CYTOLOGICAL STUDIES ON THE ANTIMETABOLITE ACTION OF 2,6-DIAMINOPURINE IN VICIA FABA ROOTS*

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INTRODUCTION

Because of its close structural resemblance to the naturally occurring purine bases adenine and guanine, 2,6-diaminopurine (DAP) has received considerable attention in antimetabolite studies on purine metabolism. Hitchings *et al.* (1) showed that DAP inhibited growth of *Lactobacillus casei* and that the inhibition could be reversed by adenine. Hertz and Tullner (2) reported an inhibition by DAP of estrogen-induced growth in the genital tract of the female chick which was almost completely reversible by adenine. Thompson *et al.* (3) obtained inhibition of vaccinia virus growth with DAP and report it reversible by purines and nucleic acid derivatives while Friend (4), studying the Russian spring summer encephalitis virus, found an adenine-reversible inhibition of virus growth in living tissues. Philips and Thiersch (5) found that sublethal doses of DAP produced depletion of bone marrow and damage to the epithelium of colon and ileum in rats and dogs.

Partly as a result of these findings interest has centered on DAP as a possible cancer-chemotheraputic agent. Burchenal *et al.* (6) showed that large doses of DAP could increase the survival time of leukemic mice. Biesele *et al.* (7) discovered that DAP differentially damaged cultures of normal and sarcomatous mouse and rat tissues and damage was prevented by adenine. The authors suggest that the primary effect was probably not through desoxypentosenucleic acid. In a cytological study using mouse tissue cultures, Biesele *et al.* (8) found that both adenine and DAP produced chromosome breakage and other mitotic abnormalities.

With plant tissues, de Ropp (9) reported that DAP (1000 p.p.m.) had no inhibitory effect on the growth of crown gall tumors on chrysanthemum stem fragments. Miller (10), however, found that DAP (1 p.p.m.) inhibited growth

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of cultured tobacco callus, formation of buds on excised tobacco stem pieces, enlargement of cultured tobacco pith, and elongation of etiolated pea stem segments and *Avena* coleoptiles. Adenine completely reversed the callus growth and budding inhibition, both adenine and guanine reversed the block of pith enlargement, and adenine, adenosine, and adenylic acid were partially effective in reversing the inhibition of pea stem elongation.

Attempts to elucidate the biochemical mechanisms of the inhibitory action of DAP have been carried out by Elion and Hitchings (11) and Balis *et al.* (12). The first authors found that in *L. casei* DAP apparently interfered with the utilization of purine ribosides. Balis and his coworkers using isotopically labelled purines in a study of DAP action in the same organism found the only inhibitory effect of DAP on pentosenucleic acid (PNA) metabolism to be an interference with adenine-guanine interconversion. The authors conclude that this is not the primary site of inhibition and suggest that the basic inhibitory action of DAP is not through PNA but rather is an interference with some fundamental metabolic function of adenine.

Diaminopurine can also function as a precursor when supplied in proper concentrations. Bendich and Brown (13) and Brown (14) report that in the rat, labelled DAP is used as effectively as adenine for synthesis of nucleic acid guanine. Kidder and Dewey (15) found that DAP could function in *Tetrahymena gelei* as a poor substitute for guanine while in *L. casei*, Balis *et al.* (12) report that DAP can be extensively converted into polynucleotide adenine and guanine.

In addition to functioning as a precursor of other purines, DAP may also be utilized directly. Kornberg and Pricer (16) partially purified from yeast an enzyme, adenosine phosphokinase, which phosphorylates adenosine and DAP-riboside. Wheeler and Skipper (17) found that labelled DAP injected into mice is converted into DAP-ribose phosphate and Skipper (18) reports that labelled DAP is fixed intact in the nucleic acids of mice.

It is evident from these results that DAP, while being a potent growth inhibitor, undoubtedly has multiple effects on cellular physiology which necessitate caution in interpretation of results obtained from its usage. However, the physiological actions of DAP seem to be primarily dependent on its structural resemblance to naturally occurring purines thereby making it a useful chemical in antimetabolite studies. This report describes the results of experiments designed to utilize the antimetabolite approach with DAP, at the cytological level, in the hope of further elucidating some of the physiological processes underlying cell division and cell enlargement.

Materials and Methods

All experiments in this investigation were performed on the primary roots of *Vicia faba* (broad bean) seedlings germinated from seeds of variety 9-B obtained from the Olds Seed

Co., Madison. The seeds were germinated by soaking them in water for 48 hours, stripping off the seed coats, and planting the naked embryos in well soaked vermiculite for 3 to 4 days. Resultant seedlings with healthy primary roots 7 to 12 cm. in length were selected for use in experiments. These seedlings were grouped into replicates on the basis of similarity in root length and thickness and the replicates were spread across all treatments. Unless otherwise noted, in all experiments similar sets of four replicates were used for each treatment at each time. At the beginning of treatment the plumules were removed from all seedlings. Four roots were always fixed directly from the vermiculite to give the initial (0 hour) mitotic frequency (see below).

Treatment was carried out by suspending the seedlings on plastic plates possessing small holes through which the roots were passed, over 800 ml. beakers containing aqueous solutions of the chemicals to be applied. Through a larger hole in the center of each plate a gas diffusion tube connected to a filtered air line was passed to the bottom of the beaker. A small stream of air passed through this tube was used to aerate and agitate the solution during treatment. All treatments were carried out in a room with an air temperature of 20 $\pm$ 1.5°C.

The following chemicals were used in the investigation: 2,6-diaminopurine sulfate from Mann Fine Chemicals, New York; adenine, guanine hydrochloride, xanthine, hypoxanthine, and adenosine from Nutritional Biochemicals Co., Cleveland; desoxyadenosine and 4-amino-5-imidazolecarboxamide hydrochloride from California Foundation for Biochemical Research, Los Angeles. For treatment, these chemicals were dissolved at the appropriate concentrations in water which had been distilled and subsequently "demineralized" (less than 1 p.p.m. ion concentration). This water alone served as the control treatment. The free purine bases were dissolved with the aid of heat while all other chemicals were dissolved at room temperature. Treatment solutions were adjusted to pH 5.8–6.0 with dilute HCl and NaOH. The pH of the solutions did not change significantly during the experiments.

Root length increase was determined by measuring, before and after treatment, the distance between the root tip and an India ink mark placed arbitrarily beyond the region of elongation (3 to 4 cm. from the root tip). All figures on length increase presented are arithmetic means of values from four roots.

Gray and Scholes (19) report that during a 20 hour period an elongating cell in a *V. faba* root increases its length tenfold while a complete mitotic cycle, during which a meristematic cell would in effect only double its size, takes about 24 hours. An analysis of control roots from several of the experiments reported here showed no correlation between variations in length increase and mitotic frequency. On the basis of these considerations it was concluded that in short term experiments of the type here used, root length increase can be taken as a reasonably good relative measure of cell elongation activity but cannot serve as even a rough indicator of mitotic activity.

