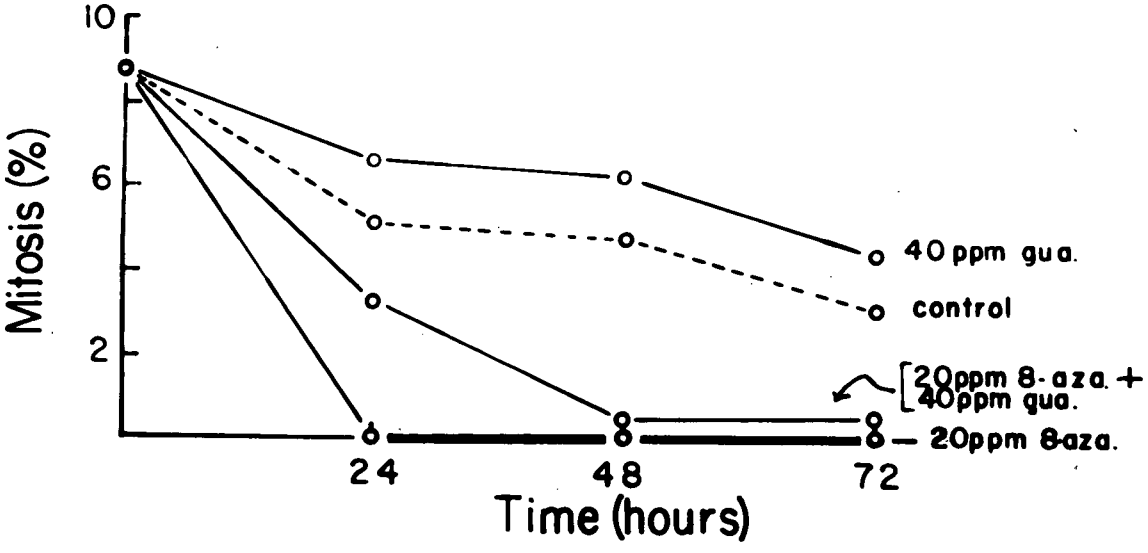
PLATE IV

Figure 6. Effects of guanine alone and guanine in combination with 20 p.p.m. 8-azaguanine on root elongation. Concentrations of guanine-HCl are equimolar with the concentrations of 8-azaguanine. Presented values are means of following number of runs: control - 4, 20 8-azaguanine - 4, 20 guanine HCl - 1, 40 guanine HCl - 1, 20 8-azaguanine plus 20 guanine HCl - 2, 20 8-azaguanine plus 40 guanine HCl - 4. All concentrations in p.p.m.; high aeration.

Figure 7. Effects of guanine alone and guanine in combination with 20 p.p.m. 8-azaguanine on mitosis in root tips. Presented values are means of following number of runs: control, 20 8-azaguanine, and 20 8-azaguanine plus 40 guanine HCl - 3 each, 40 guanine HCl - 1. All concentrations in p.p.m.; guanine equimolar; high aeration.

PLATE IV


Root elongation (mm)
70
60
40
20
24
48
72
Time (hours)
20ppm guanine
40 "
control
20ppm 8-aza.
+ 40ppm guanine
20ppm 8-aza. & gua.
20ppm 8-aza.


Mitosis (%)
10
6
2
24
48
72
Time (hours)
40ppm gua.
control
20ppm 8-aza. +
40ppm gua.
20ppm 8-aza.

ation by 20 p.p.m. 8-azaguanine was achieved with 40 p.p.m. of guanine hydrochloride; mitotic inhibition was relieved effectively at 24 hours only, while later mitotic rates sank to negligible levels. It was not possible to carry out reversal experiments with higher concentrations of guanine because of precipitation.

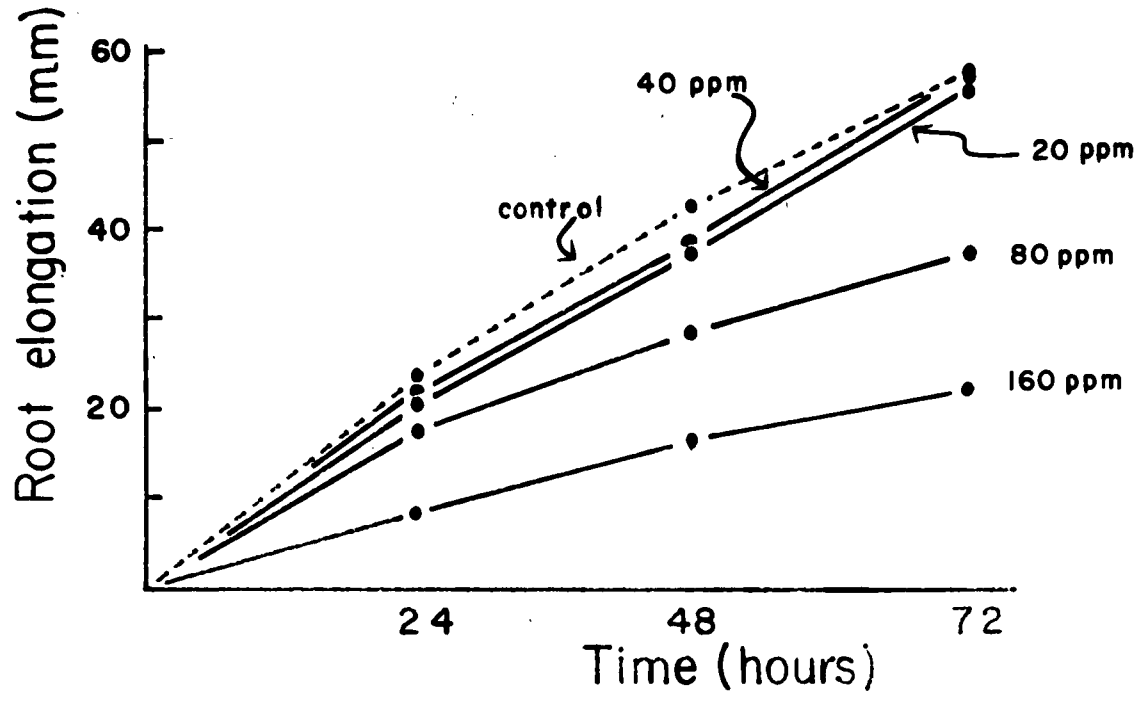
Adenine alone inhibits root elongation, the inhibition being correlated with the concentration (Fig. 8). High concentrations of adenine depressed root elongation considerably but the growth curves did not level off more than did those of the control (e.g. 160 p.p.m. of adenine reduced root elongation to 37.1, 39.6, and 38.1 per cent of control at 24, 48, and 72 hours, respectively). The highest concentrations of adenine produced swellings located about 3 to 10 mm from the root tips, and completely depressed the development of secondary roots that normally appeared at the end of runs in controls. The mitotic rates in root tips treated with adenine increased with the dosage (Fig. 9) though the effect on root elongation was in the opposite direction. From data showing the effect of adenine when applied in combination with 20 p.p.m. of 8-azaguanine, it is evident that best reversal of inhibition of root elongation was obtained with 40 p.p.m. adenine (Fig. 10). The growth curves obtained with 20 p.p.m. 8-azaguanine combined with high concentrations of adenine (80, 160, and 320 p.p.m.) closely resemble the growth curves for adenine alone and show no inhibitory effect of 8-azaguanine. The growth curve for 20 p.p.m. 8-azaguanine with 20 p.p.m. adenine levels off after 24 hours, that for 20 p.p.m. 8-azaguanine with 40 p.p.m. adenine after 48 hours. The mitotic frequency for 20 p.p.m.

PLATE V

Figure 8. Effect of adenine on root elongation. Concentrations of adenine are equimolar with those of 8-azaguanine. Presented values are means of following number of runs: control - 6, 20 adenine - 1, 40 adenine - 2, 80 adenine - 3, 160 adenine - 1. Concentrations in p.p.m.

Figure 9. Effect of adenine on mitosis in root tips. Presented values are means of following number of runs: control - 2, 80 p.p.m. adenine - 1, 160 p.p.m. adenine - 1. Concentrations of adenine are equimolar with the concentrations of 8-azaguanine.

PLATE V


Root elongation (mm)
60
40
20
40 ppm
20 ppm
control
80 ppm
160 ppm
24
48
72
Time (hours)

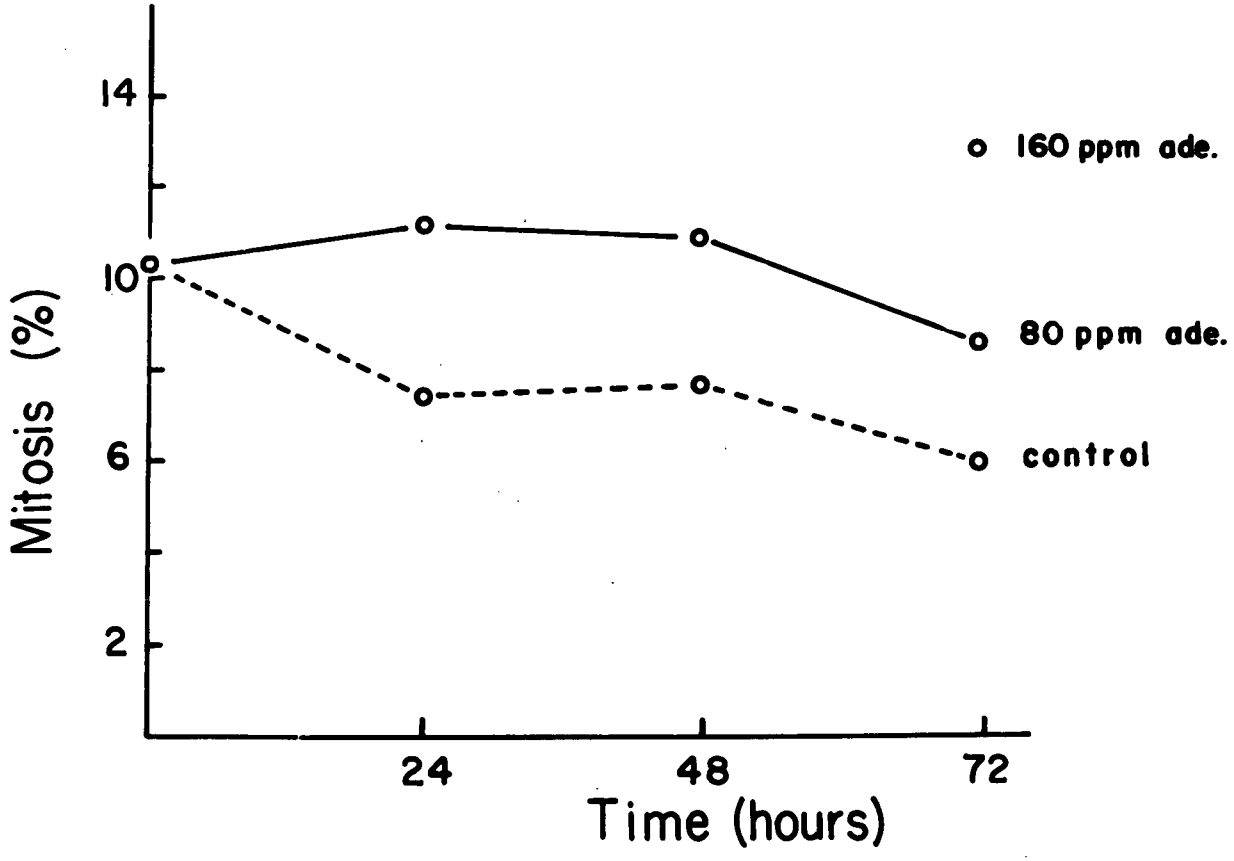
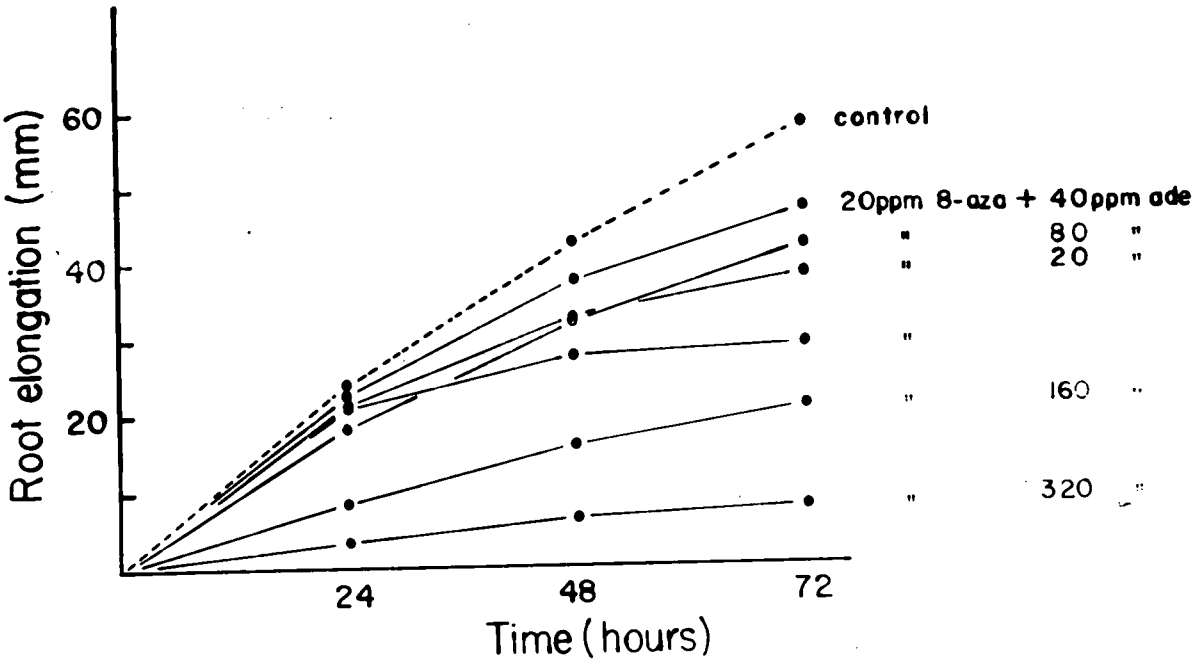

