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Effects of the Plant Amino Acid Mimosine on Cell Division, DNA, RNA and Protein Syntheses in Paramecium

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SUMMARY

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The amino acid mimosine completely inhibited cell division in *Paramecium tetraurelia* at submillimolar concentrations; DNA, RNA and protein syntheses were all found to be inhibited by mimosine. Mimosine's effect on cell division was reversible after 48 hours exposure but irreversible after 96 hours of exposure. Thymidine-5′-triphosphate was unable to protect *Paramecium* against the effects of mimosine. Binding of mimosine to purified DNA could not be demonstrated using either equilibrium dialysis or linear dichroic spectral ratios; the presence of 0.025 mM mimosine, however, caused temperature dependent abosrbance profiles of DNA to show a 3% hyperchromicity at 40° (1 × SSC, 250 nm) and an 8% hypochromicity after melting (above 80°). Adding mimosine to an *in vitro* DNA polymerase system resulted in a 30% to 50% reduction of nucleotide incorporation. DNA, mimosine and DNA polymerase may form a ternary complex that blocks both DNA and RNA syntheses; however, failure of TTP to reverse mimosine inhibition suggests that unknown factors may be involved.

INTRODUCTION

Seeds of the tropical leguminous shrub *Leucaena leucocephala* (Lam) de Wit are toxic and exhibit depilatory activity. The active agent is β-(N-(3-hydroxypridone-4))-α-aminopropionic acid (*l*-mimosine) (1). Hegarty *et al.* (2) suggested that its final effect in depilation in sheep was on the proliferative phase of fiber growth rather than on keratinization. Later work (3) demonstrated mimosine's ability to inhibit thymidine incorporation into DNA in mouse bone marrow cell culture. Tsai and Lin (4–7) supported Hegarty by showing that mimosine inhibited mitosis and DNA synthesis in H.Ep-2 hepatoma cell cultures. RNA and protein syntheses were not affected in the short term (25 hours). DeWys and Hall (8, 9) demonstrated an *in vivo* dose-related inhibition of tumor growth in male rats and mice by mimosine while reversible infertility and loss of weight of the reproductive organs in female rats were shown by Hylin and Lichton (10).

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The work of Ward and Harris (11) and Hegarty *et al.* (3), when taken with earlier findings (4, 12) that 3,5-dihydroxypyridine has over 50% of the toxicity of mimosine, strongly suggests that the presence of —OH in the 3-position and —O or —OH in the 4-position of the ring confers toxicity. Alterations to the side chain also affect activity.

Studies reported below describe the effect of mimosine on cell division, DNA, RNA and protein syntheses in *Paramecium tetraurelia*. Mimosine does not interact *in vitro* with DNA at physiological temperatures, but ultraviolet absorbance profiles at elevated temperatures indicate that mimosine-DNA interactions can occur. The data suggest that *in vivo* either mimosine does not interact directly with DNA or additional unknown substances are required for such an interaction to occur.

MATERIALS AND METHODS

Paramecium tetraurelia Stock 51 (KK sensitive) were used throughout (13). Monoxenic and axenic cultures were grown and handled as described previously (14–16).

Mimosine was supplied by the CSIRO Division of Organic Chemistry. It was further purified by 5X recrystallization from ethanol. Concentrations of mimosine solutions were determined spectrophotometrically ($E^{282}_{1cm} = 17{,}000$). Phleomycin was a gift from Dr. G. W. Grigg. Tris[1] (Trizma Base, reagent grade) was purchased from Sigma Chemical Company (St. Louis, Mo.). Radiochemicals were supplied by the Radiochemical Centre, Amersham. Studies of protein synthesis utilized *l*-[U-^{14}C]amino acid mixtures with specific activities of 55 to 57 mM/mAtom carbon. Incorporation of uridine into RNA was followed using [5-^{3}H]uridine, specific activity 5.0 Ci/mmol while DNA synthesis was followed using (6-^{3}H) thymidine, specific activity 5.0 Ci/mmol.