Mitotic frequency was determined by the method described by Setterfield *et al.* (20). Essentially, meristems are fixed in 3:1 alcohol–acetic acid, stained by the Feulgen technique, and macerated to free cell suspensions through treatment with pectinase. The free cells are concentrated by centrifuging, and are mounted on slides in a mixture of karo syrup and phosphate buffer. By taking high power (×400) microscopic transects across such slides random samples of cells can be rapidly scored as to mitotic stage. Except in the photometric studies (see below), the first 2 mm. from each root used for length increase determinations was macerated individually and a sample of 1000 to 1100 cells scored. Thus, all values reported are arithmetic means of four separate frequency determinations based on 1000 to 1100 cells each.

In scoring nuclear stage the following five categories were used:—interphase: nucleus and nucleolus intact in appearance and chromatin in a diffuse condition; prophase: from the

first appearance of distinct chromosomal threads up to and including prometaphase, in which the chromosomes are fully contracted and nuclear membrane and nucleolus have disappeared; metaphase: visibly double, contracted chromosomes lined up on the metaphase plate; anaphase: from the beginning of chromatid separation until the daughter chromosomes have formed closely packed groups, with arms protruding, at each end of the cell; telophase: the period during which dense rounded groups of daughter chromosomes lie at each end of the cell up to the point when chromosomal strands are no longer visible in the fully formed nuclei of paired daughter cells. Since in these experiments there was no significant shift in the relative proportions of the latter four stages (the actual division stages) they were often lumped together and reported as per cent nuclei (or cells) in mitosis or "mitotic frequency."

In general, mitotic frequency may be taken as a measure of relative mitotic duration. Brown (21) has pointed out that this extrapolation can be misleading since for example, a decrease in mitotic frequency (the case usually met) may be due to either a relative shortening of the division stages (stimulation) or lengthening of interphase (inhibition). Although cell counts (see below) coupled with mitotic frequency determinations helped settle this question in these experiments, several other lines of reasoning can be used to overcome the objection. First, DAP has been shown to be inhibitory toward growth of a number of organisms; second, in the results reported below, DAP inhibits other processes such as cell elongation and desoxypentosenucleic acid synthesis and it is unlikely that while inhibiting these processes it would at the same time stimulate mitosis; third, extending the time of DAP treatment causes the mitotic frequency to reach zero which must mean that no mitoses are occurring and interphase is infinite. On these bases it can be safely inferred that in the experiments reported here, decreases in mototic frequency indicate lengthening of the interphase and concomitant retardation of the over-all division rate.

One experiment was performed which utilized the cell count technique described by Brown and Rickless (22) and Brown (21). Essentially the method involves measuring increase in cell number by macerating roots to free cell suspensions and determining the absolute cell number, before and after treatment, in a counting chamber. During the counting the frequency of meristematic and of non-meristematic cells is noted and by dividing the average number of meristematic cells into the number of new cells formed an average division rate per cell is obtained. The average duration of the complete mitotic cycle can then be calculated and multiplying this duration value by the percentage frequency of cells in any defined mitotic stage gives an estimate of the duration of that stage. Patau (data in preparation) objects to this last calculation on the basis that mitotic stage duration is not directly proportional to relative frequency since one telophase gives rise to two interphases. He has computed a general correction formula: $\frac{Tm}{Tc} = \frac{\log(1 + m)}{\log 2}$ in which, m = frequency of nuclei in any mitotic phase which ends with telophase, Tm = duration of the above defined phase and Tc = duration of the complete mitotic cycle. The correction is essentially a constant and therefore is not important when only relative durations are used for comparative purposes (frequency can be used directly), but it is of value when absolute durations are determined and it was applied to the data of this type presented here.

A modification of the original Brown and Rickless maceration procedure was used in the work described here. The portion of a root to be macerated was separated into a 2 mm. tip which was Feulgen-stained and macerated in pectinase and the remaining differentiated region which was macerated in 5 per cent chromic acid for 24 hours. Separate determinations of cell number were made on each suspension and the two results combined to give data for the entire original portion of root. The suspension obtained by pectinase maceration was concentrated and slides for mitotic frequency determination made from it. It was thus possible to have information on number of cells and mitotic frequency for the same root.

In the cell number determinations four aliquots from each suspension were counted, the total number of cells and number of meristematic and non-meristematic cells being determined in each. Cells with large vacuoles and their nuclei no longer in a central position were scored as non-meristematic. Occasional total cell values showed a large deviation (probably due to faulty loading of the counting chamber), necessitating the application of an arbitrary standard of acceptance to all sets of four total cell number counts. If one of the four values differed from the mean of the other three by more than four times the average deviation of the other three from that mean, it was discarded. With this standard, the probability is only 0.007 that a meaningful result is discarded (23).

Conventional microphotometric desoxypentosenucleic acid (DNA) measurements (24, 25) were made on random samples of Feulgen-stained interphase nuclei in slides prepared by a modification of the pectinase procedure. Eight roots from each treatment were fixed 10 minutes in 3:1 alcohol–acetic acid, washed 15 minutes in water, hydrolyzed 8 minutes in 1 N HCl, stained 1 hour in leucobasic fuchsin, and washed in three 10 minute changes of SO_2 water. After removal of the root cap under a dissection microscope a 1 mm. tip was cut from each of the eight roots and all eight 1 mm. pieces were placed together in 1 ml. of a 5 per cent aqueous pectinase solution for 12 hours. The pectinase solution was then decanted and the root segments were given two washes in water, 5 minutes each, and finally were shaken vigorously in about 1 ml. of water to give a suspension of free cells. The free cells were then centrifuged down, the water decanted and replaced with 70 per cent alcohol, and the cells resuspended by stirring. This centrifuging, decanting, and resuspending procedure was repeated so that in all the free cells were passed through two changes, 5 minutes each, of 70 per cent alcohol, 95 per cent alcohol, absolute alcohol, and toluene. Following the second change of toluene the cells were resuspended in technicon mounting medium and mounted on slides.

Measurement of Feulgen dye (DNA) bound in individual nuclei was carried out using a conventional microphotometer (24), incorporating a monochromator designed by Dr. K. Patau (26). A wave length of 5000 A, which Woodard (27) found to be optimal for Feulgen dye measurements of darkly staining nuclei on this instrument, was used. Random transects were made across the slides and all reasonably homogeneous and approximately spherical nuclei encountered were measured.

Values for the relative amount of DNA per nucleus were obtained by measuring the extinction of a central core of the nucleus and multiplying this by the product of two right-angle diameters of the nucleus. This procedure gives values directly proportional to the amount of absorbing substance in spheres and spheroids. It should be added that the relatively large nucleolus of *V. faba* nuclei introduced some error although it tended to be proportionately the same in all nuclei.

An approximate measure of relative nuclear volume was obtained by cubing the average nuclear diameter. This value is related to the true nuclear volume of a sphere by the constant factor $\frac{\pi}{6}$, and since only relative values were desired this factor was disregarded.