Mitosis (%)
14
10
6
2
160 ppm ade.
80 ppm ade.
control
24
48
72
Time (hours)

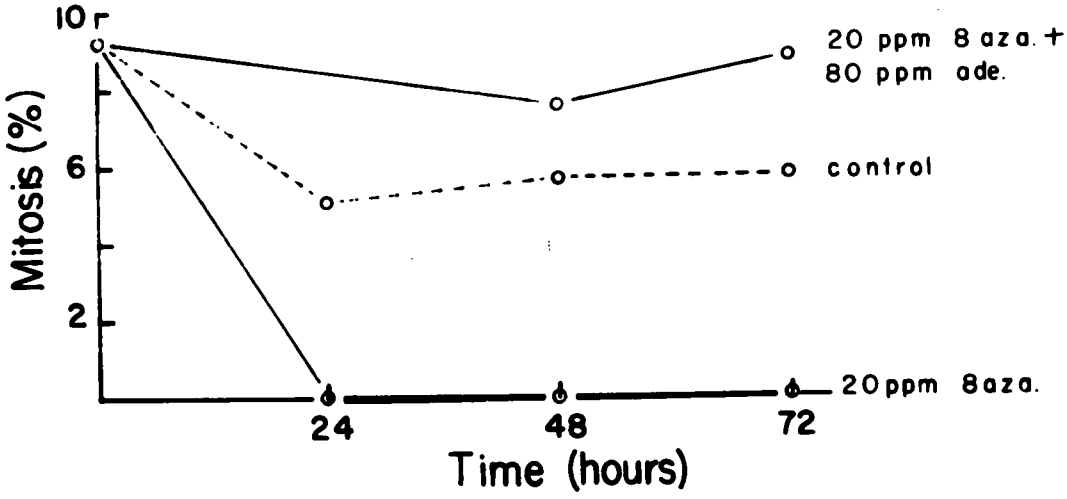
PLATE VI

Figure 10. Effects of adenine in combination with 20 p.p.m. 8-azaguanine on root elongation. Presented values are means of following number of runs: control - 6, 20 8-azaguanine - 6, 20 8-azaguanine plus 20 adenine - 3, 20 8-azaguanine plus 40 adenine - 2, 20 8-azaguanine plus 80 adenine - 4, 20 8-azaguanine plus 160 adenine - 2, 20 8-azaguanine plus 320 adenine - 1. Concentrations in p.p.m.; concentrations of adenine are equimolar with concentrations of 8-azaguanine.

Figure 11. Effect of 80 p.p.m. adenine in combination with 20 p.p.m. 8-azaguanine on mitosis in root tips. Presented values are means of 1 run per treatment.

PLATE VI


Root elongation (mm)
60
40
20
24
48
72
Time (hours)
control
20ppm 8-aza + 40ppm ade
80
20
160
320


Mitosis (%)
10
6
2
24
48
72
Time (hours)
20 ppm 8 aza. +
80 ppm ade.
control
20ppm 8aza.

8-azaguanine with 80 p.p.m. adenine was stimulated to about the same level (Fig. 11) as in treatment with 80 p.p.m. adenine alone (Fig. 9).

The preceding experiments have shown that complete inhibition of mitosis was accomplished at 24 hours, using 8-azaguanine in concentrations exceeding 5 p.p.m. The rate of descent of the mitotic rate within the first 24 hours of treatment with 20 p.p.m. of the compound was investigated in the three experiments represented in Fig. 12. It is indicated that the onset of inhibition occurred after eight hours, with the mitotic rate declining to nearly zero after 14 hours. The decrease in mitotic frequencies was accompanied by a simultaneous drop in the prophase:telophase ratio. These were the values of the ratio: 0 time - 2.83, 2 hours - 3.43, 4 hours - 2.48, 6 hours - 4.24, 8 hours - 3.80, 10 hours - 1.79, 12 hours - 1.53, 14 hours - .75, 16 hours - .40, 18 hours - .25.

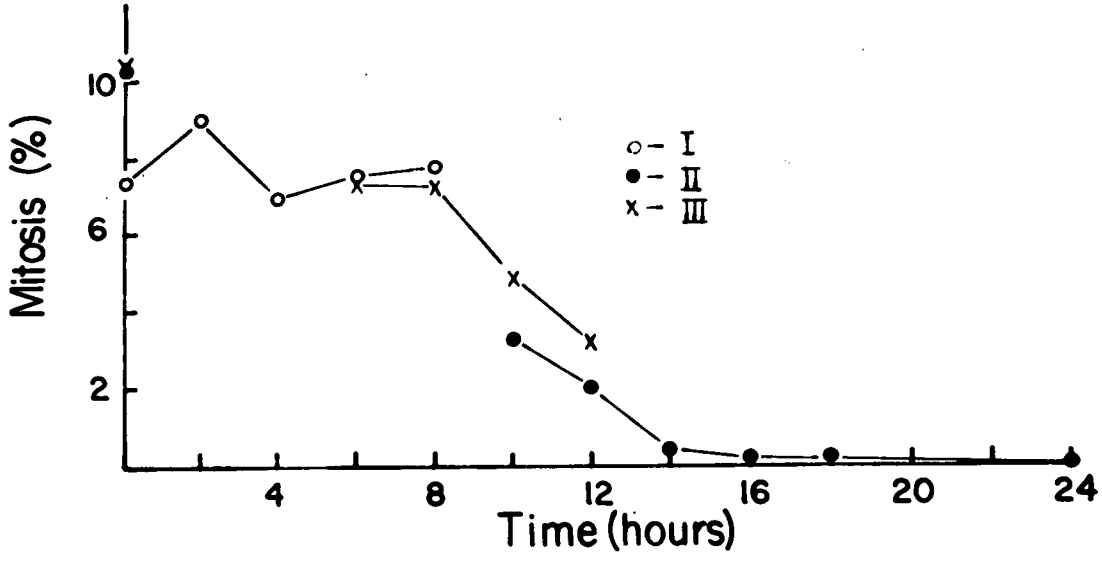
The effects of 8-azaguanine on fresh and dry weight during a treatment period of 72 hours is shown in Fig. 13. Concentrations of 10 p.p.m. and 80 p.p.m. reduced the fresh weight to 69.06 per cent and 61.70 per cent of that of the control; dry weight was reduced to 71.26 per cent and 66.13 per cent, respectively. These data were calculated from total weights of the roots to the mark; if increments only had been considered (i.e. final weight less original weight) the relative differences between treatments would be greater. The main point of interest in the results of the two experiments is that dry weight as well as fresh weight decreased with increasing dosage. This shows that

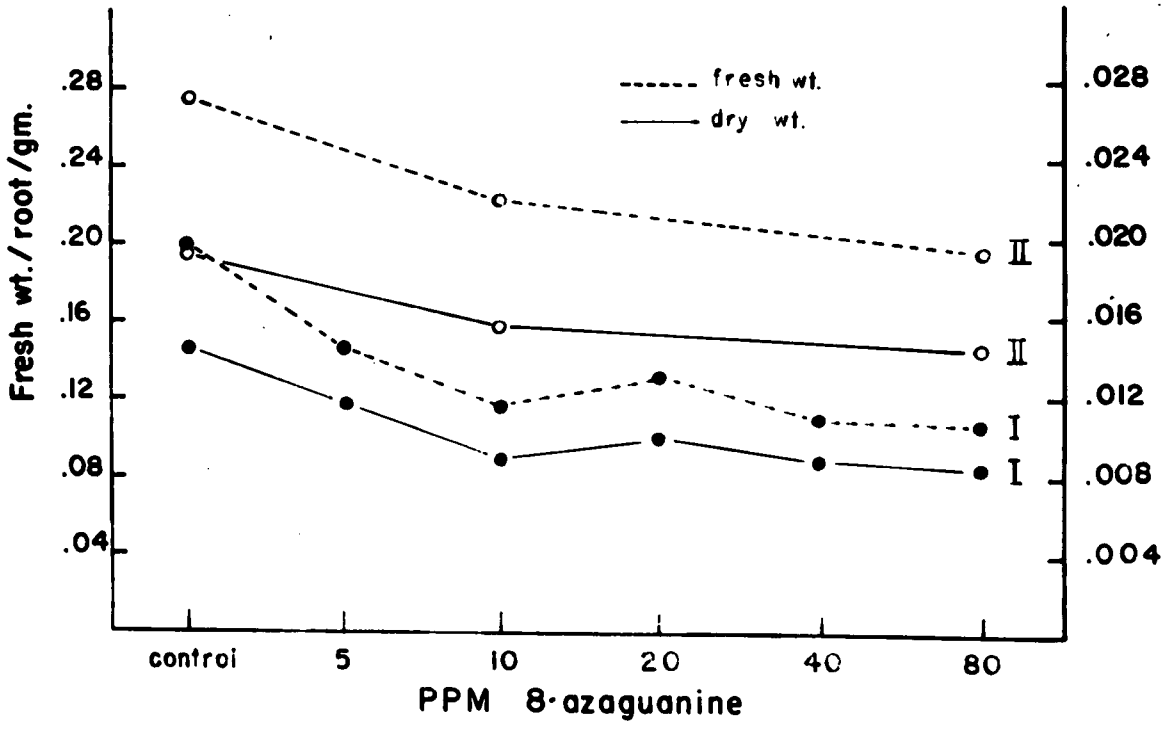
PLATE VII

Figure 12. Time effect of 20 p.p.m. 8-azaguanine on mitosis in root tips. Three different runs; mitotic frequencies were calculated from four repeated counts at each time on material prepared from two root tips each.

Figure 13. Effects of 8-azaguanine on fresh and dry weights of roots. Treatments lasted for 72 hours. Run I represents 12, run II 24 roots per treatment. Seeds from crop 1955 were used in run I, seeds from crop 1956 in run II. In run II the average fresh weight was .0965 gm, the average dry weight .0054 gm per root at 0 time in 30 mm long sections of roots.

PLATE VII


Mitosis (%)
10
6
2
o – I
● – II
x – III
4
8
12
16
20
24
Time (hours)


Fresh wt./root/gm.
.28
.24
.20
.16
.12
.08
.04
------ fresh wt.
——— dry wt.
Dry wt./root/gm.
.028
.024
.020
.016
.012
.008
.004
II
II
I
I
control
5
10
20
40
80
PPM 8-azaguanine

the inhibition of root elongation by this compound is accompanied by a reduction in dry matter synthesis and is, therefore, not principally dependent on an induced reduction in water content.

TABLE III

Effect of 6-mercaptopurine on root elongation.

The results are an average of three runs for the control, 20, and 160 p.p.m., and of two runs for the other concentrations. High aeration.

Concentration	Elongation as per cent of control		
	24 hours	48 hours	72 hours
10 p.p.m.	107.1	104.3	107.2
20 p.p.m.	105.5	99.2	98.4
40 p.p.m.	103.2	94.6	90.6
80 p.p.m.	97.3	95.4	95.6
160 p.p.m.	109.6	105.2	100.6

TABLE IV

Effect of 6-mercaptopurine on mitosis in root tips after 72 hours.

Presented data are means of four different counts of 1,700 - 1,900 meristematic cells each. Mitotic frequency at 0 time was 7.57± .33 per cent. High aeration.