Labeling of nucleic acids was performed using the method described by Sherton and Kabat (17). Samples were prepared for counting using 0.5 ml of NCS (Amersham/Searle) to dissolve damp TCA precipitates, which were transferred to counting vials with toluene, which contained 6 g/l of 2,5-Diphenyloxazole (PPO, Packard). Labeling of protein was done by adding *l*-[U-^{14}C]-amino acid mixture (0.75 μCi/ml final concentration) to one ml of mimosine solution (various concentrations) plus 5 ml of axenically growing cells (in log phase) and incubating at 25° for various intervals. Samples were prepared for counting as previously described (16). The obtaining and analyses of polyribosomes from *Paramecia* approaching log-phase growth have been described fully (16). Mimosine was added to cell cultures in a solution of 8.3 mM tris-HCl, pH 7.6.

Analyses of linear dichroic changes, developed when mimosine was added to DNA solutions, were kindly performed by Dr. Bengt Norden, (19–21). For the studies with mimosine a hydrodynamic gradient of 3000/sec was employed and because of the large absorbance shown by mimosine near the DNA absorbance maximum, the linear dichroic ratio LD_{280}/LD_{260} was determined.

Binding studies by equilibrium dialyses were set up essentially according to the methods described by McMenamy (22). Tritiated mimosine (2.1 Ci/mmole) was prepared for us by the Radiochemical Centre (Amersham) by catalytic exchange with tritium gas (20).

DNA from *Paramecia* was prepared as described (18, page 349). Differential DNA melting curves in SSC, pH 7.7, were obtained at 250 nm in order to minimize the contribution of mimosine to the absorbance readings. Runs were made by continuous heating at a rate of 0.5°/min. In order to preclude any bias, they were duplicated in such a way that the sample and blank compartments were used for all mixture combinations. Partially purified DNA polymerase from wool roots was obtained using a modification of the method of Bachmann and Lexius (24) (K. A. Ward, in preparation). Activity in the presence and absence of mimosine was assayed at 37° (60 min) in 0.5 ml buffer containing 50 mM tris-HCl

[1] The abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; PCA, perchloroacetic acid; BSA, bovine serum albumin; DEAE, diethylaminoethyl; NCS, Nuclear-Chicago solvent; LD, linear dichroism; TTP, thymidine-5'-triphosphate; CMP, cytidine 5'-monophosphate; SSC, 0.15 M NaCl, 0.015 M Na citrate.

(pH 7.6 at 37°), 2 mM $MgCl_2$, 5 mM dithiothreitol, 80 μm dATP, dCTP, dGTP, 20 μM [methyl-^{3}H]TTP (Amersham, 50 mCi/mmole), 200 μg/ml calf thymus DNA (native or heat denatured), 3% (v/v) glycerol and 0.1 ml enzyme preparation (50 to 200 μg of protein). Reactions were terminated with the addition of 1 mg BSA, 0.02 M sodium pyrophosphate-1% (w/v) TCA, precipitates collected by centrifugation, washed and then dissolved in Soluene-350 (Packard) and counted in a standard toluene based scintillant.

Studies of RNA synthesis mediated by RNA polymerase were carried out by Dr. Jane Prosser as described by Dunsmuir (25).

RESULTS

Paramecia in axenic culture (200/ml) were mixed with mimosine, at various concentrations from 0.01 mM to 5 mM; concentrations of 0.03 mM and 0.09 mM depressed the rate of cell division but did not inhibit it completely, whereas 0.25 mM and 0.75 mM completely inhibited cell division. The animals appeared healthy, though nondividing, after 11 days. At higher concentrations, the *Paramecia* had died. The introduction of TTP into the cultures at concentrations up to 0.5 mM had no effect, whether it was added together with the mimosine or after 48 hours.

In order to determine the reversibility of mimosine's effect on cell division, *Paramecia* were exposed to 0.0, 0.25 and 0.5 mM mimosine for 5 min and 24, 48, 96 and 144 hours after which the cultures were diluted 20-fold with fresh medium and the incubation continued. Exposure for 5 min and 24 hours had little or no effect on subsequent growth (Fig. 1a), but a 48 hour exposure (Fig. 1b) caused some lag in recovery. On the other hand, 96 hours exposure (Fig. 1c) had a marked effect on the ability of *Paramecia* to resume cell division. Once the mimosine was diluted many of the *Paramecia* died even though the cultures appeared normal at the time of dilution. About 40% of the cells survived the 0.25 mM treatment but less than 15% survived exposure to 0.5 mM mimosine for 96 hours once the culture had been diluted. Exposure to 0.5 mM mimosine for 144 hr caused irreversible inhibition of cell division in all cells, and they died within six days following dilution.