Microphotometric measurements of relative nuclear DNA content were also made using the sensitive two wave length method described by Ornstein (28) and Patau (29). These measurements were made on prophase nuclei in the meristematic region of 3:1 fixed (15 minutes) roots which were sectioned in paraffin and the sections passed together through hydrating solutions, HCl hydrolysis (8 minutes), Feulgen stain (1 hour), SO_2 water (3 changes, 10 minutes each), and dehydrating solutions. Technicon was used for mounting. The technical measuring procedure used was that described by Srinivasachar (26); wave lengths of 4900 A and 5132 A and a field size of 3 mm. with a × 43 objective and × 5 ocular were employed. Two "replicate" measurements of each nucleus were made, each replicate consisting of four

readings with the nucleus in the field and four of a blank area of the slide at each of the two wave lengths. Relative DNA content was calculated as described by Patau (29).

RESULTS

Cell Elongation and Mitotic Frequency Studies

Experiments indicated that 9.6×10^{-5} M DAP (20 p.p.m. DAP-sulfate) was the lowest concentration which would give extensive and reproducible inhibition of both root elongation and mitotic frequency. The effects of this

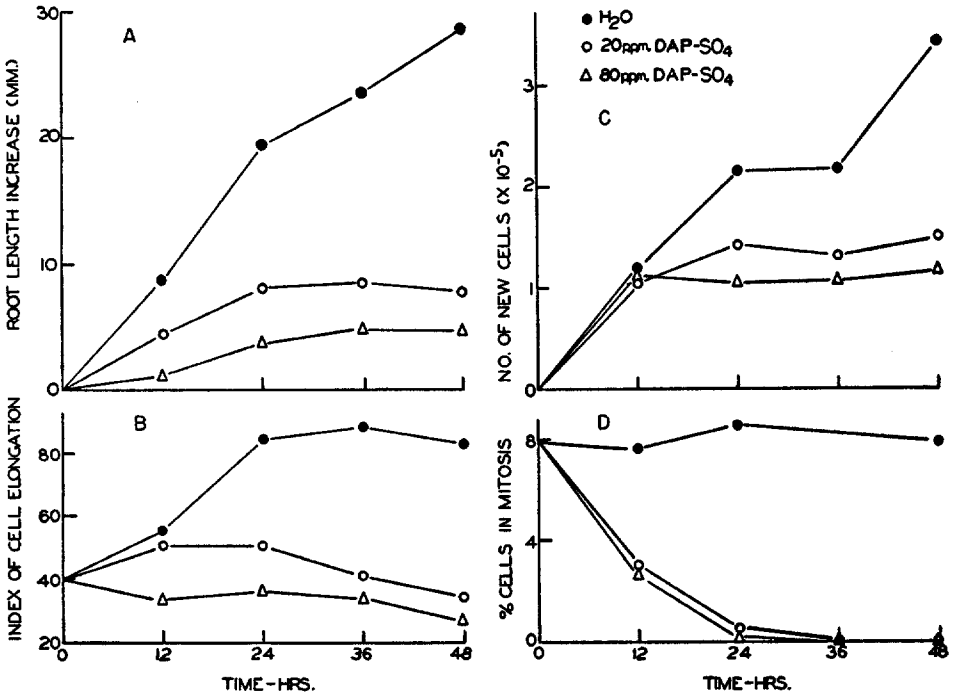

FIG. 1. Graphs showing results of cell count studies on *V. faba* roots treated with inhibitory concentrations of 2,6-diaminopurine (DAP). 20 p.p.m. DAP-SO_4 = 9.6×10^{-5} M DAP; 80 p.p.m. DAP-SO_4 = 3.8×10^{-4} M DAP.

and a second, higher concentration, 3.8×10^{-4} M DAP (80 p.p.m. DAP-sulfate), were studied by the cell count technique in an attempt to describe the inhibitory effects precisely. Fig. 1 illustrates the results of this cell count study. Four aspects of root growth were measured: over-all root length increase, cell elongation, cell production, and frequency of mitotic stages. The two concentrations of DAP gave qualitatively similar results in all respects with the higher one consistently being quantitatively somewhat more drastic.

The rate of root length increase was considerably slowed for the first 24

hours of DAP treatment and after that was completely stopped (Fig. 1 A). This growth behavior was reflected by changes in the index of cell elongation (Fig. 1 B). Index of cell elongation is a value derived by dividing the number of non-meristematic cells in a segment of root above the meristem into the length of that segment. It is a rough relative measure of the average length of the non-meristematic cells (22). The measurements were made on segments of roots below a mark placed 3 mm. from the root tip at the beginning of treatment. The meristem was assumed to be 2 mm. long (19) and thus the 0 hour determination included only the first 1 mm. of the elongation zone in which the cells are small and give a low index of cell elongation. In the control roots the index rose as growth below the original mark increased and more of the elongation zone was included in the root segment measured. Finally, fully elongated cells were in a preponderance and the index remained constant (from 24 hours on). In 9.6×10^{-5} M DAP (20 p.p.m. DAP-sulfate) the index rose to about the same extent as the controls during the first 12 hours, but remained at this level for the next 12 hours and then began a slow decline. This decline in the index was due to a continued slow increase in the number of cells non-meristematic in appearance (vacuolated, nucleus at the side of the cell) without a corresponding increase in root (cell) length. The higher DAP concentration showed a similar pattern except that the index dropped below the 0 hour value during the first 12 hours of treatment. This indicated that in this concentration of DAP the average non-meristematic cell size never reached that of cells of the first 1 mm. of the elongation zone in untreated roots.

Increase in total cell number is a direct result of the degree of mitotic activity. Fig. 1 C indicates that up until 12 hours neither concentration of DAP had great effect on cell division but during the second 12 hours of treatment cell production was stopped completely (somewhat earlier in the higher concentration than in the lower). These results are paralleled by those in Fig. 1 D, which shows the decline in mitotic frequency accompanying DAP treatment. Somewhere between 12 and 36 hours after treatment the mitotic frequency in both concentrations reached zero.

Table I shows the calculated average absolute durations of the mitotic stages obtained by combining the data on cell counts and frequency of mitotic stages. The values indicate that the primary action of DAP in inhibiting cell division was the prevention of nuclei from entering into prophase of mitosis thus causing interphase to become essentially infinite in length. There was no significant shift in either the relative or absolute duration of the actual division stages. It should be emphasized that these duration values are only average values for the whole meristem, which undoubtedly has cells with different mitotic rates within it, and are primarily of use in making comparisons. Furthermore, the values are influenced not only by treatment but also

by both variation between roots and random errors in the cell count technique. The sets of three control values show standard errors of about 20 per cent of the mean although the considerable increase in length of the mitotic cycle at 48 hours may be a reflection of the real slowing in rate which was observed in over-all root growth (Fig. 1 A). The mean control values, 22.6 hours for interphase and 2.9 hours for mitosis, agree well with those of Gray and Scholes (19) who, using a different technique, reported 20 to 25.6 hours for interphase and 2.2 hours for mitosis in *V. faba* roots.

TABLE I

The Average Duration of Mitotic Stages in V. faba Roots Grown in Water (Controls) and Two Concentrations of 2,6-Diaminopurine (DAP)

Determinations made at 12, 24, and 48 hours after treatment. Values were calculated by the method of Brown and Rickless, using correction factors developed by Patau (see Materials and Methods).

