

EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON CELL GROWTH AND ALKALOID PRODUCTION IN CELL CULTURES OF *PAPAVER SOMNIFERUM*

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ABSTRACT.—Suspension cultures of cells of *Papaver somniferum* L. metabolized ³H-tyrosine and produced labeled alkaloids including thebaine, codeine, and morphine. Protein synthesis inhibitors cycloheximide (CHI), puromycin (PUM), and actinomycin (ATM) affected cell growth and the biosynthesis of alkaloids. PUM was least inhibitory to cell growth, while CHI and ATM were potent inhibitors of cell growth. The degree of inhibition of cell growth by varied concentrations of CHI correlated with reductions in the production of ³H-labeled alkaloids in cultures. Low concentrations of PUM and ATM promoted the production of total labeled alkaloids, despite the inhibition of cell growth. High concentrations of ATM and PUM greatly inhibited cell growth without affecting the biosynthesis of labeled alkaloids. Analysis of phenanthrene alkaloid (PA) production indicated that low concentrations of PUM and ATM increased the accumulation of codeine but not morphine; CHI and high concentrations of PUM decreased both codeine and morphine content; and high concentrations of ATM increased the production of codeine and morphine slightly.

Secondary products of plant tissue cultures of *Papaver somniferum* L. have been studied recently by numerous researchers (1-4). Khanna *et al.* (3) reported the presence of several major phenanthrene alkaloids (PA's) in the poppy callus. They also indicated that exogenously supplied ascorbic acid and tyrosine were able to stimulate the production of alkaloids (5). Furuya *et al.* (2) and Ikuta *et al.* (4) reported the presence of benzophenanthridine, protopine, and aporphine type alkaloids but could not detect any morphinan alkaloids in callus tissues. In their biotransformation studies, Furuya *et al.* (6) indicated that cell cultures of *Papaver somniferum* lacked the ability to metabolize (RS)-reticuline to thebaine, codeine, and morphine but were able to metabolize (-)-codeinone to (-)-codeine. Recently, Tam *et al.* (7) were able to isolate codeine from the cell suspension cultures of *Papaver somniferum*.

The present paper describes cell suspension cultures of *Papaver somniferum* which are able to carry out the biosynthesis of labeled morphinan alkaloids when provided ³H-tyrosine. This investigation is part of our objective of controlling morphinan alkaloid production in the plant. Tissue culture methods were employed toward manipulating conditions that control alkaloid biosynthesis. Inhibitors of protein synthesis, cycloheximide (CHI), puromycin (PUM), and actinomycin (ATM), were utilized with suspension cultures of *Papaver somniferum* in this study.

MATERIALS AND METHODS^{2, 3}

PREPARATION OF SUSPENSION CULTURES.—Callus subcultures of *Papaver somniferum*, grown on agar medium for a total period of at least one year, were transferred to modified Murashige

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²A tissue culture established from seedlings of *Papaver somniferum* L. was obtained from Dr. P. Mahlberg, Department of Biology, Indiana University, Bloomington, Indiana, and maintained on Murashige and Skoog's agar medium (8) supplemented with 0.1 ppm 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 ppm kinetin. All chemicals were of high purity grade obtained from commercial sources. The plates were purchased from Analtech Inc. Tyrosine [Ring-3,5-³H] (S.A. 54 Ci/m mole) and Aquasol liquid scintillation fluid were obtained from New England Nuclear.

³Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

and Skoog's liquid medium (8) with 0.5 ppm 2,4-D and 0.1 ppm kinetin (MMS medium) to form cell suspension cultures. These cultures were grown at 25° with constant light (130 ft-C) in Psychrotherm shaking incubators (150 rpm). Suspensions were subcultured every 10 to 14 days. In order to limit the culture to small cell aggregates, the cell material was allowed to settle for 30 sec before transferring, and then only the upper part of the fine suspension was transferred into fresh medium.