Treatment	Control	10	20	40	80	160 p.p.m.
Mitotic frequency	2.00	1.91	1.87	2.06	2.11	1.11
Standard Error	±.21	±.15	±.12	±.17	±.35	±.08

TABLE V

Effect of 8-azaxanthine on root elongation.

The presented data are an average of two runs for the control, 20, and 160 p.p.m., and of 1 run for the other concentrations. High aeration.

Concentration	Elongation as per cent of control 24 hours	48 hours	72 hours
20 p.p.m.	103.5	102.3	104.8
40 "	85.4	85.5	87.5
80 "	96.6	94.7	91.0
160 "	101.00	100.7	106.0
320 "	87.0	88.1	92.4

The effects of two other purine analogs, 6-mercaptopurine and 8-azaxanthine on root elongation (both analogs) and mitosis (6-mercaptopurine only) are given in Tables III, IV and V. The former compound had no apparent effect on root elongation at any of the concentrations tested, and gave only partial inhibition of mitosis at the highest dosage (160 p.p.m.). Treatments with 8-azaxanthine had no marked effect on root elongation; the apparent slight depression at 40, 80, and 320 p.p.m. may have resulted from chance variation and from differences in aeration between beakers in the single experiment in which these three concentrations were tested.

c. Effect of aeration on root elongation and mitosis.

The preliminary experiments have shown that within a considerable range increased air flow stimulated root elongation. But it became apparent that high rates of aeration resulted, at the same time, in a progressive decrease in mitotic rates in the controls during the

incubation period (72 hours). This drop in mitotic rate was not observed in similar experiments reported by Setterfield and Duncan (1955). Therefore, experiments were undertaken in order to clarify the responses of root elongation and mitosis to the rate of aeration, the results of which are plotted in Figs. 14 and 15. The rates of aeration had opposite effects on mitosis and root elongation, i.e. increased aeration depressed the mitotic rate and stimulated elongation; the converse was true for no aeration. The pH of non-aerated water decreased during the runs from the original 6.0 to about 5.5; this change in pH is too small to account alone for any of the effects reported above.

d. Effect of aeration on the inhibition of elongation and mitosis in roots treated with 8-azaguanine.

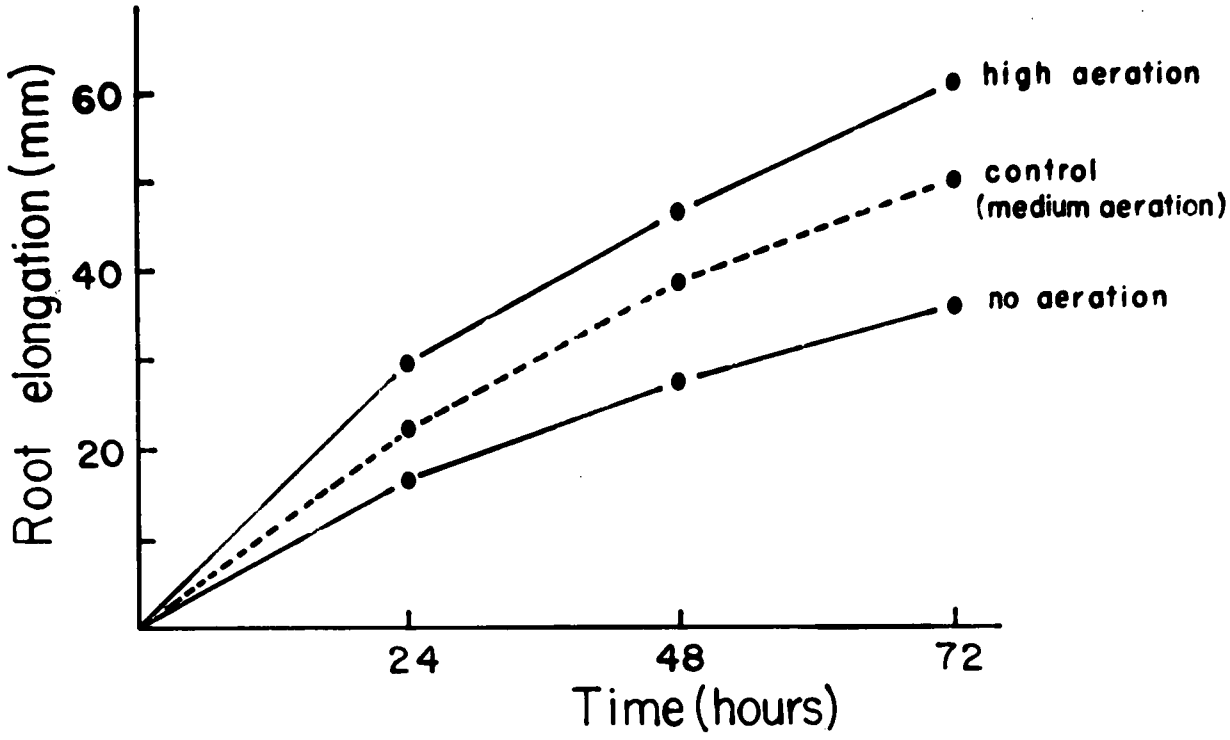
The demonstration that rate of aeration profoundly influences both root elongation and mitotic activity suggested that aeration might also influence the degree of effect of exogenous purines and their analogs on the two aspects of root growth. This possibility was tested in experiments in which 8-azaguanine was administered at different rates of air-flow. The results are plotted Figs. 16, 17, 18, and 19. In agreement with the earlier experiments control roots elongated more rapidly at the relatively higher rate of aeration (cf. Figs. 16, 17, and 18). Moreover the degree of inhibition of root elongation at both concentrations (20 and 80 p.p.m.) of 8-azaguanine increased with the rate of air flow (cf. Figs. 16, 17, and 18). Similarly, 8-azaguanine inhibited mitosis more effectively in aerated solutions: while the

PLATE VIII

Figure 14. Effect of aeration on root elongation. Presented values are means of following number of runs: control (medium aeration) - 5, high aeration - 3, no aeration - 5.

Figure 15. Effect of aeration on mitosis in root tips. Presented values are means of following number of runs: control (medium aeration) - 5, high aeration - 3, no aeration - 5.

PLATE VIII


Root elongation (mm)
60
40
20
high aeration
control
(medium aeration)
no aeration
24
48
72
Time (hours)

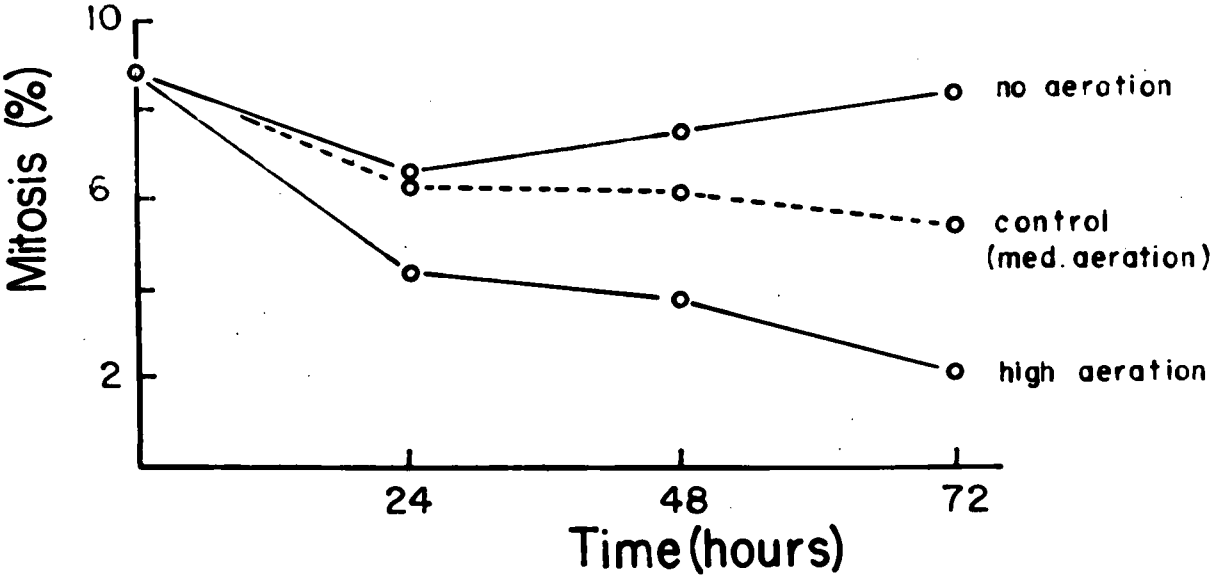
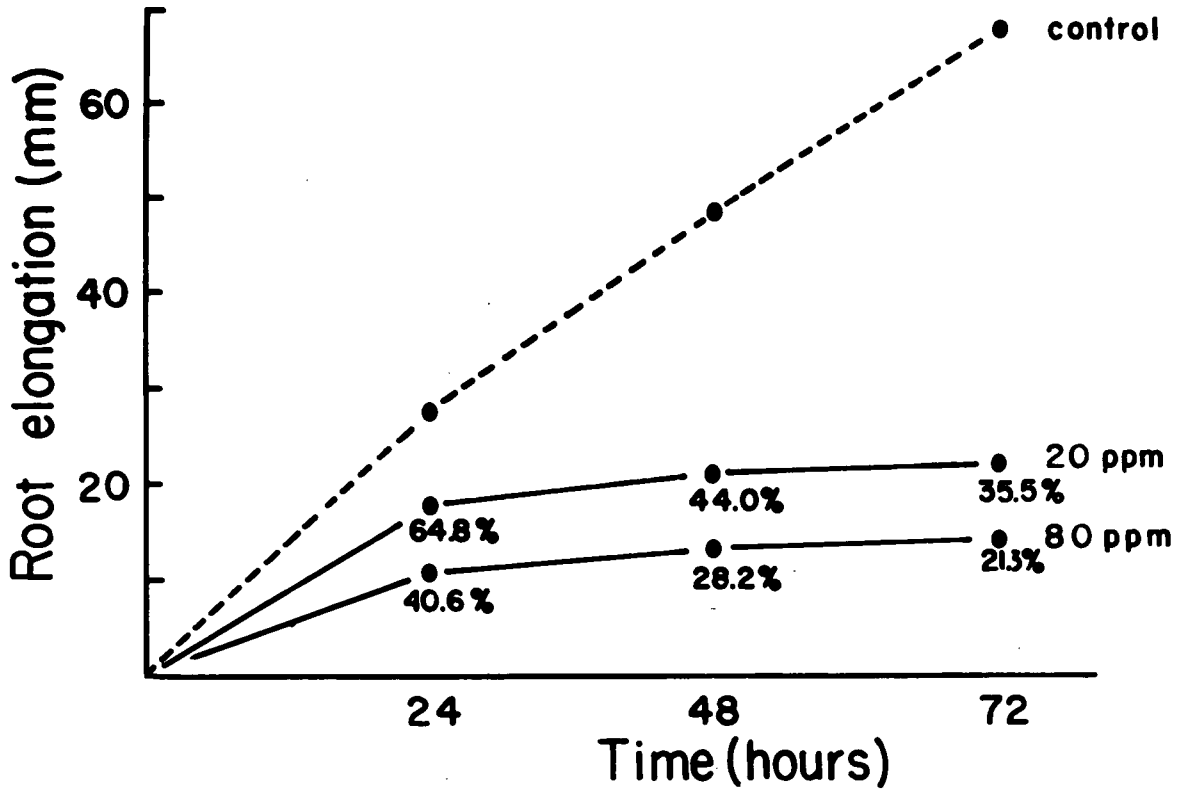

Mitosis (%)
10
6
2
no aeration
control
(med. aeration)
high aeration
24
48
72
Time (hours)

PLATE IX

Figure 16. Effect of high aeration on inhibition of root elongation by 20 and 80 p.p.m. 8-azaguanine. Presented values are means of 1 run (12 roots) per treatment.

Figure 17. Effect of medium aeration on inhibition of root elongation by 20 and 80 p.p.m. 8-azaguanine. Presented values are means of 1 run (12 roots) per treatment.