Time of determination...	H_2O			9.6×10^{-5} M DAP (20 p.p.m. DAP-SO_4)			3.8×10^{-4} M DAP (80 p.p.m. DAP-SO_4)		
	12 hrs.	24 hrs.	48 hrs.	12 hrs.	24 hrs.	48 hrs.	12 hrs.	24 hrs.	48 hrs.
Complete mitotic cycle....	18.6 hrs.	22.6 hrs.	35.3 hrs.	25.9 hrs.	46.9 hrs.	188 hrs.	27.0 hrs.	∞	253 hrs.
Interphase.	16.6 "	20.1 "	31.2 "	24.0 "	45.9 "	188 "	24.9 "	∞	253 "
Prophase...	76 min.	92 min.	139 min.	75 min.	40 min.	—	78 min.	—	—
Metaphase.	18 "	24 "	42 "	18 "	8 "	—	19 "	—	—
Anaphase..	14 "	16 "	32 "	11 "	5 "	—	11 "	—	—
Telophase..	14 "	20 "	34 "	13 "	8 "	—	15 "	—	—

As the data on duration of the individual mitotic stages would suggest, DAP at these concentrations did not structurally disturb the division process to any great extent. In the higher concentration, at 12 hours, there were some chromosome bridges and a few cases of nuclear reconstitution directly from metaphase or anaphase. These abnormalities were very infrequent at the lower concentration of DAP.

Numerous investigations have shown that adenine can reverse the inhibitory action of DAP and consequently the effects of this compound on the inhibition of cell enlargement and cell division by DAP were studied. Fig. 2 shows the results of two experiments of this type. A concentration of 4.8×10^{-5} M DAP did not affect cell elongation significantly but reduced the mitotic frequency by about 50 per cent. The mitotic inhibition was completely reversed by 3.3×10^{-4} M adenine, while this concentration of adenine alone gave no statistically significant difference from the control in either root elongation or mitotic frequency (Fig. 2 A). Fig. 2 B shows that, as found in

the cell count experiment, 9.6 × 10^{-5} M DAP gave complete inhibition of mitosis and root elongation by 36 hours. Adenine at a concentration of 3.3 × 10^{-4} M in combination with this concentration of DAP produced approximately an 80 per cent reversal of the mitotic frequency depression but gave less than 25 per cent reversal of the root growth inhibition. The chromosomes of the mitoses occurring in the mixtures of DAP and adenine showed a slight contraction in all stages but no other structural abnormalities occurred in significant frequency.

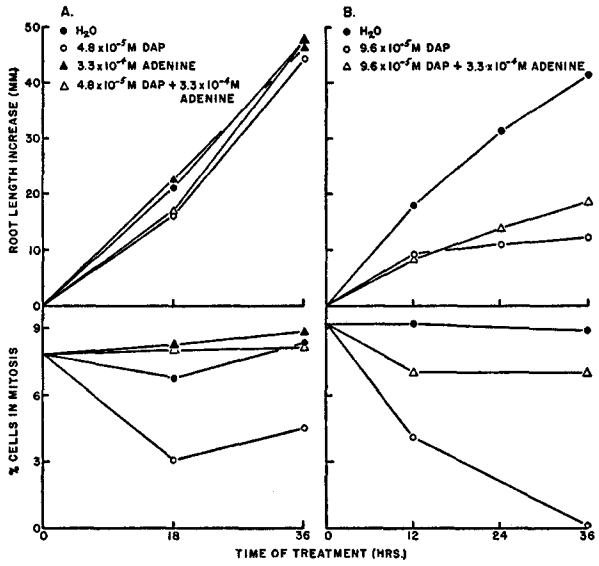

FIG. 2. Graphs showing the root length increase and mitotic frequency of *V. faba* roots treated with 2,6-diaminopurine (DAP), adenine, and DAP and adenine in combination.

Table II gives a summary of all mitotic frequency data obtained for DAP, adenine, and DAP plus adenine treatments, for 36 hours. It can be seen that mitotic frequency varies roughly inversely with the molar ratio of DAP to adenine supplied. Of particular interest is the fact that in the two mixtures of DAP and adenine which have the same molar ratio (0.28) but different absolute concentrations (one double the other), the mitotic frequency was of the same general magnitude (69.3 per cent and 78.0 per cent of the controls). A molar ratio of DAP to adenine of 0.56 gave 56 per cent inhibition of mitotic frequency indicating that the inhibition index is of the order of 0.5. Data on root length increase for these same roots showed no direct relation to the DAP-adenine ratio and the maximum reversal obtained only reached the order of 25 per cent.

The ability of several other compounds to reverse DAP inhibition of root length increase and mitosis was tested and the results are shown in Fig. 3. Solutions containing 9.6×10^{-5} M DAP were used throughout these experiments and all potential reversing agents were supplied at the concentration (3.3×10^{-4} M) at which adenine gave an 80 per cent reversal of the mitotic inhibition produced by this level of DAP. Fig. 3 A indicates that the nucleosides adenosine and desoxyadenosine produced about half maximal reversal of the DAP inhibition of root growth but were incapable of reversing the drop in mitotic frequency. Both compounds produced a slight but statistically significant depression of root growth and mitotic frequency when supplied alone (Fig. 3 B).

TABLE II

Mean Mitotic Frequencies (as Per Cent of Respective Controls) of V. faba Roots Treated with 2,6-Diaminopurine (DAP) and Adenine, Separately and in Mixture, for 36 Hours

DAP concentration	Adenine concentration	DAP concentration / Adenine concentration	Mitotic frequency
—	—	—	100.0
4.8×10^{-5} M	—	—	54.2
9.6×10^{-5} M	—	—	1.1
—	1.7×10^{-4} M	—	102.0
—	3.3×10^{-4} M	—	106.0
4.8×10^{-5} M	1.7×10^{-4} M	0.28	69.3
4.8×10^{-5} M	3.3×10^{-4} M	0.14	97.6
9.6×10^{-5} M	1.7×10^{-4} M	0.56	44.0
9.6×10^{-5} M	3.3×10^{-4} M	0.28	78.7

Buchanan (30) and Buchanan and Schulman (31) have presented evidence that the compound 4-amino-5-imadazolecarboxamide (AIC) may function as an intermediate in purine synthesis. Fig. 3 B shows that this compound was incapable of reversing inhibition of either root elongation or mitotic frequency by DAP and that when supplied alone at the same concentration was noticeably inhibitory toward mitosis by 36 hours. AIC caused an increase in chromosomal length in all stages of mitosis.

The activities of guanine, xanthine, and hypoxanthine as reversing agents are illustrated in Fig. 3 C. Guanine gave essentially complete reversal of the inhibition of root length increase while xanthine gave only about 50 per cent and hypoxanthine none. Guanine also gave a small but statistically significant reversal of the depression in mitotic frequency (20 per cent at 36 hours), while xanthine and hypoxanthine had no effect in this respect. The effectiveness of guanine in reversing root growth inhibition may be somewhat overemphasized since the control roots grew relatively slowly in this experiment.