GROWTH IN MEDIUM CONTAINING ^3H -TYROSINE.—Suspensions of the third transfer (12 days old) were combined to form the cell inoculum. After the cells had settled, the medium of the combined cultures was decanted and the cells were resuspended in MMS medium. Cell transfers were made with sterile 10 ml pipets with tip removed. Three ml of cell inoculum were transferred into 125 ml culture flasks containing 27 ml of fresh MMS medium and 5 μCi of ^3H -tyrosine. Cells in media containing ^3H -tyrosine were incubated for up to 11 days with or without protein synthesis inhibitors. A growth curve for the batch culture was established over a 20-day period of incubation. The protein synthesis inhibitors used were CHI (10^{-7}M to 10^{-3}M), ATM (10^{-7}M to 10^{-4}M), PUM (10^{-7}M to 10^{-4}M).

MEASUREMENT OF CELL GROWTH AND DETERMINATION OF ALKALOIDS.—Suspensions were filtered at the end of the incubation period. Cells recovered from the filter were lyophilized, and the cell growth was expressed as the dry weight. A 5% aqueous solution of acetic acid (15 ml) was added to the dry cells (20–200 mg), and this solution then was sonicated and adjusted to pH 9.0 and extracted three times with 20 ml portions of chloroform-isopropanol (3:1, v/v). The combined chloroform extracts were dried over anhydrous sodium sulfate then evaporated to dryness under a nitrogen stream. The dried residues were dissolved in 1 ml of absolute ethanol. Standard solutions of unlabeled thebaine, codeine, and morphine were added to an aliquot of the ethanol solution. The mixture was then applied to activated silica gel tlc plates and developed in the solvent system of acetone-xylene-methanol- NH_4OH (99:83:10:8, v/v). The plates were air dried and sprayed with modified Dragendorff's reagent (9). The silica gel spots showing positive Dragendorff reaction were scraped from the plates and assayed for radioactivity with a Beckman liquid scintillation counter (model LS 8100) after the addition of Aquasol liquid scintillation fluid.

RESULTS AND DISCUSSION

CELL GROWTH AND BIOSYNTHESIS OF LABELED ALKALOIDS IN CONTROL CULTURES.—The dry weight of cells and the total content of ^3H -labeled alkaloids determined from cultures harvested at various times during the 20-day period of incubation increased linearly during the first 14 days and then declined slightly (fig. 1A). Tlc analysis revealed three radioactive areas corresponding to thebaine, codeine, and morphine. The maximum accumulations of thebaine and morphine occurred after 6 or 7 days of incubation; the maximum accumulation of codeine occurred after 10 or 12 days (fig. 1B). The accumulations of all three of these

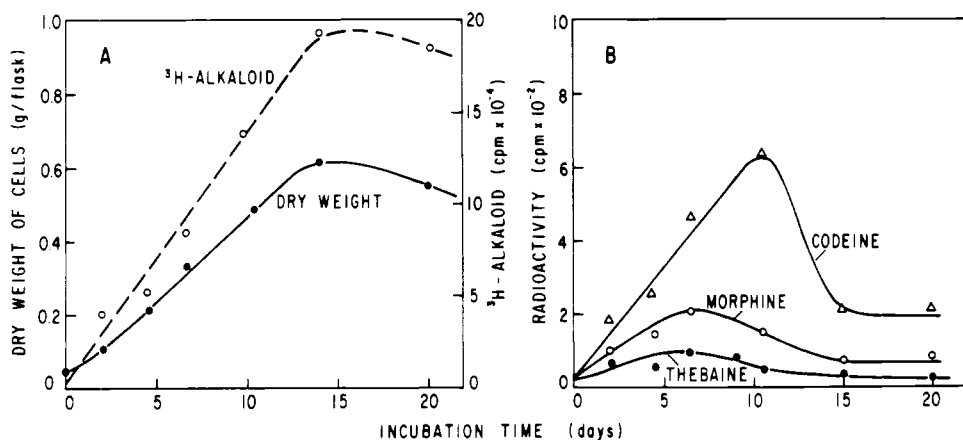


FIG. 1A and 1B. (A) Growth curve and labeled alkaloid accumulation of cells of *Papaver somniferum* in suspension culture. (B) The accumulation of labeled alkaloids for thebaine, codeine, and morphine. The incubations were carried out for 20 days and harvested at various times.

alkaloids decreased substantially after 15 days of incubation and remained at about the same level at 20 days.