PLATE IX


Root elongation (mm)
60
40
20
control
20 ppm
80 ppm
64.8%
44.0%
35.5%
40.6%
28.2%
21.3%
24
48
72
Time (hours)

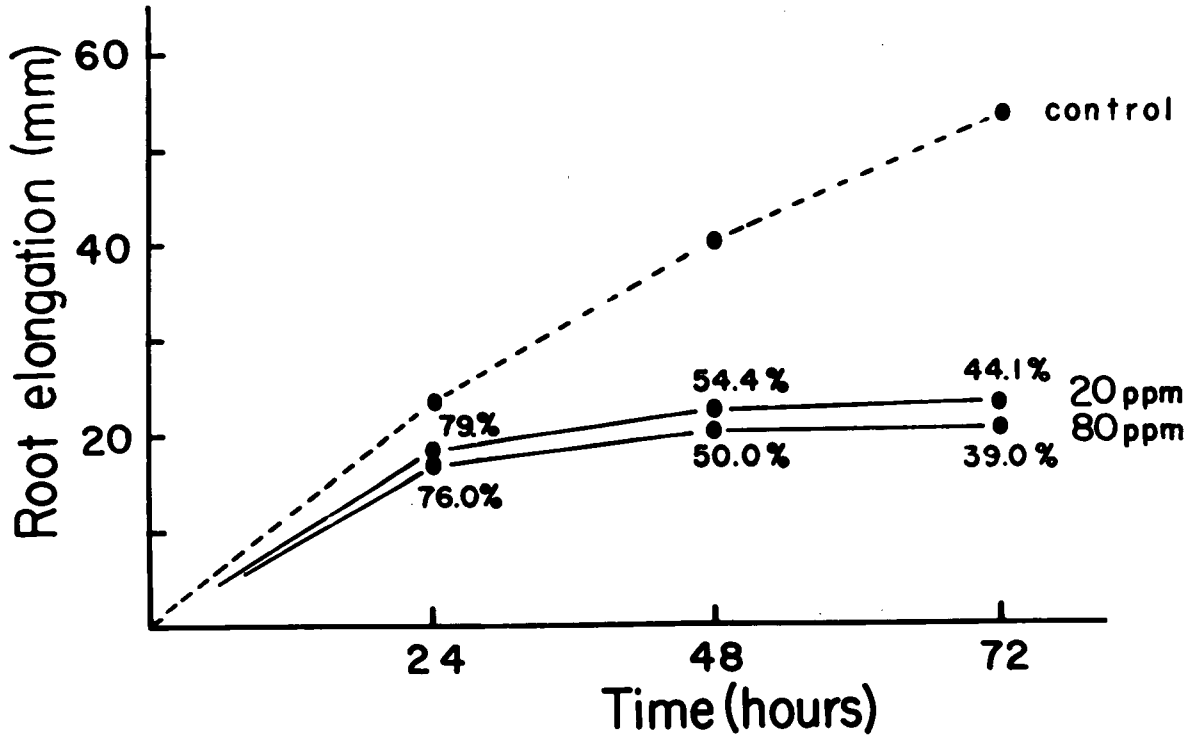
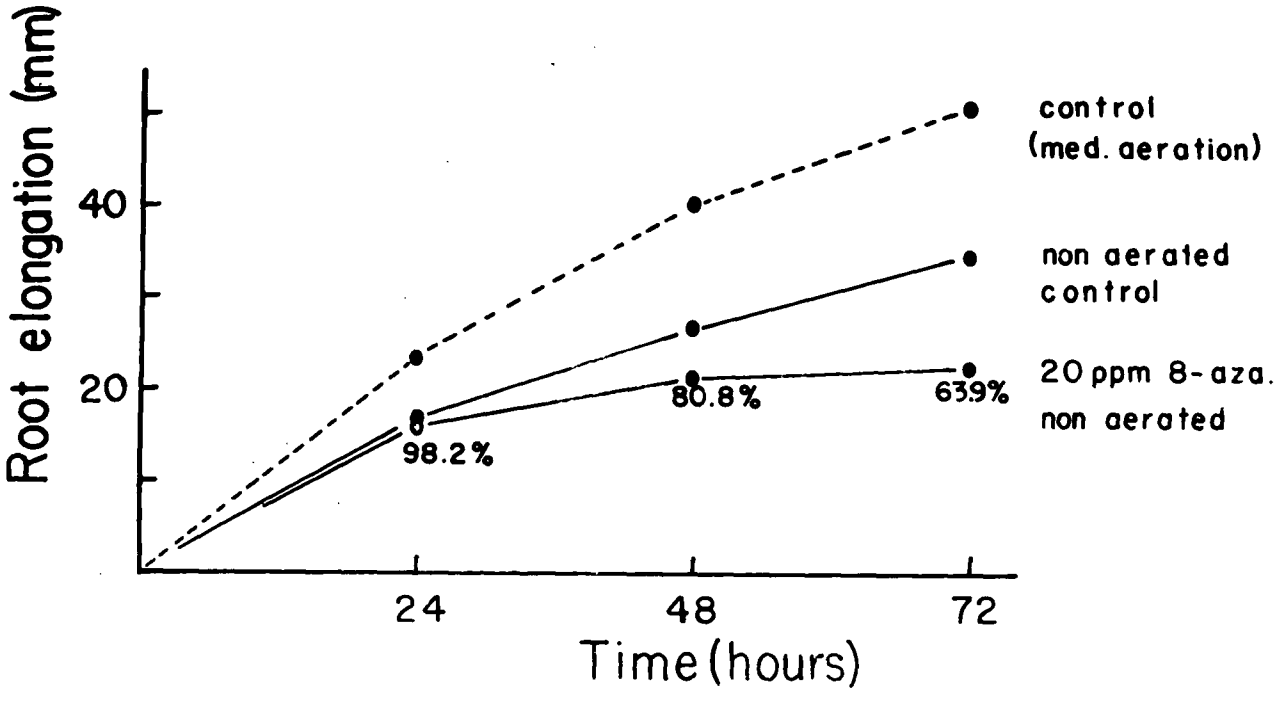

Root elongation (mm)
60
40
20
control
54.4%
44.1%
79%
50.0%
39.0%
76.0%
20 ppm
80 ppm
24
48
72
Time (hours)

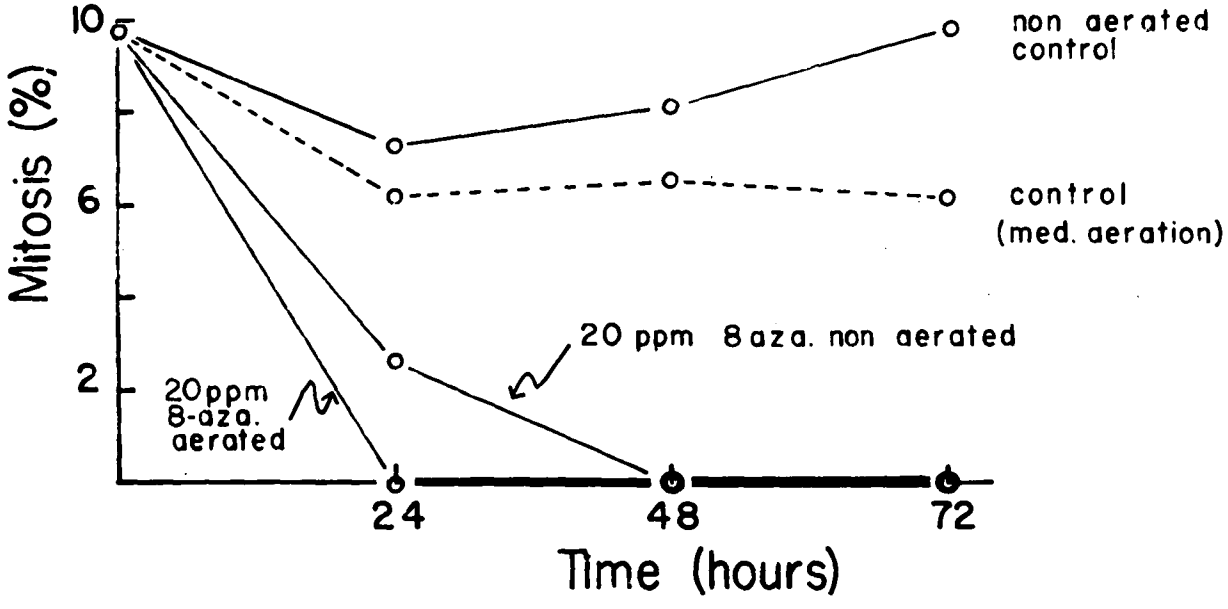
PLATE X

Figure 18. Effect of lack of aeration on inhibition of root elongation by 20 p.p.m. 8-azaguanine. Presented values are means of two runs per treatment.

Figure 19. Effect of aeration on mitosis in root tips treated with 20 p.p.m. 8-azaguanine. Presented values are means of two runs per treatment.

PLATE X


Root elongation (mm)
40
20
control
(med. aeration)
non aerated
control
20 ppm 8-aza.
non aerated
98.2%
80.8%
63.9%
24
48
72
Time (hours)


Mitosis (%)
10
6
2
non aerated
control
control
(med. aeration)
20 ppm 8 aza. non aerated
20ppm
8-aza.
aerated
24
48
72
Time (hours)

mitotic rate reached zero at 24 hours in both high and medium aerated runs (and also with 10 p.p.m. 8-azaguanine - Fig. 3) it stayed above two per cent in non-aerated solution of 20 p.p.m. 8-azaguanine at 24 hours (Fig. 19).

e. Reversal with adenine after inhibition by 8-azaguanine.

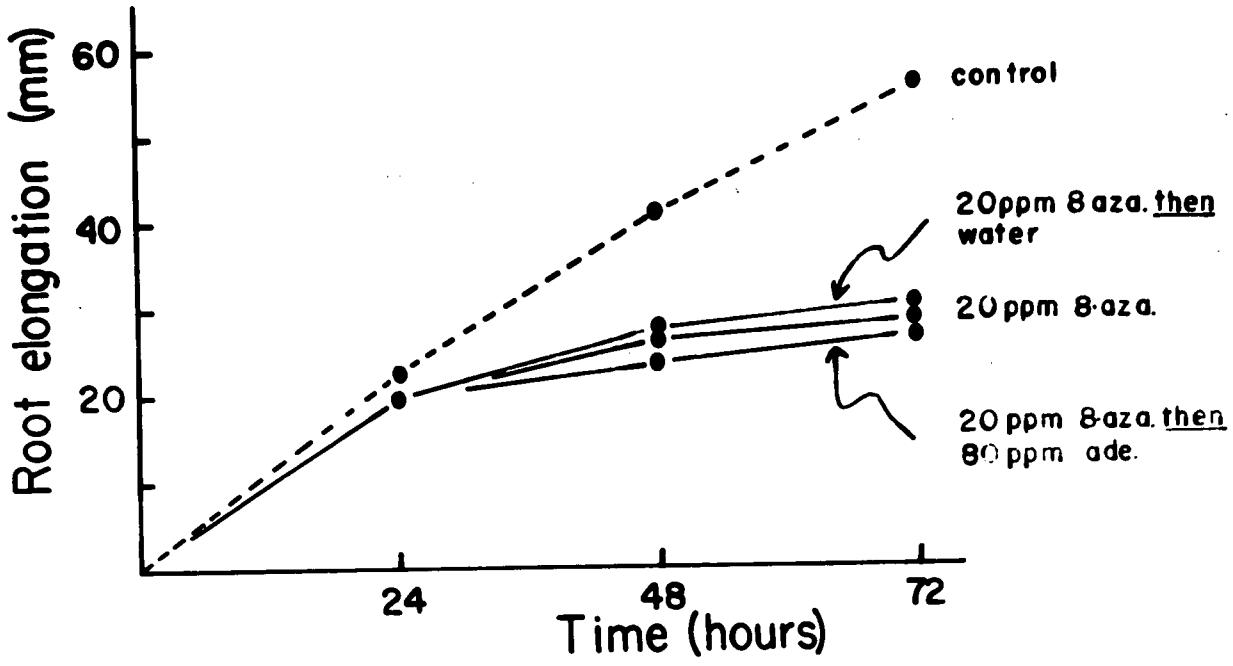
The preceding experiments have shown that the inhibition of root elongation and mitosis by 8-azaguanine could be reversed by either adenine or guanine when both the analog and the metabolite were administered together. While this suggested that the two compounds acted competitively in one or more critical metabolic processes leading to cell growth and nuclear division, the possibility could not be excluded that the reversal resulted merely from an interaction of the two substances in the culture solution. This possibility was tested in an experiment in which roots were held in 20 p.p.m. of 8-azaguanine for 24 hours, and then placed in water or in a solution of 80 p.p.m. adenine for 48 hours. If reversal by adenine were dependent principally on interaction with 8-azaguanine in solution, reversal should not occur in this experiment. The results plotted in Fig. 20 show that water slightly relieved the inhibition of root elongation, while the growth curve for roots transferred into adenine somewhat resembled those in Figs. 8 and 10 for roots treated with adenine alone, or with 8-azaguanine plus adenine: a depressed but steadily ascending curve. However, within the period of reversal in this experiment, adenine had a rather small effect on root elongation. Interestingly, the reversal of mitosis by adenine in this experiment was very marked (Fig. 21). Partial recovery (0.27 per cent mitosis) was