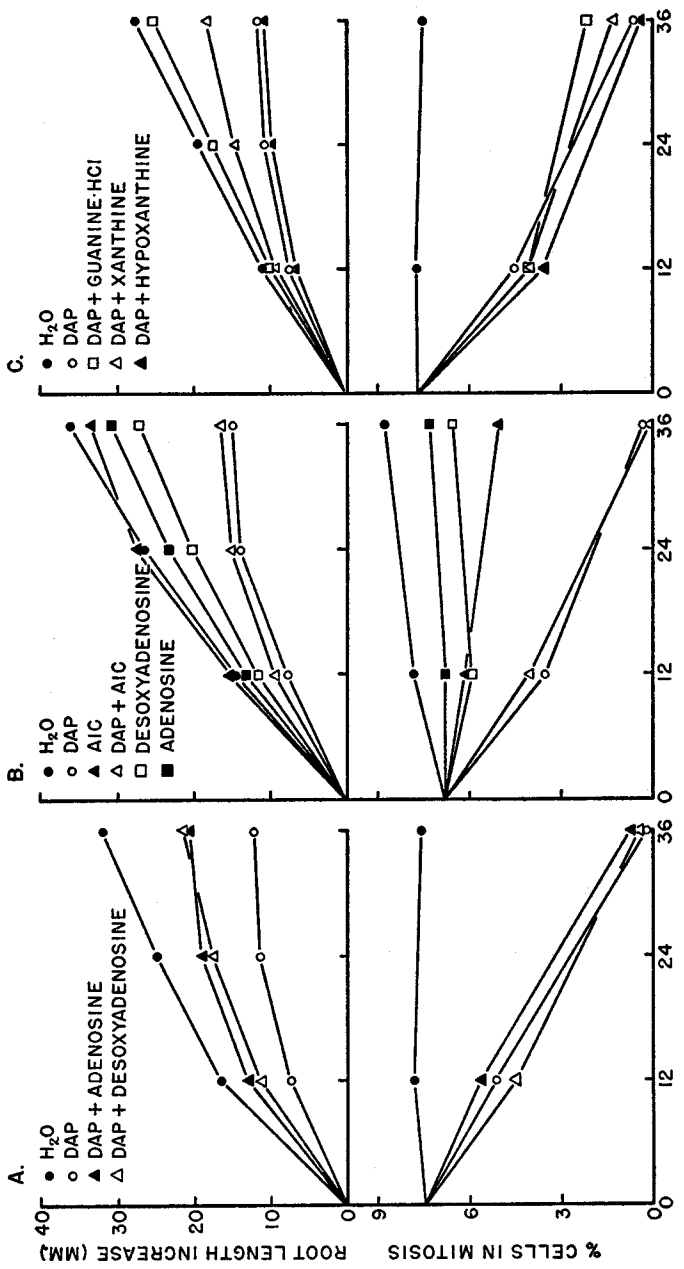

FIG. 3. Graphs showing the root length increase and mitotic frequency of *V. faba* roots treated with 2, 6-diaminopurine (DAP) alone and in combination with a variety of possible reversal agents. DAP was supplied at a concentration of 9.6×10^{-5} M in all cases; all other compounds were supplied at a concentration of 3.3×10^{-4} M.

Microphotometric Studies

Figs. 4 and 5 show the relative DNA content of randomly chosen individual interphase nuclei from treated and control roots plotted against the cube of their relative mean nuclear diameter. Mean nuclear diameter cubed is directly proportional to nuclear volume which in turn serves as a rough, though not necessarily strictly linear, time scale of interphase development (26). Control roots plotted in this manner (Figs. 4 C and 5 C) showed nuclei with the basic or 2 C (32) amount, intermediate amounts, and the doubled or 4 C amount of DNA in an approximately sigmoid distribution. The intermediate values represent nuclei undergoing DNA synthesis preparatory to the next cell division.

Meristems treated with DAP contained only two very striking groups of interphases with the 2 C and 4 C amount of DNA respectively (Fig. 4 B). The total absence of nuclei with intermediate values indicates a blockage of DNA synthesis. Adenine, adenosine, and desoxyadenosine supplied in combination with DAP (Figs. 5 A, 5 D, and 4 A, respectively) all caused an extensive appearance of intermediate DNA values without conspicuous retention of the sigmoid distribution found in the controls. Treatment with the combination of AIC and DAP on the other hand, showed the presence of primarily 2C and 4C nuclei and only a few intermediates (Fig. 5 B). The three reversal treatments, DAP plus adenine, adenosine, and AIC, were all repeated and the second series of measurements showed essentially the same distribution of nuclear DNA content as the first.

Mitotic frequencies determined in the slides used for microphotometric measurements are also presented in Figs. 4 and 5. Since only the first millimeter of the root tip was used for the photometric determinations, and since this segment contains the most active region of the meristem (19), these mitotic frequencies tended to be somewhat higher than those in the previous experiments, in which 2 mm. tips were used. It should be noted that adenosine and desoxyadenosine produced a small but statistically significant mitotic frequency reversal.

A further significant facet of the DNA content–nuclear volume scatter diagrams is a slight over-all depression, in comparison to the controls, of the DNA content values in the four combination (reversal) treatments, DAP plus desoxyadenosine, plus adenine, plus adenosine, and plus AIC (Figs. 4 A, 5 A, 5 D, and 5 B, respectively). The general drop is only of the order of 10 per cent, which is close to the limits of accuracy of the conventional microphotometric method, but it was consistently obtained in repeat experiments with these same treatments. Absorption curves from nuclei in controls and these reversal treatments were not significantly different, indicating that the depression of DNA values was not due to a change in the absorption characteristics of the bound Feulgen dye.