The analysis of the chloroform extract of the cells harvested after 11 days of growth (fig. 1A) demonstrated the co-migration of radioactive components with authentic thebaine, codeine, and morphine (visualized with Dragendorff's spray reagent) (9). In addition, ^3H labeled product(s) as a wide spot of a low R_f (0.06) was also detected but not identified (fig. 2A). Hplc retention volumes were established for thebaine, codeine, and morphine by uv absorption (285 nm). Co-elution of radioactive components with the three authentic alkaloids substantiated the identity of some of the ^3H -labeled components as these three alkaloids. Analysis of the chloroform extract of the growth medium revealed that 10 to 15% of the total ^3H -labeled, chloroform-soluble compounds were in the medium, but none of these components co-migrated to any detectable extent with authentic thebaine, codeine, or morphine (fig. 2B).

Previous work (10, 11) demonstrated that isolated latex of the poppy can synthesize morphine and other alkaloids from a simple precursor such as tyrosine

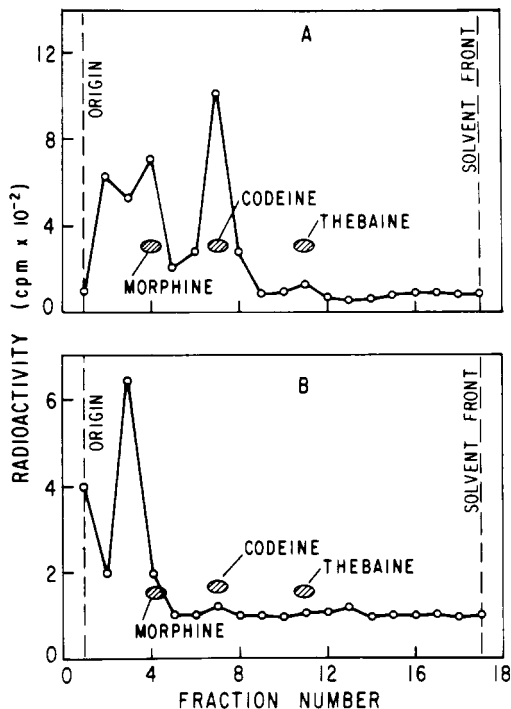


FIG. 2A and 2B. Thin layer chromatography of (A) intracellular and (B) extracellular alkaloids accumulated in suspension cultures of *Papaver somniferum*. The 11-day cultures were filtered to separate the cells (intracellular) and medium (extracellular); both of two fractions were subjected to extraction, and the analysis of alkaloid was as described in Materials and Methods. The plates were cut into 1 cm segments. Each segment received 10 ml of Aquasol liquid scintillation fluid and was analyzed for radioactivity.

or dihydroxyphenylalanine. Morphine alkaloids (3) and other benzophenanthridine, protopine, and aporphine type alkaloids (6) have been isolated from callus tissue of *Papaver somniferum*. The present report indicates that callus tissues of *Papaver somniferum* are capable of synthesizing thebaine, codeine, and morphine from radioactive tyrosine. Khanna *et al.* (5) showed that the exogenous supply of tyrosine stimulates cell growth and alkaloid formation in cultured tissues of *Papaver somniferum*. These data support the contention that callus tissue of *Papaver somniferum* has the biosynthetic capacity of carrying out the biotransformation of thebaine→codeine→morphine. On the other hand, Furuya *et al.* (6) had indicated that callus tissue of *Papaver somniferum* lacks the capacity to synthesize thebaine, codeine, and morphine. Fairbairn *et al.* (12, 13) showed that the biosynthetic sequence of thebaine→codeine→morphine does not terminate with morphine, but morphine is further metabolized. Each of these alkaloids exists in a dynamic pool with a rapid turnover in the intact *Papaver somniferum* plant or fresh latex from the plant. A similar condition probably exists in these calluses which might explain why morphine disappeared faster than codeine (fig. 1B).