Figures 2 and 3 summarize the evidence of mimosine effects on nucleic acid and protein synthesis. It has been shown that mimosine inhibits DNA synthesis in tumor cultures and sheep skin sections (4, 5, 11): the same is the case in *Paramecium*. However, the data reported in Figure 2 indicate that contrary to previous reports on other systems (4–7), the RNA synthesis was also suppressed; indeed, a concentration of 2 mM completely blocked uridine incorporation after 48 hours. For both nucleic acids, inhibition was greater after 48 hours of exposure than during the first few hours. No reduction of protein synthesis was observed within the first 18 hours of mimosine treatment (Fig. 3a), but 75% to 80% inhibition could be demonstrated with 0.5 mM mimosine after 48 hours (Fig. 3b, c). Furthermore, cells starved overnight in the presence of mimosine were still able to utilize the 80s ribosomal pool to form polyribosomes in a manner indistinguishable from controls (16). Of possible interest was our finding that in some experiments, short exposure of cells in the log phase of growth seemed to cause an increase in the very large polyribosomes—as if mRNAs became fully packed with 80s ribosomes. However, it must be emphasised that the effect was transitory and was not always observed.

A number of different approaches were used to determine whether or not a direct interaction between DNA and mimosine could be demonstrated. Absorbance spectra between 220 and 400 nm (22°) of mixtures of DNA and mimosine were indistinguishable from the sum of their spectra when measured separately; linear dichroic measurements (19–21) showed that with a shear gradient of 3,000/sec, LD_{280}/LD_{260} was not significantly altered by the presence of 0.6 mM mimosine in a 300 μg/ml DNA solution (0.53 vs 0.54 under the experimental conditions [19]), and the results from equilibrium dialyses using either denatured or double stranded DNA were negative. Finally, the use of mimosine in the phleomycin-stationary phase *E. coli* system (26, 27)

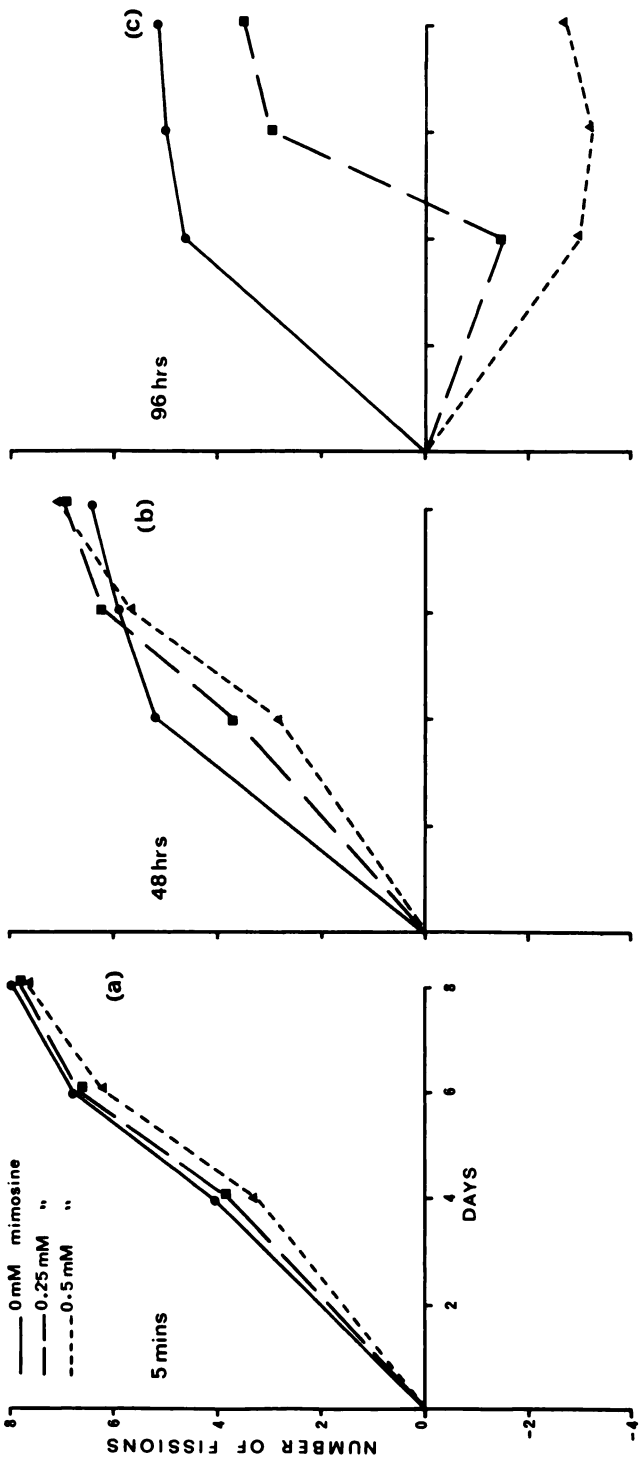

FIG. 1. *The effect of exposure to mimosine for various times on ability of the animals to resume cell division following dilution of the drug*

