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INFLUENCE OF PYSCHOACTIVE AND NONPSYCHOACTIVE CANNABINOIDS ON CELL PROLIFERATION AND MACROMOLECULAR BIOSYNTHESIS IN HUMAN CELLS

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Abstract—We have examined the influence of psychoactive [Δ^9-tetrahydrocannabinol (Δ^9-THC), Δ^8-THC, and 11-OH-Δ^9-THC] and nonpsychoactive (cannabinol) cannabinoids on proliferation of human cells (HeLa S_3 cells grown in suspension culture) and on biochemical events associated with the proliferative process. The four cannabinoids studied brought about a dose-dependent inhibition in cell growth. Pulse-labeling studies indicated a cannabinoid-induced decrease in incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine into DNA, RNA and protein, respectively, in intact cells; in most cases a comparable decrease in the acid-soluble intracellular precursor pools was observed. Results from *in vitro* nuclear and chromatin transcription experiments did not suggest a drug-induced change in RNA synthesis. It appears, therefore, that quantitative alterations in the synthesis and/or turnover of nucleic acids and proteins do not result from cannabinoid treatment of HeLa cells. Our data, however, do not preclude the possibility that cannabinoids affect the synthesis of specific molecules.

Elucidation of the influence of cannabinoids on cell proliferation and on macromolecular biosynthetic events required for cell division is important from several standpoints. Because cannabinoids are drugs of abuse, it is instructive to determine their effects on cell proliferation which is associated with a broad spectrum of essential biological processes including wound healing, tissue regeneration, erythropoiesis, the immunological response, and replacement of epithelial cells that line the gastrointestinal tract. Cannabinoids were recently shown to be effective clinically as anti-emetics in conjunction with cancer chemotherapy; it is necessary, therefore, to determine whether cannabinoids and chemotherapeutic agents function synergistically or antagonistically to influence cell proliferation. Other clinical applications that further necessitate evaluation of possible cannabinoid-induced aberrations in the proliferative process include treatment of glaucoma.

We reported previously that Δ^9-tetrahydrocannabinol, the major psychoactive component of marijuana, brings about a dose-dependent depression in proliferative activity as well as in the incorporation of radiolabeled precursors into nucleic acids and proteins [1]. This observation is consistent with data from several laboratories [2–13]. In this paper, which represents a direct extension of our earlier work, we consider the influence of two naturally occurring psychoactive cannabinoids [Δ^9-tetrahydrocannabinol (Δ^9-THC) and Δ^8-tetrahydrocannabinol (Δ^8-THC)], a psychoactive cannabinoid metabolite [11-OH-Δ^9-tetrahydrocannabinol (11-OH-Δ^9-THC)], and a nonpsychoactive cannabinoid [cannabinol (CBN)] on cell division in exponentially growing human cervical carcinoma cells (HeLa cells) maintained in suspension culture. Because progression through the cell cycle requires a complex and interdependent series of biochemical events involving changes in gene expression, we focus on cannabinoid-induced effects on DNA, RNA and protein metabolism.

MATERIALS AND METHODS

Cell culture

Exponentially growing (log phase) HeLa S_3 cells, a hypotetraploid line of human cervical carcinoma cells, were maintained in suspension culture in Joklik-modified Eagle's Minimal Essential Medium (Grand Island Biological Co., Grand Island, NY) supplemented with 7% calf serum and 1.0 mM glutamine and containing 75 units/ml of potassium penicillin G and 50 μg/ml of streptomycin sulfate [14].

Drug administration

The cannabinoids selected for these studies included (−)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC), (−)-*trans*-Δ^8-tetrahydrocannabinol (Δ^8-THC), 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-Δ^9-THC), and cannabinol (CBN). All four compounds were supplied by the National Institute on Drug Abuse; their purities, determined by gas–liquid chromatography, were 99 per cent for Δ^9-THC, 98 per cent for Δ^8-THC, 97 per cent for 11-OH-Δ^9-THC, and 99 per cent for CBN. In the subcellular fractionation studies the following radiolabeled cannabinoids were used: Δ^9-THC-[1′,2′-$^3H_2$] (137 μCi/mg), Δ^8-THC-[4′,5′-$^3H_2$] (159 μCi/mg), 11-OH-Δ^9-THC-[4′,5′-$^3H_2$] (320 μCi/mg), and CBN-[1′,2′-$^3H_2$] 121 μCi/mg).

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The drug in 95% ethanol (as vehicle) was added directly to the suspension cultures; the final ethanol concentration in all experiments was adjusted to 0.15% (v/v). The specific drug doses used in these studies were determined from growth curve studies of each compound, as described below. The drug concentrations chosen were those that had caused a 30–40 per cent growth depression in continuously dividing HeLa S_3 cell cultures. We compensated for the 'vehicle effect' in each *in vitro* system tested, if necessary, by carrying out a 'vehicle only' control or by delaying monitoring of biochemical and biological parameters until reversal of the 'vehicle effect'.