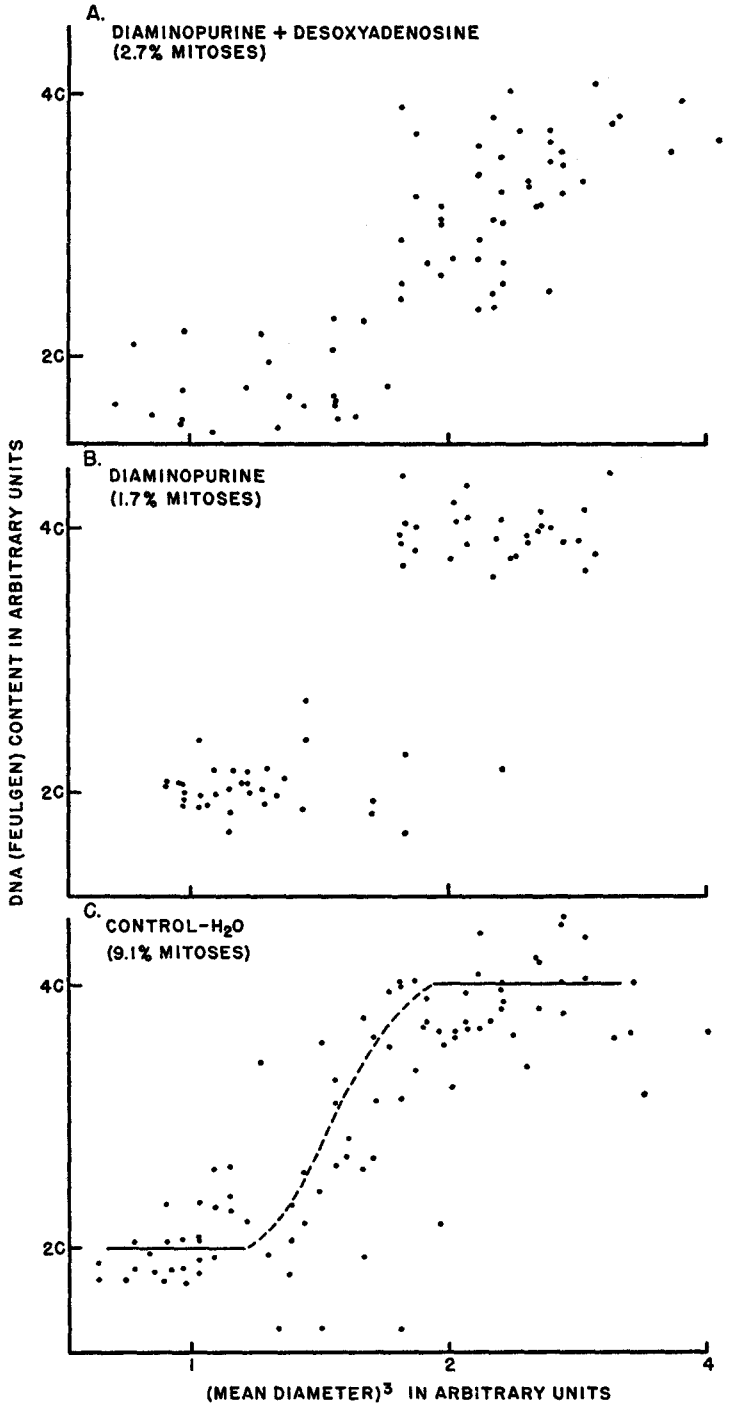

FIG. 4. Relative desoxypentosenucleic acid (DNA) content of randomly chosen individual interphase nuclei, in control and treated *V. faba* roots, plotted against the average nuclear diameters (in arbitrary units) cubed of the same nuclei. Scales on both axes are logarithmic. Determinations made using conventional microphotometric measurements (λ = 5000 A) of bound Feulgen dye. 2,6-diaminopurine (DAP) and desoxyadenosine supplied at concentrations of 9.6×10^{-5} M and 3.3×10^{-4} M, respectively.

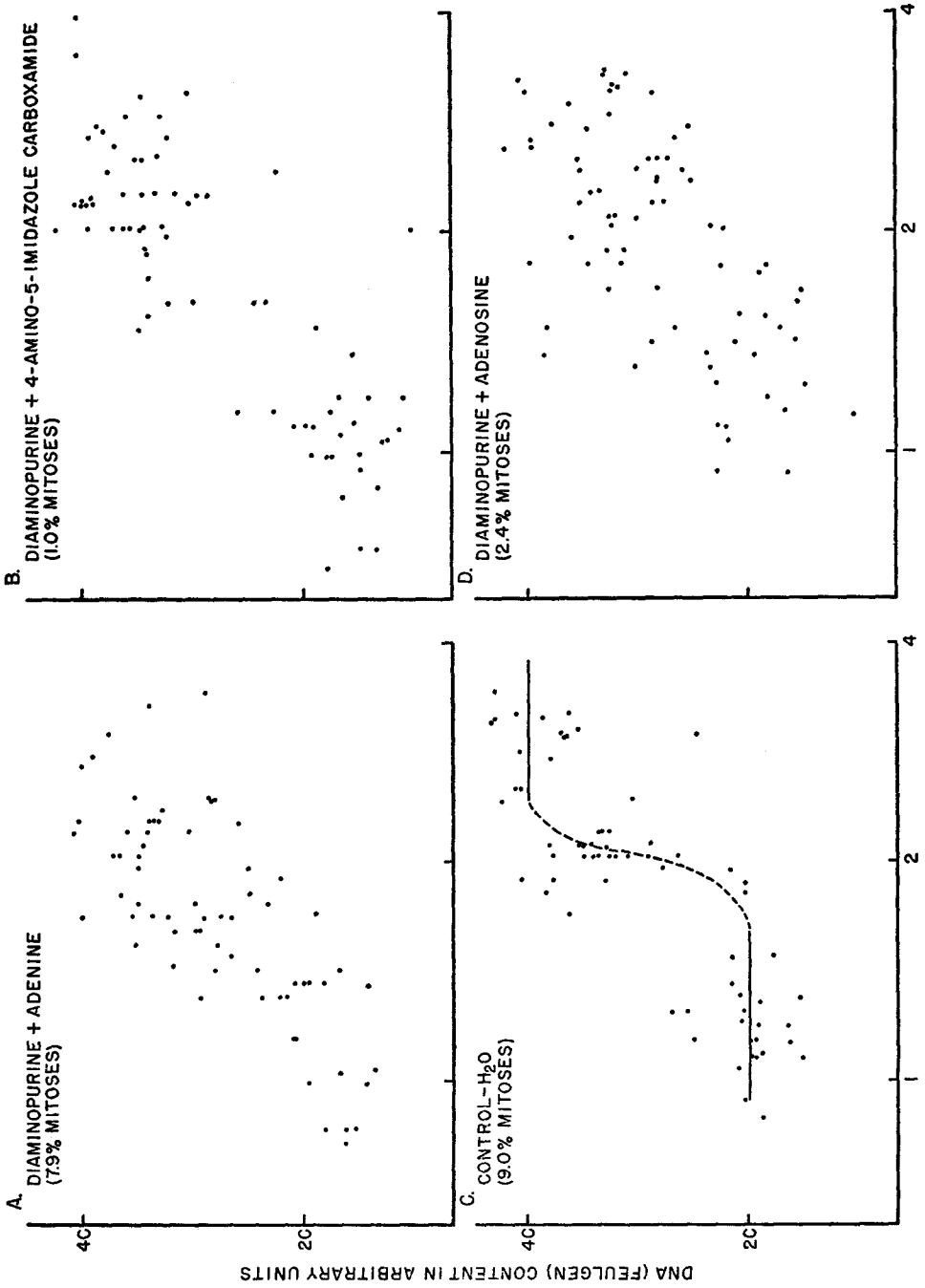

FIG. 5. Relative desoxypentosenucleic acid (DNA) content of randomly chosen interphase nuclei in control and treated *V. faba* roots plotted against the average nuclear diameters (in arbitrary units) cubed, of the same nuclei. Scales on both axes are logarithmic. Determinations made using conventional microphotometric measurements (λ = 5000 A) of bound Feulgen dye. 2,6-diaminopurine was supplied at a concentration of 9.6×10^{-5} M; all other compounds were supplied at a concentration of 3.3×10^{-4} M.