Paramecia grown in axenic culture were exposed to mimosine for varying lengths of time. The cultures were then diluted 20-fold and each culture divided into triplicate. Population densities (P) were determined 4, 6, and 9 days after dilution and converted to fission numbers ($\log_1 [P - P_0]$) where P_0 = population/ml immediately after dilution.

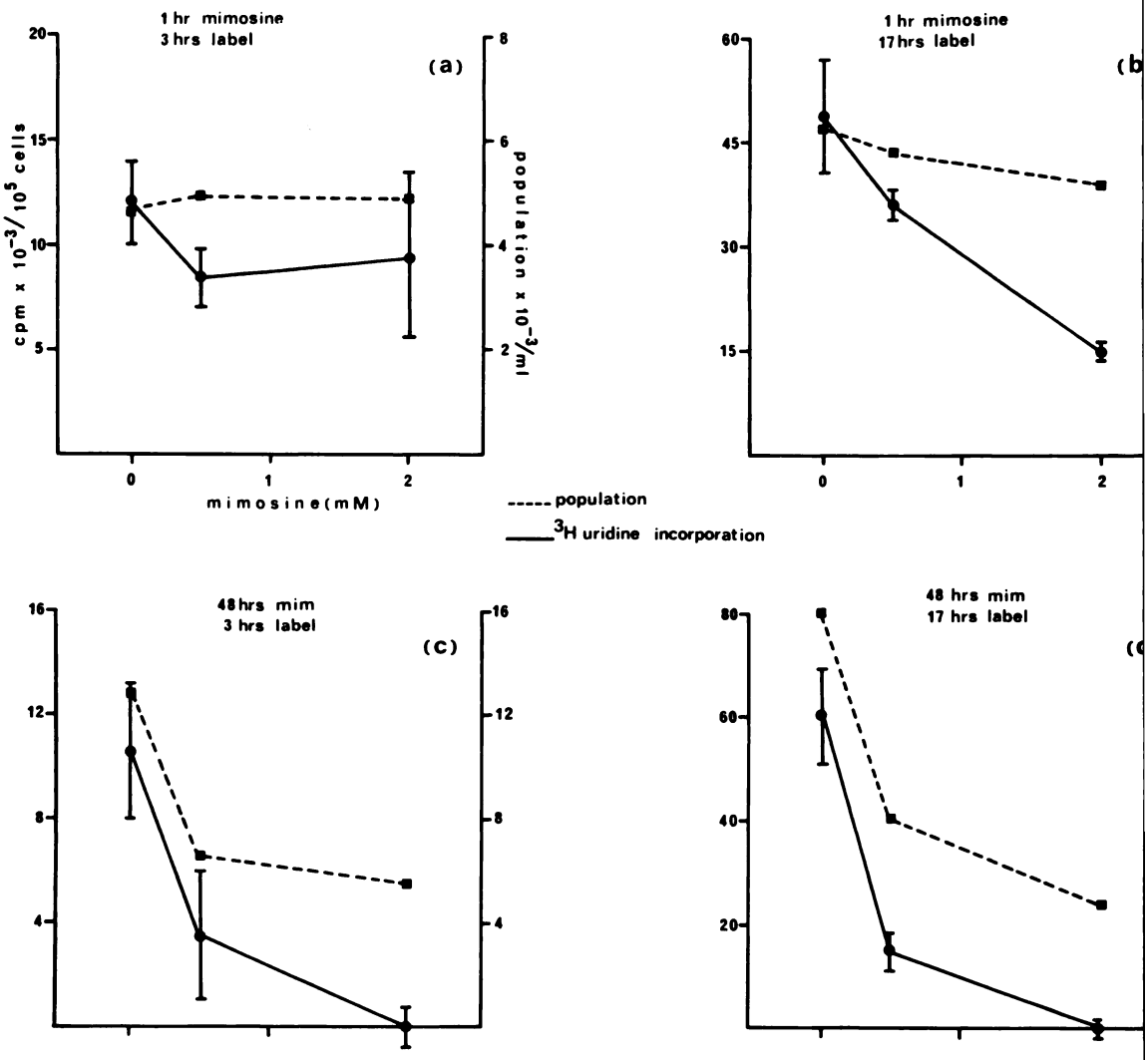

FIG. 2. *Incorporation of uridylic acid into RNA in the presence of mimosine*

Triplicate 3 ml axenic cultures were set up starting with *Paramecia* in log phase. The animals had been grown to a cell density of about 3,200/ml. Mimosine was then added to give final concentrations of 0.5 mM and 2.0 mM and the cultures incubated at 25° for 1 hr or 48 hr prior to the introduction of label. Following the introduction of label the cultures were incubated for an additional 3 hr or 17 hr and then prepared for determination of incorporated label into RNA, as described in METHODS. Ordinate scales are adjusted to improve data presentation. Bars in this figure and Figures 4 and 5 indicate SD.

had no potentiating effect on DNA breakage, even when used at concentrations 2,000 times that required of crystal violet.