EFFECT OF PROTEIN SYNTHESIS INHIBITORS ON CELL GROWTH AND ³H-ALKALOID PRODUCTION.—The effect of various concentrations of cycloheximide (CHI), puromycin (PUM), and actinomycin (ATM) on cell growth, as determined from the dry weight of cells harvested from cultures on the eleventh day of incubation, is shown in fig. 3A. CHI and ATM were potent inhibitors of cell growth. At 10⁻⁴ M, both CHI and ATM inhibited cell growth by 90%. ATM at 5 × 10⁻⁶ M and CHI at 6.5 × 10⁻⁶ M produced 50% inhibition of cell growth, PUM produced the same effect at 5 × 10⁻⁵ M, a 10- to 15-fold increase in concentration.

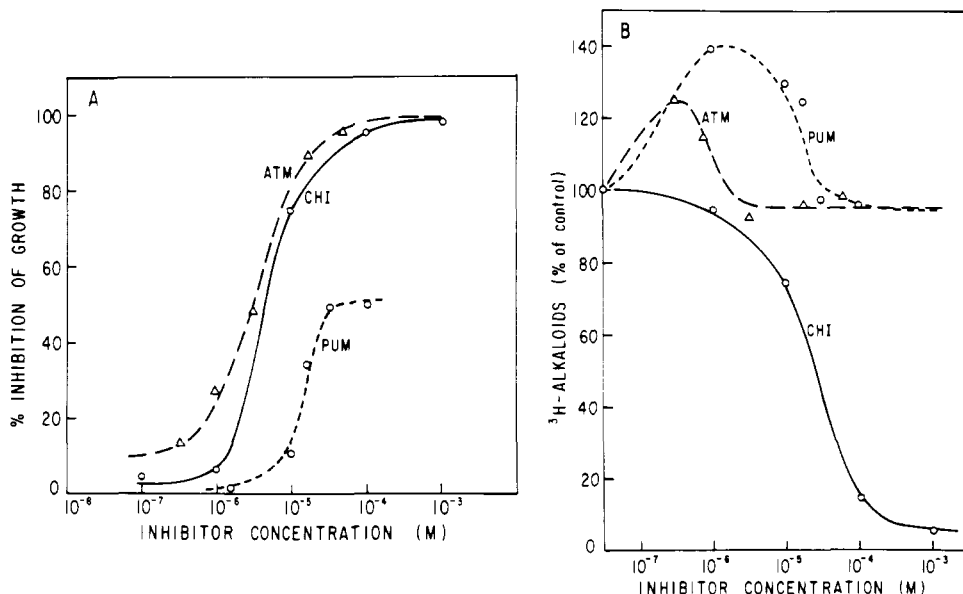


FIG. 3A and 3B. Dose-response curve for (A) growth inhibition and (B) ³H-alkaloid accumulation by CHI, ATM, and PUM. Inhibitors were added to the inoculum 18 h prior to the addition of ³H-tyrosine. The incubations were further carried out for 10 days as described in Materials and Methods. The growth of the cells and ³H-alkaloid accumulation were compared to those of the control incubation (expressed as 100 percentage).

The effect of these inhibitors on the accumulation of ^3H -alkaloids in cultures incubated for 11 days is shown in fig. 3B. The decrease in alkaloid accumulation paralleled the inhibition of cell growth in cultures treated with CHI. At lower concentrations of about 10^{-6} M, PUM and ATM promoted the accumulation of higher levels of ^3H -alkaloids than were present in control cultures; even at the highest concentrations of PUM and ATM, where cell growth was significantly inhibited, alkaloid accumulation was equivalent to that of the controls.