To investigate this phenomenon further, measurements of the Feulgen dye content of prophase nuclei in paraffin sections of roots were carried out using the sensitive two wave length microphotometric method. Prophases in control roots were compared with those in roots treated with DAP plus adenine; the results are shown in Table III. Despite the variation and slight overlapping in extreme values, the prophases in the reversal treatment had a mean relative DNA content about 9 per cent below the controls, the difference being statistically highly significant ($p = 0.005$).

TABLE III

Desoxypentosenucleic Acid (Feulgen Dye) Content, in Arbitrary Units, of Individual Prophase Nuclei in Roots Treated with H_2O (Controls), and 1.3×10^{-4} M 2,6-Diaminopurine (DAP) + 3.3×10^{-4} M Adenine, for 48 Hours

Determinations made on nuclei in paraffin sections using the two wave length microphotometric method ($\lambda_1 = 4900$ A, $\lambda_2 = 5132$ A).

	Treatment	
	Control, H_2O	DAP + adenine
	120.6	118.8
	134.6	122.6
	136.5	123.5
	138.8	125.7
	141.6	128.5
	142.2	134.6
	152.5	135.5
	154.9	138.2
Mean and S.E.	141.3 ± 3.1	128.4 ± 2.5
P (based on "*t*" test)	0.005	

DISCUSSION

The results on the inhibitory action of DAP are in general agreement with, and in some areas go beyond, the findings reported for other organisms. General growth inhibition by DAP as measured in most previous experiments is obviously an extremely complex and diverse process and needs further elucidation. In this investigation DAP inhibition was studied at three distinct points in cellular development; cell enlargement, cell division, and DNA synthesis. Although all three processes were completely inhibited by DAP treatment they responded differentially to the various reversing agents used.

Adenine, guanine, xanthine, adenosine, and desoxyadenosine all gave partial reversal of the inhibition of cell elongation, guanine and the nucleosides being more effective than adenine. This pattern of response differs from that generally reported for DAP inhibition of growth (and obtained here for

the inhibition of mitosis), in which adenine is usually the only or at least the most effective reversing agent. It does, however, somewhat parallel the reports by Miller (10) that adenine, adenosine, and adenylic acid were effective in reversing DAP inhibition of pea stem elongation and that adenine and guanine reversed inhibition of tobacco pith cell enlargement by DAP. The superiority of guanine to adenine as a reversing agent is a somewhat unique finding although Elion and Hitchings (11) found a similar effect with *L. casei* grown on a medium containing folic acid.

The relative superiority of the nucleosides as reversing agents strongly suggests that DAP inhibition of cell enlargement is through interference with nucleoside or nucleotide metabolism. The possibility that the supplied nucleosides are degraded to give free adenine as the active agent is ruled out by their superior reversing ability in respect to root elongation and the fact that they gave no reversal of DAP inhibition of cell division, in which adenine was very effective.

Inhibition by DAP through nucleoside or nucleotide metabolism could be brought about by disruption of the synthesis or physiological action of several cellular compounds; desoxypentosenucleic acid (DNA), pentosenucleic acid (PNA), the adenylic acid system, or the adenine-containing coenzymes. It has been shown by several workers that DNA occurs in a constant basic amount (or in some cases simple multiples of the basic amount) in all cells of the organism (33). Furthermore, autoradiographic studies have shown that little or no P^{32} uptake occurs in the DNA of nuclei in the elongation zone of the root (34). These facts tend to indicate that DNA is not a metabolite directly utilized in cell elongation, which in turn suggests that the DAP inhibition of cell enlargement is not primarily through DNA metabolism.

Although complete reversal of the inhibition of cell elongation was not obtained with any of the agents tried, the fact that guanine was considerably superior to adenine in reversing DAP inhibition of cell elongation suggests that the inhibition may be at least in part through adenine-guanine interconversion. This interpretation would be in agreement with the finding by Balis *et al.* (12) that the only effect of DAP on PNA metabolism in *L. casei* is blockage of the adenine-guanine interconversion. That PNA is probably an active substance during cell enlargement is indicated by the finding of Silberger and Skoog (35) that the PNA content of cultured tobacco pith increases in direct relation to increases in cell size. It is thus possible that at least part of the inhibition of cell elongation caused by DAP is through a disruption of PNA metabolism.

Inhibition of cell division by DAP, as measured here, takes the form of a prevention of entrance into prophase. Once division starts it proceeds at a normal rate to completion. The inhibition is not caused by a failure of DNA synthesis since about half of the nuclei in the meristem possess the doubled (4C) amount of DNA necessary for cell division (Fig. 4 B).

Inhibition of cell division through an inhibition of entrance into prophase is given by numerous types of treatment including compounds which inhibit various areas of the glycolytic and tricarboxylic acid energy systems (36). Bullough and Johnson (36) have designated this sensitive period of the interphase immediately preceding prophase, the antephase. They feel that it is a period when sufficient energy-rich compounds are accumulated to carry the cell completely through division. It is possible that DAP interferes with the accumulation of energy-rich compounds such as adenosinetriphosphate during the antephase but it is also possible that the inhibition is through a separate system which serves to "trigger" entrance into mitosis.

The inhibition of entrance into prophase of mitosis was reversed only by adenine and guanine. The reversal by adenine ranged up to complete and varied with the molar ratio of DAP to adenine supplied. This along with the fact that the same molar ratio of DAP to adenine at different absolute concentrations produced roughly the same degree of reversal, indicates a competitive relationship between DAP and adenine in respect to the onset of mitosis. Reversal by guanine was only slight and was probably due to conversion into adenine. The specific, competitive reversal by adenine strongly suggests that the action of DAP in inhibiting cell division is a direct interference with utilization of free adenine. If this effect is on the formation of nucleosides and nucleotides it is difficult to understand why adenosine and desoxyadenosine showed complete inability to reverse inhibition of cell division. It would seem that there is a basic need for free adenine in the physiological systems responsible for the onset of mitosis. Both Elion and Hitchings (11) and Balis *et al.* (12) noted an apparently similar basic need for free adenine in the growth of *L. casei.* Howell and Skoog (37) report that adenine sulfate exerts a marked growth promoting effect on excised *Pisum* epicotyls grown *in vitro* and they suggest that a specific factor, to which adenine is related, is synthesized in roots.

It is interesting that the inhibition index obtained in respect to the DAP-adenine competition in the inhibition of mitosis was less than one, suggesting that DAP is a very efficient competitor for enzymes utilizing adenine. Miller (10), who found adenine to be specific in reversing DAP inhibition of tobacco callus growth and bud formation, was also forced to use considerably higher molecular concentrations of adenine for reversal than those of DAP necessary for inhibition. This would indicate an inhibition index of less than one and may suggest that the same systems had been interfered with. Results with other organisms generally show inhibition indices greater than one although Biesele *et al.* (7) found a range of 0.5 to 256 in respect to the ability of adenine to prevent damage to mouse and rat tissues by DAP.