The effect of mimosine on the DNA melting profile suggests, however, that such an interaction can occur at elevated temperature. Figure 4 shows that mimosine appears to have two distinct effects on DNA structure. At temperatures below 76°, the 0.025 mM mimosine–15 mg/ml DNA mixtures were hyperchromic relative to the control of mimosine and DNA in separate cuvettes. However, above 67°, hypochromicity occurred (8–10%) and T_m was lowered 1.5°.

Concentrations of mimosine up through 2 mM had no effect on RNA synthesis *in vitro*; indeed, 10 mM mimosine caused only a 35% reduction of incorporation of CMP

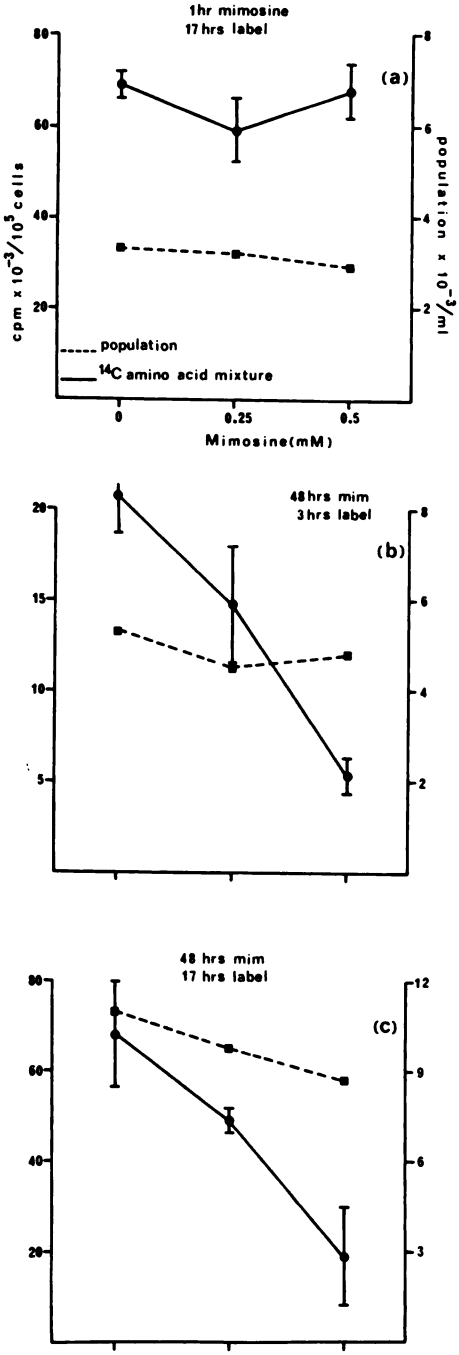

FIG. 3. *Incorporation of amino acids into protein in the presence of mimosine*

Cultures were set up as described for Figure 2 but grown to a cell density of about 3,600 cells/ml. Following incubation the cultures were prepared for radioactive counting. Ordinate scales are adjusted to optimize data presentation.

into RNA. The effect of mimosine at a concentration of 2 mM on DNA polymerase activity *in vitro* is reported in Table 1. The data support the suggestion that mimosine is able to interfere with DNA polymerase activity. The addition of 2 mM mimosine resulted in 30%–50% inhibition of the [^{3}H]TTP incorporation.