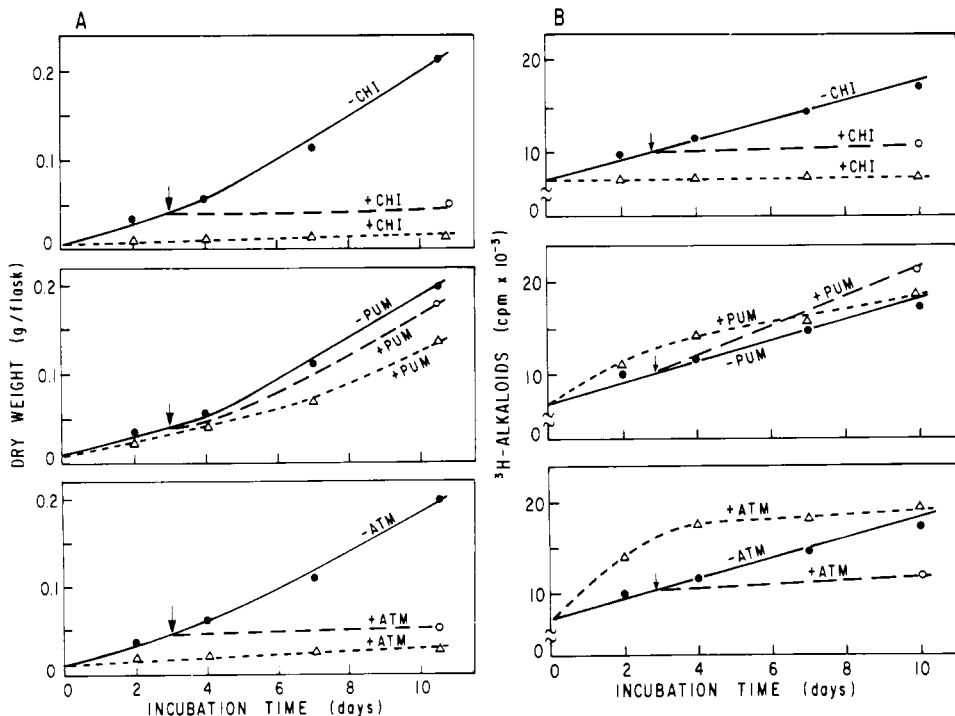


Fig. 4A and 4B. Time course of the inhibition: (A) cell growth and (B) ^3H -alkaloid accumulation by CHI, PUM, and ATM. The suspension cultures were incubated with CHI (10^{-4} M), PUM (10^{-4} M), and ATM (7.5×10^{-5} M) at zero time in the presence of ^3H -tyrosine. The same concentrations of inhibitors were added to the incubation after 3 days (arrow).

KINETICS OF CHI, PUM, AND ATM INHIBITION ON CELL GROWTH AND ^3H -ALKALOID PRODUCTION.—The kinetics of the inhibition of cell growth and ^3H -alkaloid accumulation were studied with cultures incubated with CHI (10^{-4} M), PUM (10^{-4} M), and ATM (7.5×10^{-5} M) added either 16 h prior to the addition of the ^3H -tyrosine substrate (zero time) or after 3 days of incubation with the ^3H -tyrosine substrate. Cells were harvested periodically, and dry weight and ^3H -alkaloid content were determined. Figures 4A and 4B show that cell growth and ^3H -alkaloid accumulation increased in a nearly linear manner in the control cultures. The incorporation of CHI in the medium caused a cessation of cell growth and ^3H -alkaloid accumulation; when CHI was added after 3 days of incubation, the dry weight of cells and alkaloid content remained nearly constant throughout the remainder of the time of incubation. When added at zero time or after

3 days of normal incubation, cell growth was much less inhibited by PUM than by ATM. When inhibitors were added (at zero time), both PUM and ATM enhanced the production of ^3H -alkaloids throughout the entire eleven day incubation period, with the most dramatic increase occurring prior to the fourth day. When inhibitors were added on the third day of incubation, ATM unexpectedly depressed and PUM enhanced ^3H -alkaloid production.

TABLE 1. The effect of cycloheximide, puromycin, and actinomycin on the accumulation of labeled phenanthrene alkaloids in suspension cultures of *Papaver somniferum*.^a

Experiments ^b	Weight of dry cells	Total ^3H -alkaloid	^3H -Codeine	^3H -Morphine
Control.....	100	100	100	100
PUM (10^{-6} M).....	95	140	126	95
PUM (10^{-4} M).....	55	95	28	70
CHI (10^{-4} M).....	5	5	10	12
ATM (5×10^{-7} M).....	90	123	132	94
ATM (7.5×10^{-8} M).....	5	96	120	105

^aData present as a percentage of control.