Growth curves

The HeLa S_3 cells were grown to a cell density of 5.0×10^5 cells/ml in suspension culture at 37°. Fresh warm medium was added to achieve a cell density of 2.5×10^5 cells/ml, and 350 ml of cell culture was aseptically transferred to individual, sterile, prewarmed culture flasks. Each drug, in 95% ethanol, was added directly to the culture medium to achieve the desired final concentration. Δ^9-THC was added to obtain final concentrations of 0.5, 5, 10, 15, 20 and 40 μM; Δ^8-THC was added to final concentrations of 1, 5 and 10 μM; and 11-OH-Δ^9-THC and CBN were added to achieve final concentrations of 5, 10 and 15 μM. An equal amount of 95% ethanol was added to one of two control cultures. All experiments were done in duplicate. Periodic cell density measurements were made by the packed cell method [1]; results were plotted as percentages of initial cell density versus time incubated with the various cannabinoid concentrations.

A 'reversal treatment' growth curve was also plotted for Δ^9-THC. The HeLa S_3 cells were grown for 24 hr in a medium containing a 30 μM concentration of Δ^9-THC. The cells were then harvested by centrifugation at 500 *g* for 5 min in sterile centrifuge bottles, resuspended in warm fresh medium without Δ^9-THC to a cell density of 2.5×10^5 cells/ml, and allowed to grow for another 24 hr. The cells were harvested and resuspended a second time and then allowed to grow for another 24 hr in warm fresh medium without Δ^9-THC. Periodic cell density measurements were done throughout the entire procedure. The results of duplicate cultures were plotted as percentages of initial cell density (at the beginning of each 24-hr period) versus time incubated.

Cell viability determination

The effects of the four cannabinoids on cell viability were estimated by trypan blue exclusion studies [15] done 10 and 40 min after the addition of drug and/or vehicle to exponentially growing HeLa S_3 cells. Samples of duplicate cultures were mixed with an equal volume of 0.4% trypan blue solution (in 0.15 M NaCl–0.01 M Tris, pH 8.0) for 5 min and then counted in a hemocytometer. The percentage of viable cells was the number of unstained cells per total number of cells × 100.

Subcellular fractionation

Exponentially growing HeLa S_3 cells in 100 ml suspension cultures were exposed for 30 min at 37° to 30 μM concentrations of [^{3}H]-Δ^9-THC, [^{3}H]-Δ^8-THC, [^{3}H]-11-OH-Δ^9-THC, or [^{3}H]-CBN (sp. act. 20 μCi/mg for all four compounds). Cell densities were determined, and the cells were pelleted by centrifugation at 500 *g* for 5 min. The remainder of the fractionation procedure was carried out at 4°. Cells were washed in 20 vol. of Earle's balanced salt solution (Grand Island Biological Co.) and pelleted again by centrifugation at 500 *g* for 5 min. The supernatant fractions were combined with 95% ethanol washings of the culture flask; the combination is referred to as the 'culture flask and medium' fraction. The culture flasks were washed with 95% ethanol to recover the portion of the cannabinoids that strongly adhered to the glass. The cells were lysed twice with 40 vol. of 80 mM NaCl, 20 mM EDTA, and 1% Triton X-100 (pH 7.2) by vortexing. 'Nuclei' were pelleted by centrifugation at 1400 *g* for 5 min and then washed in 25 vol. of 0.15 M NaCl–10 mM Tris (pH 8.0) and pelleted again by centrifugation at 1400 *g* for 5 min. The supernatant fractions from both the lysing medium and the salt washing were combined and are referred to as the 'cytoplasmic' fraction. Nuclei isolated in this manner were largely free of cytoplasmic material when examined by phase contrast microscopy. Nuclei were then lysed by suspending the nuclear pellet in double glass-distilled water. The chromatin was allowed to swell at 4° for 30 min and then was pelleted by centrifugation at 20,000 *g* for 15 min. The supernatant fraction was decanted and is referred to as the 'nucleoplasmic' fraction. The chromatin was solubilized in 5 vol. of NCS™ tissue solubilizer (Amersham/Searle Corp., Arlington Heights, IL) and is referred to as the 'chromatin' fraction. Aliquots of the 'culture flask and medium', 'cytoplasmic', and 'nucleoplasmic' fractions were counted in Triton–toluene scintillation fluid containing 2500 ml toluene, 1333 ml Triton X-100, and 168 ml liquifluor (New England Nuclear Corp., Boston, MA). Aliquots of the 'chromatin' fraction were counted in a dioxane–toluene scintillation fluid containing 188 mg of 1,4-di[2-phenyloxazolyl)-benzene (POPOP)], 240 g of naphthalene, 15 g of 2,5-diphenyloxazole (PPO), 1 l. of toluene, 1 l. of ethanol, and 1 l. of dioxane. A Beckman liquid scintillation counter was used for assaying the radioactivity of the samples along with the appropriate internal standards. Fractionations were done in triplicate and the results are reported as percent of total [^{3}H]cannabinoid added (at a 30 μM concentration) in the subcellular fractions, and as picograms of cannabinoid per 10^3 cells.