While fractionation of the crude cytosol by DEAE-cellulose column chromatography did not result in increased specific activity, some fractionation was indicated by the finding that fraction II utilized heat-denatured DNA as a template whereas fraction I and the crude cytosol preparations did not (K. A. Ward, unpublished data).

DISCUSSION

The short term effects of mimosine on cell division in *Paramecium* were comparable to its effects in other systems in that it was able to block cell division completely at concentrations of 0.25 mM while removal of the amino acid allowed cell division to recommence. However, new findings in the present study require interpretation. (Prolonged exposures (48 hr) of *Paramecia* to mimosine inhibited RNA and protein syntheses as well as DNA synthesis but inhibition of protein synthesis was significantly delayed.) *In vitro*, mimosine up to a concentration of 2 mM had no effect on RNA polymerase activity, but caused a 30% to 50% inhibition of DNA polymerase activity. In addition, while the phleomycin-*E. coli* system did not indicate any DNA breakage effect of mimosine in bacteria in stationary-growth phase, and neither linear dichroic measurements nor equilibrium dialyses suggested direct DNA-mimosine interaction, the temperature dependent DNA profile did indicate that such interaction can occur at elevated temperatures. Control experiments showed that mimosine in 1 × SSC showed a 3% linear increase in absorbance (250 nm) over the 22° to 95° range examined (DNA from *Paramecium* has a T_m of 78° and exhibits a hyperchromicity of 37%). Sarocchi and Guschlbauer (28) have shown that in the premelting region, DNA shows at 250 nm a characteristic hypochromicity which reaches −0.5%