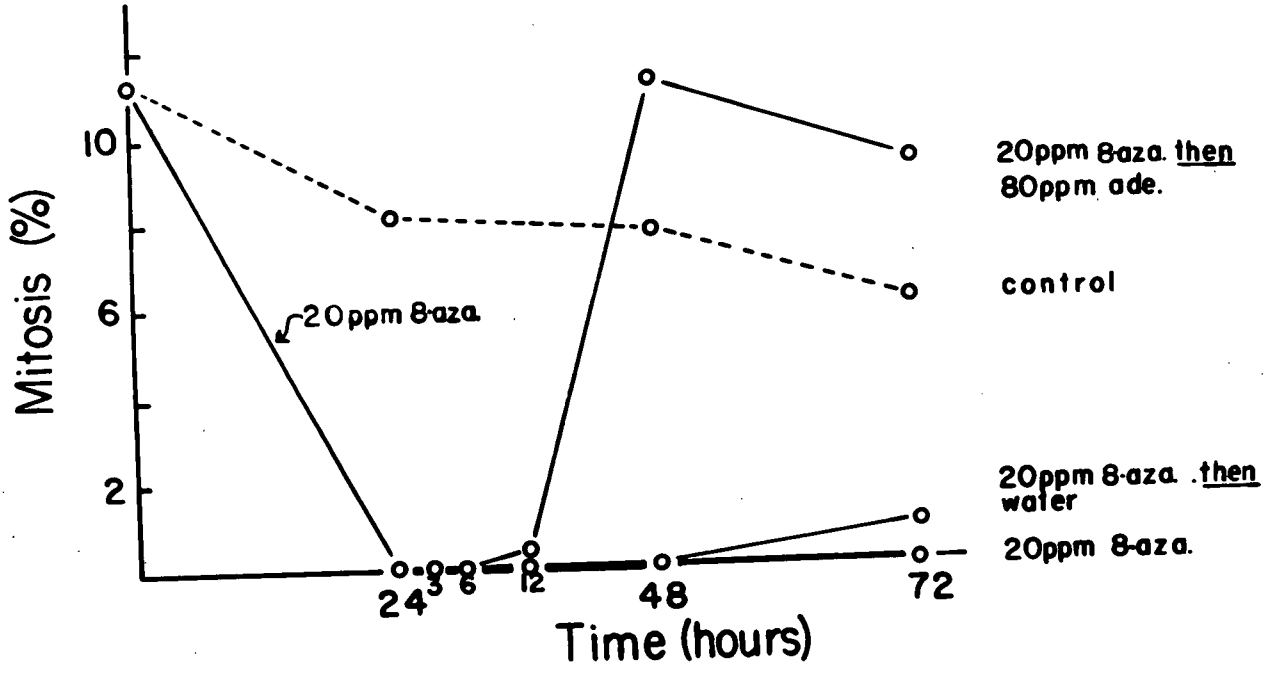
PLATE XI

Figure 20. Reversal of the inhibition of root elongation by 80 p.p.m. adenine (equimolar) after the roots had been inhibited by 20 p.p.m. 8-azaguanine. The values are means of following number of runs: control - 2, 20 p.p.m. 8-azaguanine - 1, 20 p.p.m. 8-azaguanine then 80 p.p.m. adenine - 3, 20 p.p.m. 8-azaguanine then water - 2. The values at 24 hours in different treatments with 20 p.p.m. 8-azaguanine (range 17.2 - 19.8 mm) were adjusted to the value of the control treatment with 20 p.p.m. 8-azaguanine (19.8 mm) and the differences were added up at 48 and 72 hours.

Figure 21. Reversal of mitotic inhibition in roots treated for 24 hours with 20 p.p.m. 8-azaguanine by transferring into solution with 80 p.p.m. (equimolar) adenine. Presented values are means of following number of runs: control - 2, 20 p.p.m. 8-azaguanine - 1, 20 p.p.m. 8-azaguanine then 80 p.p.m. adenine - 2, 20 p.p.m. 8-azaguanine then water - 1.

PLATE XI


Root elongation (mm)
60
40
20
24
48
72
Time (hours)
control
20ppm 8 aza. then water
20ppm 8-aza.
20 ppm 8-aza. then 80 ppm ade.


Mitosis (%)
10
6
2
20ppm 8-aza
24
3
6
12
48
72
Time (hours)
20ppm 8-aza. then 80ppm ade.
control
20ppm 8-aza. then water
20ppm 8-aza.

first observed 12 hours after the transfer from 8-azaguanine to adenine; mitotic figures observed at this time were all at prophase. Complete recovery had been established a few hours later as indicated by the rapidly rising curve. The rate of mitosis levelled off after its ascent (at 24 hours after transfer), but remained well above the control until the termination of the experiment. In roots transferred from 8-azaguanine into water some recovery of mitosis was observed only 48 hours after transfer. In these roots mitotic figures were restricted to the elongated provascular cells. The ratio of prophases:telophases in controls at all times and in roots transferred from 8-azaguanine into adenine at 24 and 48 hours after transfer was between 2.5 and 3.5; the ratio in roots transferred from 8-azaguanine into water was 5.62 at 48 hours after transfer, indicating a sharply rising mitotic frequency at the end of the experiment.

f. Spectrophotometric measurements.

This aspect of the work was undertaken primarily to determine whether or not DNA synthesis occurred in cells in which mitosis was blocked by 8-azaguanine. In principle, frequency distributions of DNA content per nucleus were determined in both treated and control roots after various periods of incubation.

The results of microspectrophotometric measurements in control roots fixed at 24 and 72 hours are shown in Figs. 22 and 25, respectively. The essential characteristics of these curves are very

similar: in each case there are two well marked peaks representing DNA values in a ratio of approximately 1:2, and there are a few intermediate values lying between the peaks. It will be noted that the lower peak for interphasic distribution coincides with the modal DNA value for telophasic and anaphasic nuclei. In common usage (e.g. Swift, 1950a, b, Deeley et al., 1957) this amount is designated as "2C", and the amount characteristic of prophasic and metaphasic nuclei, "4C". There were no values exceeding 4C level indicating that there were no polyploid nuclei in the root tip.

The curves representing treated roots fixed at 24, 48, and 72 hours are plotted in Figs. 23, 24, and 26, respectively. These differ from those of controls chiefly in one respect, namely that intermediate values are rare or absent. It is apparent that DNA synthesis decreases prior to 24 hours and reaches zero before 48 hours. This finding is also supported by the fact that the proportion of nuclei containing 4C level of DNA remained constant (16 per cent at 24, 16 per cent at 48, and 18 per cent at 72 hours) throughout the treatment while no mitoses were observed.

TABLE VI

DNA values of nuclei in different mitotic stages in control at 24 hours.

DNA in arbitrary units; values presented here are plotted in Fig. 22

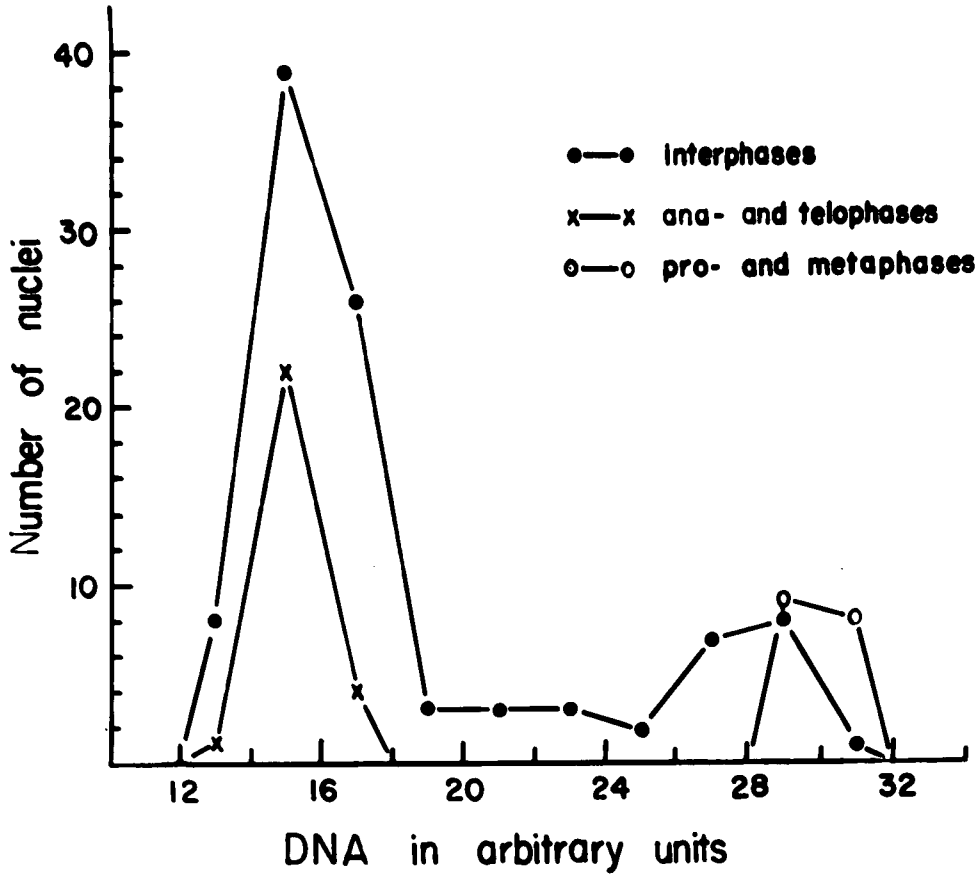
Mitotic stage	Number measured	DNA content		
		Range	Mean	S.E.
Prophase	13	28.23-31.43	29.95	±.29
Metaphase	4	28.69-31.37	29.63	±.59
Anaphase	6	14.37-16.52	15.51	±.35
Telophase	21	13.24-16.23	15.05	±.14

PLATE XII

Figure 22. DNA content of the nuclei in apical portion of the root in control at 24 hours. Mitotic frequency 7.44 per cent. Number measured: interphases - 100, prophases - 13, metaphases - 4, anaphases - 6, telophases - 21.

Figure 23. DNA content of the nuclei in apical portion of the root treated with 20 p.p.m. 8-azaguanine for 24 hours; 100 nuclei measured.

PLATE XII


40
30
20
10
Number of nuclei
●—● interphases
x—x ana- and telophases
o—o pro- and metaphases
12
16
20
24
28
32
DNA in arbitrary units

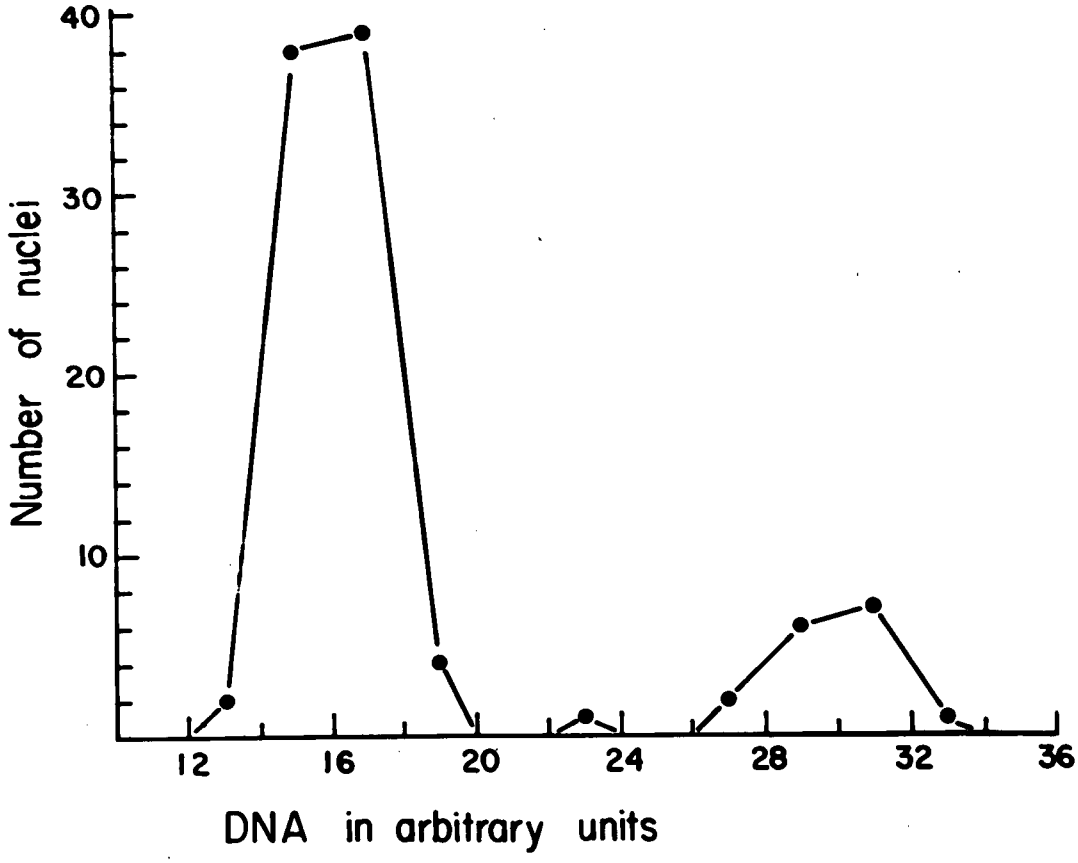

40
30
20
10
Number of nuclei
12
16
20
24
28
32
36
DNA in arbitrary units

PLATE XIII

Figure 24. DNA content of the nuclei in apical portion of the root treated with 20 p.p.m. 8-azaguanine for 48 hours and DNA content of telophases from control at 48 hours; 51 treated nuclei and 4 telophases (control) were measured.