Concerning the studies on DNA metabolism, the complete absence of nuclei with DNA contents intermediate between the 2C and 4C (basic and doubled) levels in meristems treated with DAP indicates that DAP prevents

the initiation of DNA synthesis while allowing synthesis which is already in progress to reach completion. A similar type of inhibition of DNA synthesis has been obtained in onion root tips by Duncan and Woods (38), using benzimidazole, and by Swift (39), using x-rays and nitrogen mustard treatment. Swift considers the sharp separation of nuclei with the basic (2C) amount of DNA from those with the doubled (4C) amount to be evidence for a fairly rapid doubling of DNA content (perhaps occurring in all chromosomes of a nucleus at the same time) in normal roots. He suggests that DNA synthesis is an all-or-nothing reaction.

That DNA synthesis does take up a relatively short period of the entire interphase is evidenced by the sigmoid distribution, in control roots, of 2C, intermediate, and 4C nuclei, when plotted against relative nuclear volume ("age" of interphase). Swift (39), using the conventional microphotometric method, and Srinivasachar (26), using the more accurate two wave length method, obtained similar distributions in onion root tips. Synthesis may even be faster than the scatter diagrams indicate since it may not always start at the same point in relation to nuclear volume. This, in effect, would mean that the ascending portion of the sigmoid is multiple.

Adenine, adenosine, and desoxyadenosine all apparently reverse the blockage of DNA synthesis by DAP since in root tips treated with DAP in combination with these chemicals a high frequency of nuclei with intermediate DNA contents is present. However, these intermediate values extend over a much wider volume range than in control roots and no conspicuous indication of a sigmoid distribution is present. Furthermore, no exceptional increase in 4C nuclei occurred in the nucleoside reversals which have a very low mitotic rate. These facts suggest that in the reversal treatments synthesis is proceeding at a slower rate than normal.

Since DNA is a polynucleotide compound it is not surprising that reversal of DAP inhibition of synthesis is given by both free adenine and the adenine nucleosides. With the type of analysis used it is impossible to say whether or not the reversal was competitive or which, if any, of the reversing agents is superior. It is thus difficult to decide at what point in DNA synthesis DAP is acting; purine utilization, nucleoside and nucleotide synthesis and/or nucleotide polymerization could all be affected. It should also be pointed out that in these experiments, adenosine and desoxyadenosine gave slight reversals of the mitotic frequency, probably due to liberation of adenine from the nucleosides during the longer treatment time used in the microphotometric studies. It is therefore conceivable, although unlikely, that the reversal caused by the nucleosides was due to free adenine.

The consistent small depression of DNA content of interphase nuclei in the reversal treatments and the lowered DNA content of the prophases of the adenine reversal are somewhat unique findings. Interpretation of the phe-

nomenon is difficult since it has been generally established that DNA occurs in constant amounts (or simple multiples of the basic constant amount) in all cells of organisms of the same species. Further, Swift (33) lists a number of treatments (*e.g.*, low protein diet and vitamin B_{12} deficiency) which induce severe metabolic changes in rats but which have no effect on the basic "constancy" of DNA.

Several tentative explanations of the phenomenon are possible. The rate of DNA synthesis may be so slowed that relatively few nuclei have reached the 4C condition in the time of treatment used, DNA synthesis may be completely unable to go to completion, or the DNA may be qualitatively changed so that a fraction of it is now unmeasurable by the Feulgen technique. Any of these effects could conceivably come about through direct incorporation of DAP into the DNA. It is noteworthy that the depression effect found here occurred only when inhibition of DNA synthesis by DAP was reversed; that is, when DNA synthesis was allowed to proceed in the presence of normally inhibitory concentrations of DAP. However, a slow degeneration of the DNA or undetected systematic errors in determination could also account for the decline in relative amount. If the effect is real, it is surprising that in the adenine reversal, mitosis could start and proceed relatively normally to completion (only a general contraction of the chromosomes was observed), with a reduced amount of Feulgen-measurable DNA.

The results from the attempts at reversal with 4-amino-5-imidazolecarboxamide (AIC) show that this compound is incapable of reversing DAP inhibition of cell enlargement and cell division and gives only a slight possible reversal of DAP inhibition of DNA synthesis. This general lack of response indicates that AIC is unable to give rise to adenine in the presence of DAP. The lack of activity may be due to a failure of the compound to enter the cells, the absence of an enzyme system which utilizes the compound in purine synthesis, or a blockage by DAP of its conversion into purine. The latter interpretation agrees somewhat with the finding by Elion and Hitchings (11) that DAP inhibition of *L. casei* growth is stronger in a medium containing folic acid than in one with a purine and thymine. However, it is not possible to say whether the inhibition is directly on the folic acid system or through energy systems which may be connected with purine synthesis. Since AIC gave no reversal, inhibition does not appear to be competitive interference with utilization of AIC.

It is also worthy of note, that throughout these experiments, adenosine and desoxyadenosine gave exactly the same responses, suggesting that they are directly interconvertible. This might indicate that desoxyribonucleosides (or nucleotides) are derived from ribonucleosides (or ribonucleotides) rather than through a direct coupling of purines and desoxyribose.

SUMMARY

At a concentration of 9.6×10^{-5} M, 2,6-diaminopurine (DAP) completely inhibited cell enlargement, cell division, and DNA synthesis (determined by microphotometric measurement of Feulgen dye) in *Vicia faba* roots.

Inhibition of cell enlargement was partially reversed by adenine, guanine, xanthine, adenosine, and desoxyadenosine. Guanine and the nucleosides gave the greatest reversal, suggesting that one point of DAP action upon cell enlargement is a disruption of nucleoside or nucleotide metabolism, possibly during pentosenucleic acid synthesis.

DAP inhibited cell division by preventing onset of prophase. At the concentrations used it had no significant effect on the rate or appearance of mitoses in progress. Inhibition of entrance into prophase was not directly due to inhibition of DNA synthesis since approximately half of the inhibited nuclei had the doubled (4C) amount of DNA. Adenine competitively reversed DAP inhibition of cell division, giving an inhibition index of about 0.5. Guanine gave a slight reversal while xanthine, hypoxanthine, adenosine, and desoxyadenosine were inactive. A basic need for free adenine for the onset of mitosis was suggested by this reversal pattern.

Meristems treated with DAP contained almost no nuclei with intermediate amounts of DNA, indicating that DAP prevented the onset of DNA synthesis while allowing that underway to reach completion. The inhibition of DNA synthesis was reversed by adenine, adenosine, and desoxyadenosine although synthesis appeared to proceed at a slower rate in reversals than in controls. Inhibition of DNA synthesis by DAP is probably through nucleoside or nucleotide metabolism.

A small general depression of DNA content of nuclei in the reversal treatments was observed. This deviation from DNA "constancy" cannot be adequately explained at present although it may be a result of direct incorporation of DAP into DNA.

The possible purine precursor, 4-amino-5-imidazolecarboxamide gave no reversal of DAP inhibition of cell elongation and cell division and only a slight possible reversal of inhibition of DNA synthesis.

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