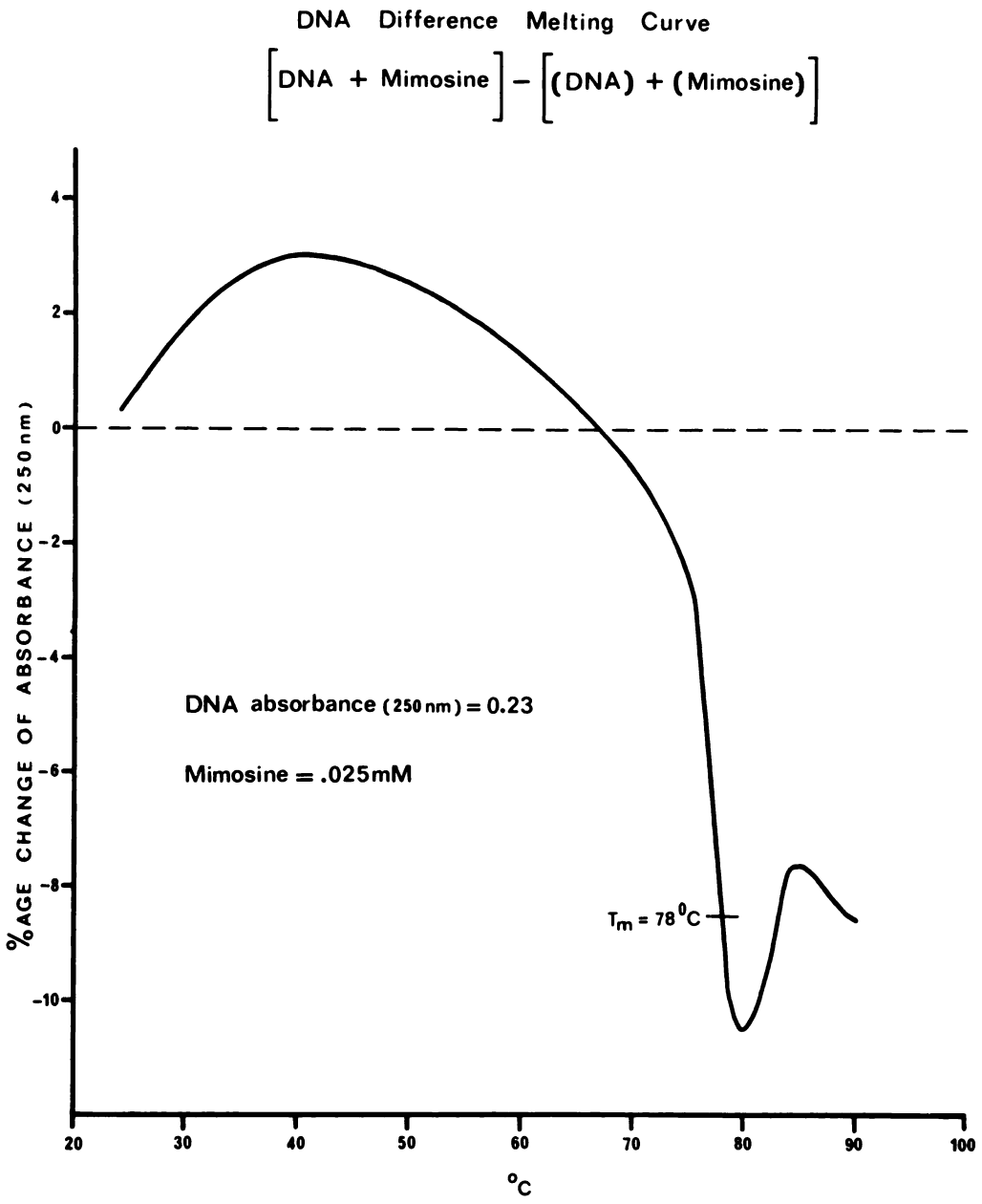

FIG. 4. *Difference spectrum of the temperature-absorbance profile of DNA from Paramecium in the presence of mimosine*

The reference side of the spectrophotometer held mimosine and DNA (15 mg/ml) in separate cuvettes while the sample side had mimosine and DNA mixed. The second cuvette on the sample side held only buffer (1 × SSC). Percentage change of absorbance (250 nm) ≡ ([(absorbance at t° = absorbance at 25°)/absorbance at 25°] × 100). Details are given in the METHODS section.

at near neutral pH, 60°. The hyperchromicity we detected at 40° suggests that perturbation of the DNA or the mimosine, or both, occurs. Paleček (29) recently pointed out that temperature correlated changes of DNA structure in the premelting region are complex. However, the 9% hypochromic effect of mimosine-DNA interaction shown

TABLE 1

The effect of mimosine on DNA polymerase activity

Preparation of the various DNA-polymerase-containing fractions were as previously described (24). Fraction I, unbound by DEAE-cellulose; Fraction II, eluted with 0.4 M KCl.

Enzyme	Template	Mimosine (2mM)	TTP (nmoles/mg)	Inhibition (%)
Crude cytosol	None	–	0.012	95.0
	Native DNA	–	0.241	——
		+	0.110	54.4
Fraction I	Native DNA	–	0.116	——
		+	0.080	31.0
Fraction II	Native DNA	–	0.059	——
		+	0.040	32.2
	Denatured DNA	–	0.091	——
		+	0.056	38.5

during and following melting of the DNA strands suggests a significant effect on electron motion in the denatured DNA. In other words, once a region of native DNA is "unzipped" a mimosine-DNA interaction can occur. Apparently, however, the mode of unwinding RNA polymerase induced into DNA (30) does not allow mimosine to interfere directly with *in vitro* RNA synthesis. Formation of a ternary complex of mimosine with DNA and DNA polymerase, which would either preclude RNA polymerase from being able to complex with DNA or prevent it from moving down the DNA once attached, can be suggested as a possible mechanism. However, the fact that TTP does not reverse the effect of mimosine while simple dilution of the drug does, indicates that more complex interactions are involved. The finding that protein synthesis is inhibited by mimosine some hours after RNA synthesis has declined is in agreement with the relatively long half lives of eukaryotic mRNAs.

Finally, DNA and RNA syntheses are more affected after 48 hours exposure to mimosine than during the first few hours, which suggests (since the cultures are asynchronous) that during the cell cycle a specific sensitive period exists, such as the S phase. Up to 48 hours of exposure to mimosine, the effects are reversible, while after 96 hours exposure to the drug, over 60% of the cells exposed to 0.25 mM mimosine never recover, and at 0.5 mM mimosine about 90% of the cells die (Fig. 1). The reason for the change from a reversible to an irreversible state is not known.

In conclusion, these results show the feasibility of using *Paramecia* (a naturally-occurring homogeneous and continuously dividing tissue) for the study of the effects of mimosine and similar agents. From a practical point of view, mimosine, if carefully applied, might prove a useful tool for obtaining synchronously dividing clones. In view of these reported effects, however, its possible use as a depilatory agent in mammals should be viewed with caution.

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