PLATE XIII

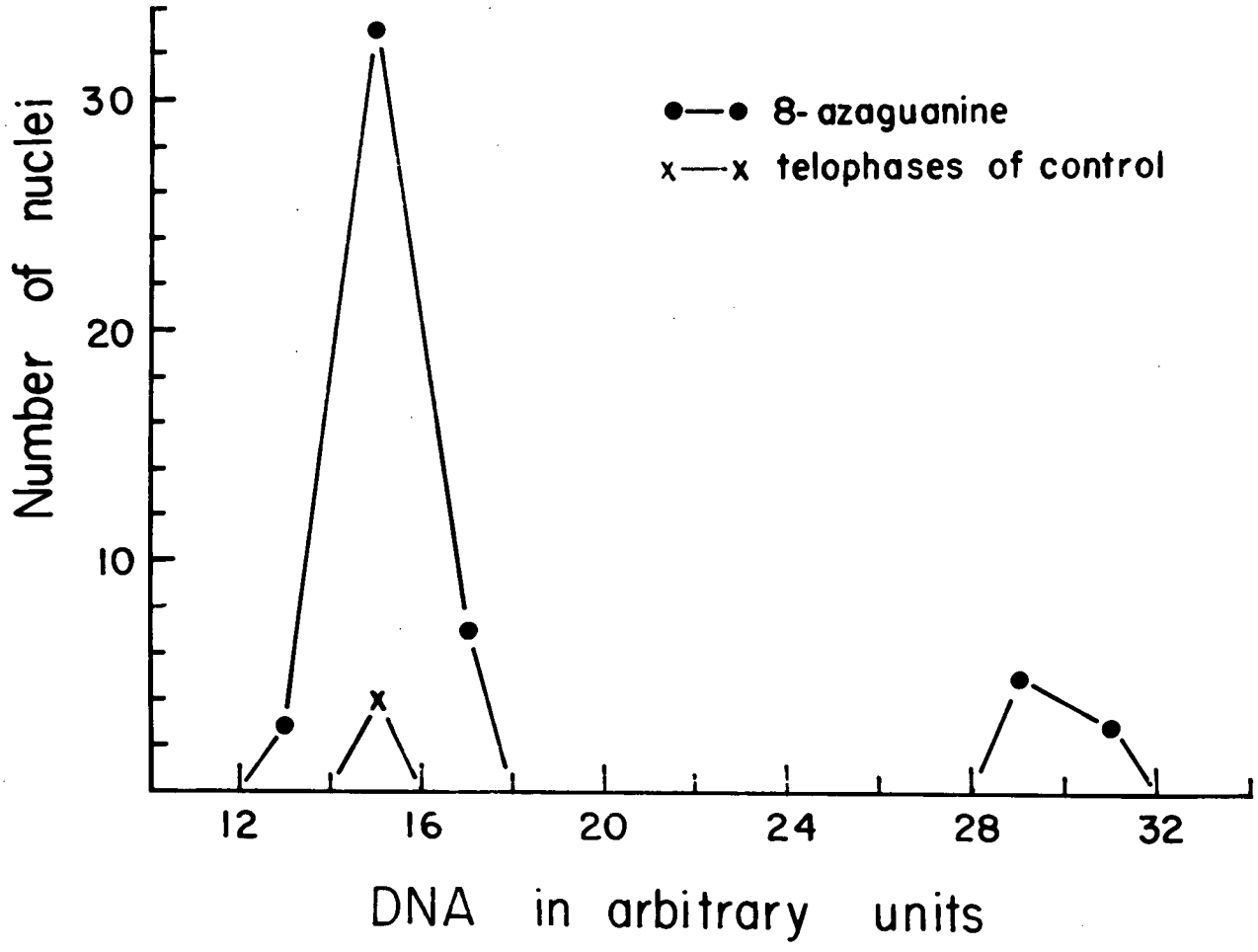
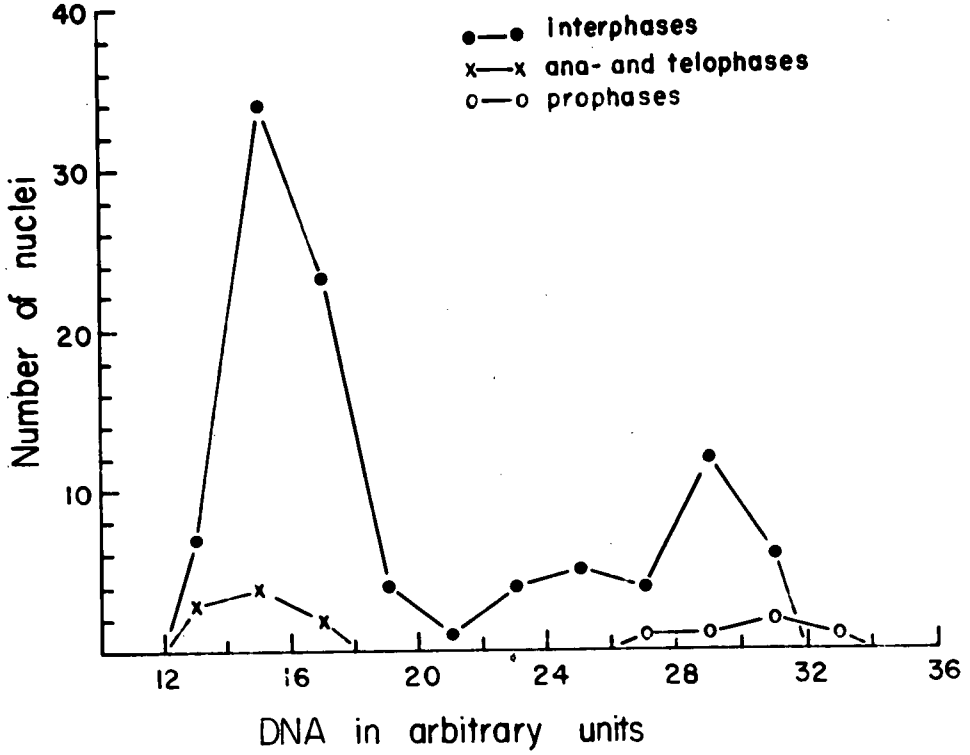

Number of nuclei
30
20
10
●—● 8-azaguanine
x—x telophases of control
12
16
20
24
28
32
DNA in arbitrary units

PLATE XIV

Figure 25. DNA content of the nuclei in apical portion of the root in control at 72 hours. Mitotic frequency 6.87 per cent. Number measured: interphases - 100, prophases - 5, anaphases - 3, telophases - 6.

Figure 26. DNA content of the nuclei in apical portion of the root treated with 20 p.p.m. 8-azaguanine for 72 hours; 100 nuclei measured.

PLATE XIV


Interphases
ana- and telophases
prophases
Number of nuclei
40
30
20
10
12
16
20
24
28
32
36
DNA in arbitrary units

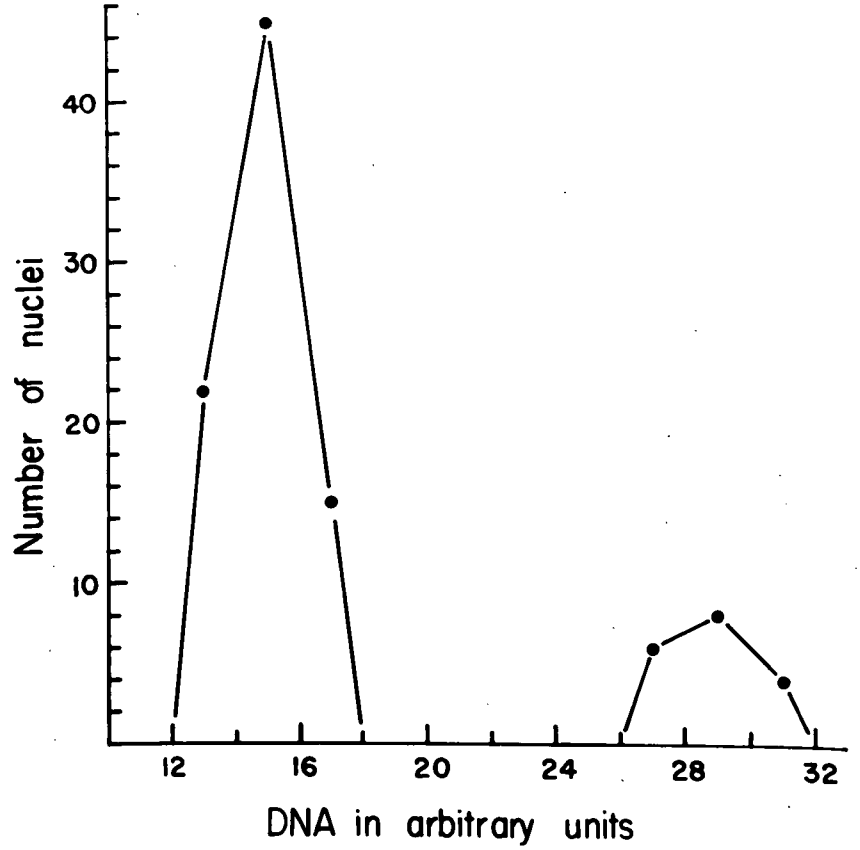

Number of nuclei
40
30
20
10
12
16
20
24
28
32
DNA in arbitrary units

TABLE VII

DNA values of four telophases in control at 48 hours.

DNA in arbitrary units; values presented here are plotted in Fig. 24.

Mitotic stage	Number measured	DNA content		
		Range	Mean	S.E.
Telophase	4	14.21 - 15.65	15.05	±.30

TABLE VIII

DNA values of nuclei in different mitotic stages in control at 72 hours.

DNA in arbitrary units; values presented here are plotted in Fig. 25.

Mitotic stage	Number measured	DNA content		
		Range	Mean	S.E.
Prophase	5	27.67 - 33.35	30.19	±1.04
Anaphase	3	14.13 - 16.90	15.12	± .88
Telophase	6	13.20 - 16.23	14.38	± .54

Tables VI, VII, and VIII contain the DNA values for 2C (anaphase and telophase) and 4C (prophase and metaphase) nuclei in control at the three different times (24,48, and 72 hours). These data show that the internuclear variation in each class is rather small and that the ratio of DNA content between 2C and 4C nuclei closely approximates to the theoretical ratio of 1:2. The average values of 2C and 4C groups in treated nuclei agree well with the values obtained by measuring mitotic groups of chromosomes in controls; for example the mean of the 2C group in roots treated with 8-azaguanine for 48 hours equals 15.15±.13, C.V.*= 5.83 (43 nuclei), and that of 4C group 29.85±.35, C.V. = 3.31 (8 nuclei). These values differ very slightly

* C.V. = coefficient of variability.

from the values obtained at different mitotic stages of controls, e.g. telophase at 24 hours where mean = 15.05± .14, C.V. = 4.26 (21 telophase groups). The close agreement between the theoretical and obtained ratios and also between the DNA values obtained at different times shown in the three tables indicates the accuracy of the method as well as a satisfactory standardization of fixation, hydrolysis, and staining procedures.

The data presented in Figs. 23, 24, and 26 were obtained in random measurements and for this reason the possibility could not be excluded that occasional nuclei in treated roots contained amounts of DNA substantially greater than 4C level. This might be considered to occur as a consequence of the direct suppression of the onset of prophase by 8-azaguanine, provided that DNA synthesis was not also entirely suppressed. To test this possibility slides prepared from treated root tips at different times were searched for large, deeply stained nuclei which might contain unusually large amounts of DNA. These were the "pseudo-prophases" described previously.