^bSuspension cultures were grown for 11 days with or without inhibitors.

The effects of some concentrations of CHI, PUM, and ATM on the production of the total labeled alkaloids, codeine, and morphine in 11 day-old cultures are shown in table 1. While total ^3H -alkaloid accumulation was increased by the presence of lower concentrations of PUM and ATM in the medium, only the accumulation of ^3H -codeine, but not ^3H -morphine, was enhanced to any extent. High concentrations of PUM greatly inhibited ^3H -codeine formation, inhibited ^3H -morphine less, and only slightly depressed the total labeled alkaloids. Higher concentrations of ATM increased the accumulation of ^3H -codeine, slightly increased the accumulation of ^3H -morphine, and slightly decreased the total ^3H -alkaloids. CHI had a severe depressing effect on all alkaloid accumulation.

There are conflicting reports from several laboratories (3-7) on the ability of callus tissues of *Papaver somniferum* to synthesize morphinan alkaloids. Nessler and Mahlberg (14) have examined the ultrastructure of undifferentiated callus of *Papaver somniferum* and indicated the absence of laticifers, which might account for Furuya's reported absence of morphinan alkaloids. This would tend to support the hypothesis that laticifers are the primary site of morphinan alkaloid synthesis (15). In their work (14), they also have shown that callus tissue redifferentiated into shoots or roots. The ultrastructure of laticifers is similar to that of the intact plant. Recently Kamo *et al.* (16) have indicated callus frequently differentiated into white nodules, shoots, or roots. They have detected the morphinan alkaloids in both callus and differentiated organs and indicated thebaine was the predominate alkaloid present in organs and callus. Differentiated plant shoots contained higher alkaloid content than callus tissue. We have induced suspension cultures from the callus provided by Kamo *et al.* (16). These suspension cultures formed a large proportion of white nodules of embryoid-type cells. The white cells were not examined microscopically; therefore, it is not known whether they possessed laticifers. In our studies, we used a suspension culture consisting of a mixture of cells and "white nodules." We are not certain which cells are capable of synthesizing labeled morphinan alkaloids when provided with ^3H -tyrosine. Our results also indicate that codeine is the predominate

alkaloid in the suspension cultures. These results are in agreement with the result from Tam *et al.* (7) who have isolated codeine from a large quantity of suspension cell cultures of *Papaver somniferum*.

Fundamental information on the capacity of the callus to produce secondary products is needed. Precursors, hormones, metabolic cofactors, or other regulators are known to be related to the synthesis of plant secondary metabolites (17). In this report, we wished to investigate whether protein synthesis inhibitors can influence the production of alkaloids in the suspension cultures of *Papaver somniferum*. Three classical inhibitors of protein synthesis were chosen because of their unique mechanisms of inhibition. CHI is known to inhibit peptide formation in the 80S ribosomes of eucaryotic cells. ATM blocks the formation of mRNA by the inhibition of RNA polymerase. Puromycin terminates the elongation of peptidyl-RNA because it resembles aminoacyl tRNA. The finding that certain concentrations of PUM and ATM are able to stimulate the accumulation of alkaloids while depressing cell growth suggests that cell growth is not necessary for the alkaloid accumulation and these inhibitors may inhibit the *de novo* synthesis of enzymes responsible for the catabolic degradation of alkaloids. Hence, reduction in alkaloid accumulation in suspension cell cultures of *Papaver somniferum* may not be a direct consequence of the inhibition of *de novo* synthesis of enzymes that are necessary for the conversion of tyrosine to alkaloids. The inhibitor CHI has been reported to inhibit a wide variety of metabolic processes, some of which seem unrelated to protein synthesis (18, 19).

Our experiments do not provide a basis for an explanation how any of the three classical inhibitors of protein synthesis function in affecting alkaloid accumulation. It is clear, however, that CHI, PUM, and ATM affect both cell growth and alkaloid accumulation in suspension cell cultures of *Papaver somniferum*.

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