TABLE IX

DNA values of selected "pseudo-prophases" in roots treated with 20 p.p.m. 8-azaguanine.

DNA in arbitrary units.

Time of 8-aza treatment	Number measured	DNA content		
		Range	Mean	S.E.
24 hours	3	29.35 - 33.52	31.70	±1.23
48 hours	4	28.38 - 32.37	30.88	± .87
72 hours	9	27.45 - 30.53	28.99	± .34
Total	16	27.45 - 33.52	29.97	± .44

The results given in Table IX show that this variation does not occur, since the mean DNA content agrees closely with that of the unselected populations (Figs. 22-26, Tables VI and VIII).

DISCUSSION OF METHODS

The Calgon-metasilicate method of washing glassware is now being used generally in animal tissue culture. The method has the advantage over the usual acid cleaning that it avoids the risk of residual chromium ions left on the glassware. Unfortunately it cannot be used for the cleaning of volumetric glassware.

In preparing seeds for germination, removal of the seed coats after preliminary soaking results in more uniform germination and reduces the contamination of the seedlings by molds. While the seeds could be treated with antibiotics, this might introduce the possibility of effects of these substances on growth and mitosis, as well as interaction with the tested chemicals. Though some fungal growth was often found on the naked cotyledons toward the end of an experiment, this did not seem to affect either growth of the roots or mitosis.

Since the diffusion of oxygen into solution depends not only on the air flow but also on the size of air bubbles released by the sintered glass tubes, it was necessary to select tubes of uniform performance. A voltage stabilizer was introduced to eliminate variation in the speed of the pump due to changes in the line current.

It was stated in Materials and Methods that growth rate curves in the cases where some roots were collected during the treatment, were

constructed on the basis of daily average increments rather than from an average total length of 24, 48, and 72 hours. This was done in order to eliminate a distortion of the curves introduced by withdrawing roots for mitotic counts during the experiment. If, for example, the three roots collected at 48 hours were, by chance, considerably longer than the remainder, the growth curve constructed from the average total root length would be depressed in the 48-72 hour interval and might even appear to reflect a negative growth rate. This is particularly important in treatments where the growth rate declines sharply toward the end of the experiment (e.g. in 8-azaguanine treatments). This problem is avoided when the growth curves are obtained by summation of daily average increments.

Under the given experimental conditions the period of 72 hours appeared to be the maximal time span for which the root elongation and mitotic counts in the root meristem could give reliable results; after 72 hours considerable development of secondary roots usually appeared on the upper portion of the roots (the development of secondary roots was sometimes observed as early as at 48 hours) and roots in some treatment solutions became necrotic. An initial drop in mitotic rate during the first 24 hour period was generally observed in both treated and control roots. This was probably due to the change in the environment (transfer of seedlings from vermiculite into water) and due to handling of the seedlings at the beginning of the experiments. This effect would have been eliminated by allowing the roots a 24 hour recovery period in water prior to the treatment period. However,

in this case it would have been necessary to shorten the duration of treatment for the reasons given above.

Apical, 1 mm segments of root tips were used in mitotic frequency determinations, though Setterfield and Duncan (1955), Pryzina (1956), and others have used 2 mm portions for this purpose. Jensen (1955) has shown that cell elongation for the majority of cells begins at about 1.7 mm. Errors in cutting of the order of about 0.2 mm are to be reckoned with since the Feulgen-stained roots are soft and easily deformed in handling. If 1 mm sections are cut the variation in actual length of the cut portions is restricted to an area of more or less homogeneous, dividing cells while the variation at 2 mm distance will add to the sample greater or smaller amount of non-dividing cells and thus increase the error in counting of mitotic frequencies. Fresh roots could be cut more accurately but short sections are difficult to handle during staining procedures. The apical 1 mm segment in the Vicia faba root tip does not consist entirely of meristematic cells, though several authors (Holmes et al., 1955, Deeley et al., 1957) considered even 2 mm sections to be completely meristematic. As noticed by Jensen (1955), the elongation and differentiation of some central provascular cells starts as close to the tip as 0.8 mm. Partially elongated, non-vacuolated cells which have commonly been seen to divide on controls, together with isodiametric cells, were scored as "meristematic". Greatly elongated cells, occasional root cap cells, and epidermal cells were omitted in the counting.

For microspectrophotometric measurements it is necessary to standardize carefully the times and temperatures of fixing, hydrolysis,

and staining, since these factors influence the density of developed Feulgen dye and, consequently, the amount of measured DNA (Lessler, 1953, Swift, 1955). Mounting in oil of refractive index matching that of the cytoplasm was recommended by Pollister and Ornstein (1955) to diminish the non-specific light loss in the specimen. In material prepared by this method the cytoplasm was nearly invisible and its absorption negligible (less than 2 per cent of the specific absorption of the nucleus).

The two-wavelength method of microspectrophotometric measurement of DNA (Patau, 1952, Patau and Swift, 1953, Swift and Rasch, 1956) was used in these experiments. While the procedure is somewhat more tedious than the conventional one-wavelength method, it has several important technical advantages. Accuracy of measurement is not dependent on the distribution of the absorbing material, hence interphase nuclei of all shapes can be included because size, number, and position of nucleoli does not matter. Moreover, chromosomal groups of all mitotic stages can be measured accurately by the two-wavelength but not by the one-wavelength method. The superior accuracy of the method was considered to be particularly important in this type of study, since it was necessary to detect changes of a few per cent in DNA content.

The discussion of photometric errors as far as instrumentation is concerned is omitted, because, firstly, these have been discussed many times (e.g. Ris and Mirsky, 1949, Patau, 1952, Grundmann and Marquardt, 1953b, Pollister and Ornstein, 1955, Swift and Rasch, 1956) and, secondly, the results obtained here indicate that these errors

had a very minor effect. It should be mentioned only that the alignment of the apparatus is critical. The remark of Swift and Rasch (1956) that "the alignment of microphotometers is simple in principle but in practice takes patience and empirical manipulation" is rather an understatement. Suitable tests for checking the alignment are given in the above-mentioned excellent discussion. The final check on the performance of the instrument consisted of a series of measurements on nuclei of known relative DNA content. Thus in diploid dividing cells of a growing tissue the Feulgen dye uptake, and DNA content, of telophases and prophases is in the ratio of 1:2 (review in Swift, 1953, Vendrely and Vendrely, 1956). Preliminary measurements of nuclei in these two stages in several Vicia root tips gave a very satisfactory fit to this ratio. Furthermore, the results were not influenced by dye distribution, since variation in the size of the measured area surrounding the nuclei had no effect on the results.

DISCUSSION OF RESULTS

Patau and Patil (1951) proposed that the relative number of cells in prophase and telophase could be used as a sensitive indicator of changes in the rate of nuclear division. When the mitotic rate is steady the ratio is constant because equal number of nuclei will be entering prophase and telophase per time unit and the ratio will be given by the relative duration of each stage. If the mitotic rate increases the change will first appear as an increase in relative number of prophases, and, consequently, an increased prophase:telophase ratio; and when the mitotic rate decreases the ratio will be depressed as well. By comparing the ratio in treated nuclei with that of control, where the mitosis proceeds at a more or less steady rate, it can be determined, in a single count, whether at the time of collection the mitotic rate was increasing, steady, or decreasing in the treated material. Data presented here are in agreement with the above considerations; the ratio was increased at the beginning of periods of increasing mitotic rate, and was correspondingly depressed when the rate was decreasing (Table II, results of Figs. 12 and 21).

Mitosis was more sensitive than root elongation to inhibition by 8-azaguanine. With the concentrations as low as 10 p.p.m. in aerated solutions, mitosis was completely inhibited within 24 hours while root elongation was decreased in the first 24 hour period by only about 10 per

cent and some elongation was still evident at 72 hours. This result was the first indication that mitosis and root elongation were not necessarily correlated. Still more striking were the results obtained with the concentration of 1.0 p.p.m. 8-azaguanine: mitotic frequency was inhibited to about 50 per cent of control, though root elongation was slightly stimulated. The finding that small amounts of antimetabolites stimulate growth is quite common. For example Goldacre (1957) found that very low concentration (3×10^{-9}M) of 2,6-diaminopurine stimulated the growth of both isolated root tips and roots of intact seedlings of Subterranean clover while higher concentrations (1.5×10^{-8}M) were inhibitory. The treatment in these experiments lasted for 15 days and root elongation only, but not mitosis, was investigated. Similarly, Hohn (1955) noticed growth stimulation of cress roots by very low concentrations of thiouracil. Taking into consideration the demonstrated greater sensitivity of mitosis than of root elongation to inhibition by 8-azaguanine, the results can be interpreted in the following way: substrates and/or energy that cannot be utilized for cell multiplication can still be utilized for increase in cell size. It should be stressed that stimulation of growth occurs only when very low concentrations of antimetabolites are used; when the concentrations are increased both mitosis and growth are inhibited.

The responses of root elongation and mitosis to aeration observed here are interesting in the light of previous experiments on the respiratory capacity of different root tissues. It had been found repeatedly that the respiration rate is higher in elongating cells

adjacent to the meristem of roots than in meristematic cells of the root tip if oxygen consumption is calculated per unit weight of protoplasm (or N content). Thus Kopp (1948) reported a nearly threefold O_2 consumption by cells of zone of elongation as compared to that in meristematic cells in primary roots of wheat seedlings. Brown and Robinson (1955) agreed with these findings and also showed that the activity of several enzymes (invertase, dipeptidase, phosphatase, glycine oxidase, and proteinase) is low in the meristematic region of bean root. Similar results in respect of respiration were obtained by Betz (1955) in experiments with primary roots of corn and peas; the author also demonstrated the presence of fermentation in root meristems. Jensen (1955) found very low O_2 uptake in root meristem of Vicia faba and suggested that "the low oxygen consumption could indicate that the energy required for the synthetic activities is derived either from glycolysis or the utilization of high energy compounds produced by other cells". There are several papers reporting opposite results but in all these cases O_2 consumption was calculated on the basis of fresh weight or volume unit of tissue; this would bias the results substantially, since differentiated plant cells contain relatively much more water (central vacuole) than meristematic cells. Lettre (1951) suggested that while aerobic respiration is not necessary for cell division, glycolysis* is sufficient to supply the energy, and is probably indispensable. This author also demonstrated the decrease of glycolytic processes if respiration is increased, and suggested that the reaction of ribose with lactic acid to produce

* The terms glycolysis and fermentation are used interchangeably in accordance with the terminology of the original authors.

desoxyribose, pyruvic acid, and water, is a part of the glycolytic process. The results presented here may indicate that cell elongation is principally dependent on aerobic respiration, and that the energy of glycolysis can be more readily utilized in cell division. Stimulation of mitosis by lack of oxygen has been reported by Wagner (1957) in many animal tissues; mitosis in at least some embryonic tissues in animals also is independent of aerobic respiration (rev. in Ris, 1955). On the other hand, aerobic respiration is necessary for mitosis in adult mammalian epidermis (Bullough, 1952), and in the cleavage divisions in echinoderm eggs (Drahl, 1950). While similar detailed information on the metabolic characteristics of dividing plant cells is not available, the data presented here suggest that the former mechanism, namely stimulation of mitosis by lowered tension of oxygen, is operating in roots of Vicia faba.

The observed relationship between rate of aeration and mitosis might also be explained in the following way: the increased root elongation is probably, at least partially, due to the increased uptake of water dependent on enhanced respiration (Rosene and Bartlett, 1950); Swann (1957) suggested that a certain concentration rather than an absolute amount of some unknown substance is necessary for triggering of mitosis. In cells that take up greater amount of water the establishment of the critical concentration, and consequently, the mitosis in individual cells would be delayed, and the mitotic rate in the tissue would be decreased.

The rate of aeration also exerted a considerable influence on the degree of inhibition obtained with a given dose of 8-azaguanine

(Figs. 16 - 19). This agent has been relatively more inhibitory under high aeration. This was true for both root elongation and mitosis. With high or medium aeration the time required for complete mitotic inhibition with 20 p.p.m. 8-azaguanine was less than 24 hours, though only about 70 per cent inhibition had been established with this concentration at 24 hours in unaerated cultures. Though the physiological basis of this relationship is not clear, the increased inhibition probably reflects an enhanced uptake of 8-azaguanine in aerated solutions, i.e. an "active" uptake correlated with the magnitude of respiration. This sensitivity of inhibition emphasizes the importance of reproducibility of experimental conditions in experiments where the effects of different compounds at different concentrations are compared.

The effects of adenine alone on mitosis and root elongation were similar to those produced by low aeration, i.e. mitosis was stimulated whereas root elongation was depressed. Setterfield and Duncan (1955) also reported a slight stimulation of mitosis with adenine but not with adenosine, and suggested that this effect resulted from a specific requirement of free adenine for nuclear division. In all these studies aerated solutions were used. Woll (1953), using non-aerated solutions, reported inhibition of both root elongation and mitosis with comparable concentrations of adenine in roots of Vicia faba; in addition, nearly all dividing cells in these adenine-inhibited roots were in prophase. Under the conditions prevailing in the present experiments, mitotic stimulation is proportional to dose over a rather wide range of adenine concentrations, as indicated by results of experiments in which 80 and

160 p.p.m. of adenine were administered (Figs. 8 and 9). Interestingly, where either adenine concentration or the level of aeration were changed, the rates of mitosis and root growth were both influenced, but in opposite directions (Figs. 8, 9, 14, and 15). This may reflect a physiological balance between the rates of cell growth and mitosis which can be disturbed by several means, with the result that the specific stimulation of one of the processes (i.e. cell growth or mitosis) results in the suppression of the other, and vice versa.

While both 8-azaguanine and adenine inhibited root elongation with increasing concentrations (Figs. 2 and 14), the growth curves for these two treatments are basically different. The depressed growth rate obtained with adenine was approximately constant throughout the 72 hour period of incubation. On the other hand, inhibition by 8-azaguanine increased with time, indicating that the inhibition in this case was cumulative. This would be expected on the assumption that this antimetabolite progressively displaces natural purines from active sites in the cell. The curves show a modification of the metabolic processes in the former, and a progressive disruption of these processes in the latter case.

Inactivity of 6-mercaptopurine in concentrations equivalent to highly inhibitory levels of 8-azaguanine possibly indicates that the mechanism of action of these two purine analogs is different.* Biesele (1955) reported prevention of mitotic inhibition of sarcoma 180 cells caused by 1.0 mM 6-mercaptopurine by as little as 0.02 mM

* It is not possible to compare the concentrations of analogs used in work with animals with those used here since the concentrations in former studies are usually expressed as mg/day/kg weight; besides diffusion factors are quite different.

coenzyme A, and suggested that 6-mercaptopurine might act as an anti-metabolite of coenzyme A, interfering with the introduction of two-carbon units into the respiratory cycle. Accordingly, the concentration of coenzyme A in roots can be considered as being high enough to prevent the inhibition. The differences in concentration of endogenous coenzyme A might be also responsible for different effects of this analog on duckweed and virus tumor tissue reported by Nickell (1955).

The absence of inhibition of root elongation by 8-azaxanthine agrees with the findings of Hirschberg et al. (1952) that this compound had no carcinostatic potency. The results are also compatible with the demonstrated deamination of 8-azaguanine to 8-azaxanthine carried out by cells resistant to this analog (Hirschberg et al., 1953, Mandel, 1955). Since the meristematic cells were not resistant to 8-azaguanine, it may be suggested that root tips of Vicia faba are low in 8-azaguanine deaminase (guanase) content.

The demonstration that the inhibition of mitosis by 8-aza-guanine can be reversed by subsequent treatment with adenine (Fig. 21) is of some significance in that it eliminates the possibility that the reversal is dependent on a non-metabolic interaction between the two compounds in culture solution. It should ne noted that complete mitotic inhibition and its complete reversal each took about the same period of time, somewhat less than 24 hours. Since the average duration of one complete mitotic cycle in root tips of Vicia faba was reported to be 25.9 hours by Setterfield and Duncan (1955) and about 30 hours by Pelc and Howard (1955), complete mitotic inhibition and also complete reversal of the inhibition was accomplished in a time period shorter

than the duration of one complete mitotic cycle. In roots transferred from 8-azaguanine into water, mitosis first appeared 48 hours after transfer but only in the partially elongated provascular cells. This suggests that endogenous substances capable of reversing the inhibition of mitosis by 8-azaguanine were carried downwards to the root tips through the vascular tissue.

The results of spectrophotometric measurements of controls are in excellent agreement with the recently published data of Deèley et al. (1957) who measured the DNA content of root tip nuclei of Vicia faba using the technique of photoelectric scanning of crushed cells. There is also a good general agreement with the results of Jensen (1956) obtained by microchemical analysis. On the other hand, there is a discrepancy with the results presented by Grundmann and Marquardt (1953 a, b). These authors used the one-wavelength method, and concluded that DNA synthesis started immediately after telophase and was followed, again immediately upon the completion of 4C amount of DNA, by mitosis. The curves presented here for controls have to be interpreted in the following way: there is a rather long lag period at the beginning of interphase before DNA synthesis starts and another, shorter, lag period before beginning of the prophase, after completion of DNA synthesis.

The microspectrophotometric measurements yielded the expected frequency distribution of DNA per nucleus at 24, and 72 hours in the control roots. This bimodal distribution, in which the DNA values representing the frequency maxima are in the ratio 1:2 (2C, 4C) has been found typical of dividing tissues where the chromosome number per

cell is constant (for review see Vendrely and Vendrely, 1956, Swift, 1953). The high reproducibility of these measurements is indicated in the small error calculated for nuclei in known states of division (Tables VI to VIII).

Comparison of the distribution of DNA values for treated and control roots indicates two main effects of 8-azaguanine on the mitotic cycle. The virtual absence of intermediate values after 48 hours shows that this agent blocks synthesis of at least one chromosomal constituent - DNA. Moreover, in spite of the fact that "new" 4C nuclei were not produced after 48 hours, the proportion of the nuclei in this class did not decline during the 48 - 72 hour interval (Figs. 24 and 26). This shows that the entrance into prophase from 4C was also prevented.

Perhaps the best evidence that no DNA synthesis occurs in inhibited interphasic nuclei is found in the close agreement between the DNA values of inhibited 2C nuclei and the telophases and anaphases of the controls. The means are almost identical and the coefficient of variation (standard deviation in per cent of the mean) in each group is less than 6 per cent. Errors in measurement can be expected to provide an observed variation of at least 3 per cent (Swift and Rasch, 1956). Therefore, the actual internuclear variation is no greater than 3 per cent in the inhibited nuclei.

Since the mean DNA values for the inhibited nuclei and control telophases are in close agreement, it is evident that little, if any, DNA synthesis occurred in the former. Pryzina (1956) also investigated

the influence of 8-azaguanine in Vicia faba roots using the single-wavelength method of microspectrophotometric measurement. Though he found complete mitotic inhibition at 24 hours using 10 p.p.m. of the anti-metabolite, the observed DNA values of the interphase nuclei formed a continuous distribution from 2C to 4C. These data do not exclude the possibility that 8-azaguanine may block DNA synthesis at intermediate values. However, the magnitude of errors in measurement cannot be calculated from the presented data, and consequently this result cannot be evaluated.

The present results permit of the following comment on the physiological basis of 8-azaguanine-induced inhibition of the mitotic cycle. Two lines of evidence indicate that inhibition is not accomplished through incorporation of this compound into DNA. Firstly, no increase in DNA measurable by the microspectrophotometric method could be shown in inhibited interphases. Secondly, in view of considerable evidence that DNA is metabolically stable, it would not be expected that an incorporated purine antimetabolite could be rapidly replaced by natural purines. Therefore, the finding that inhibition established by 8-aza-guanine could be completely reversed within 24 hours by subsequent treat-ment with adenine provides strong additional evidence that the inhibition did not depend on incorporation of this compound into DNA. It should be pointed out that the biochemical studies on 8-azaguanine incorporation into the nucleic acids of various animal tissues have not yielded consistent results. For example, the incorporation of labelled 8-aza-guanine into RNA of both rumor and normal tissues has been reported

(rev. in Parks, 1955), the incorporation being sometimes higher in the latter. Using a similar technique Parks (1955) found incorporation of the antimetabolite into RNA, but not into DNA, in the protozoa Tetrahymena gelei. On the other hand, Mandel (1955), after injecting labelled 8-azaguanine into mice, found that this substance could be located in the liver nucleic acid hydrolysate, mainly in RNA, but also in DNA. However, there appears to be no conclusive evidence that the carcinostatic effect of this compound depends chiefly on the incorporation into either of the nucleic acids or free nucleotides. The present experiments and those of Parks (ibid.) provide two examples where the inhibition of cell reproduction is not dependent on the incorporation of the antimetabolite into DNA.

Though the incorporation of 8-azaguanine into RNA cannot be eliminated on the basis of the present results, other possible mechanisms, such as interference with adenine-containing coenzymes and especially interference with the synthesis or action of ATP, should be considered. It is known that interconversion of guanine into adenine occurs (Mandel, 1955); since the same enzyme, guanase (8-azaguanine deaminase), deaminates both guanine and 8-azaguanine, it is possible that conversion of 8-azaguanine into 8-azaadenine also occurs. Adenine was found to be superior to guanine in reversal of inhibition by 8-azaguanine (Pryzina, 1956). This might mean that 8-azaguanine interferes with the metabolism of adenine or adenine-containing compounds. It is now generally accepted that ATP or a similar high-energy compound has to be accumulated before prophase can begin, i.e. until the amount of energy is sufficient to

support complete mitosis (Bullough, 1952, Swann, 1957). The results obtained here are compatible with this hypothesis because even with the highest concentration of 8-azaguanine (80 p.p.m.) mitosis was completed and the mitotic process was interrupted in interphase. The presence of a similar mechanism which would accumulate energy before DNA synthesis can start might be indicated by the lag period of nuclei containing 2C amount of DNA. A definite correlation between the utilization of ATP and the synthesis of DNA has been reported recently (Kanazir and Errera, 1956). If the energy supply were suppressed, either by interference with the synthesis of high energy compounds (presumably ATP) or by producing "fraudulent" high energy compounds, the mitotic process would be interrupted only in the two critical stages when the energy is being accumulated. This was exactly the situation found in nuclei inhibited with 8-azaguanine.

SUMMARY

An investigation was made of the effects of the purine analogs, 8-azaguanine, 8-azaxanthine, and 6-mercaptopurine, and the purines adenine and guanine, on cell growth, mitosis, and desoxyribonucleic acid (DNA) synthesis in primary roots of Vivia faba seedlings grown in aerated solutions. Root elongation was used as a measure of cell elongation; mitotic frequency was determined in free cell suspensions prepared from 1 mm-long root tips; the relative content of DNA was determined micro-spectrophotometrically by the two-wavelength method.

It was shown that the balance between root elongation and mitosis in the root tip could be influenced by the amount of aeration and by adenine. Increased aeration stimulated root elongation and depressed mitotic frequency; adenine stimulated mitosis, inhibiting, at the same time, root elongation.

8-azaguanine, in concentrations of 10 p.p.m. and higher, stopped mitosis within 24 hours and greatly reduced root elongation and the fresh and dry weights of roots within 72 hours. This inhibitory effect on both root elongation and mitosis was positively correlated with aeration. 8-azaguanine in a concentration of 1 p.p.m. significantly reduced mitotic frequency but slightly stimulated root elongation. The inhibition of root elongation could be best, though incompletely, reversed by 40 p.p.m. adenine or guanine. The mitotic inhibition could be partially reversed by 40 p.p.m. guanine; adenine, at a concentration of 80 p.p.m., not only completely relieved mitotic inhibition, but

increased the mitotic frequency to a level higher than that of the water controls. Concentrations of 6-mercaptopurine and 8-azaxanthine comparable with those of 8-azaguanine had no inhibitory effects.

Roots treated with 20 p.p.m. 8-azaguanine for 24 hours and then transferred into 80 p.p.m. adenine showed a higher mitotic frequency than the control within 24 hours after transfer. Roots transferred from 8-azaguanine into water showed some mitosis 48 hours after transfer; in this case mitosis was restricted to the partially differentiated and elongated cells of the provascular bundles.

DNA content of interphase nuclei in the controls showed this distribution: a sharp 2C peak (about 65 per cent nuclei), a much lower 4C peak (about 20 per cent nuclei), and intermediates (about fifteen per cent). There were no polyploid nuclei in the apical meristem of the root. The DNA content of chromosomal groups in different mitotic states demonstrated the accuracy of the two-wavelength method which was used. The DNA content of nuclei in roots treated with 20 p.p.m. 8-azaguanine was distributed in a 2C peak (about 80 per cent nuclei) and a 4C peak (about 20 per cent nuclei). There were no intermediates in treated roots and no nuclei contained a higher amount of DNA than 4C. The percentage of 4C nuclei did not increase with time.

From the evidence that the mitotic inhibition induced by 8-azaguanine could be completely reversed within 24 hours by subsequent treatment with adenine, and from the findings concerning the distribution of DNA in inhibited nuclei, it may be concluded that 8-azaguanine was

not incorporated into DNA. The possibility that 8-azaguanine exerts its inhibitory effects through interference with ATP metabolism is discussed.

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