Isolation of crude nuclear fractions

Exponentially growing HeLa S_3 cells in 100 ml suspension cultures were treated with a 30 μM concentration of [^{3}H]-Δ^9-THC (sp. act. 20 μCi/mg) for 30 min at 37°. A 25-ml aliquot of cell culture was centrifuged at 500 *g* for 5 min and the cell pellet was washed twice in 20 vol. of Earle's balanced salt solution and pelleted again by centrifugation at 500 *g* for 5 min. The washed cell pellet was then solubilized

in 1 ml of NCS[tm] at room temperature and is referred to as the 'cellular' fraction. The remaining 75 ml of cell culture was centrifuged at 500 *g* for 5 min and the cell pellet was washed twice in 20 vol. of Earle's balanced salt solution and pelleted again by centrifugation at 500 *g* for 5 min. Crude nuclear fractions were prepared by a sucrose procedure according to the method of DeRobertis *et al.* [16, 17]. The cell pellet was homogenized in 3 ml of 0.32 M sucrose (10 μM Ca^{2+}) at 0–4° for 2 min using a Teflon homogenizer driven by a drill press. The homogenate was centrifuged at 1400 *g* for 10 min, and the crude nuclear pellet was resuspended twice in 3 ml of 0.32 M sucrose (10 μM Ca^{2+}) and recentrifuged. The supernatant fractions were pooled and are referred to as the 'cytoplasmic' fraction. The crude nuclear pellet was solubilized in 1 ml of NCS[tm] at 50°, and an additional 1 ml of cold NCS[tm] was then added; this is referred to as the 'crude nuclear' fraction. Isolations were carried out in quadruplicate and the results are reported as percentages of total cellular [^{3}H]-Δ^9-THC in the 'cytoplasmic' and 'crude nuclear' fractions.

Total cellular protein, DNA and RNA synthesis

Exponentially growing HeLa S_3 cells, in triplicate 50-ml suspension cultures, were exposed to various micromolar concentrations of cannabinoids for 10 hr at 37°. The cannabinoid concentrations used were as follows: 5, 10, 20, 30 and 40 μM Δ^9-THC; 5 and 10 μM Δ^8-THC; 5 and 15 μM 11-OH-Δ^9-THC; and 5 and 15 μM CBN. In each experiment two controls were used, one untreated and one treated with vehicle alone (95% ethanol). Aliquots (4 ml) from each of the cultures (in the presence of cannabinoid) were pulse-labeled for 15 min at 37° with 2 μCi of [4,5-^{3}H]-L-leucine (46 Ci/mmole), 2 μCi of [methyl-^{3}H]thymidine (13 Ci/mmole) or 1 μCi [5-^{3}H]uridine (23 Ci/mmole). All samples (in triplicate) were continuously agitated in a water bath at 37° during incubation with the radioisotopes. All radioisotopes were obtained from Schwarz/Mann, Orangeburg, NY. At the end of the incubation the samples were rapidly cooled to 4° by addition of cold Earle's balanced salt solution and immersion in an ice–water bath. The cells were pelleted by centrifugation at 900 *g* for 5 min, washed in 4 ml of Earle's balanced salt solution at 4°, and pelleted again by centrifugation at 900 *g* for 5 min. The supernatant fractions were decanted, and the cell pellets were resuspended in 20% trichloroacetic acid (TCA) at 4°. The acid-washed cell pellets were then centrifuged at 900 *g* for 5 min, and the supernatant fractions were saved as the 'acid-soluble' fractions. The 'acid-insoluble' precipitate fractions were solubilized in 500 μl NCS[tm] tissue solubilizer. Aliquots of both fractions were dissolved in dioxane–toluene scintillation liquid (188 mg of POPOP, 240 g of naphthalene, 15 g of PPO, 1 liter of toluene, 1 liter of ethanol, and 1 liter of dioxane) and assayed for radioactivity in a Beckman liquid scintillation counter. The results were plotted as cpm per cell versus cannabinoid concentration for each of the isotopes incorporated into both the acid-soluble and acid-insoluble fractions.

Isotope incorporation was also studied as described above with HeLa S_3 cell cultures treated for 1 hr at 24, 48, 72 and 96 mM concentrations of ethanol vehicle. In addition, the effect of exposure to 0.15% (v/v) ethanol on the incorporation of the radiolabeled precursors into protein, DNA and RNA was determined at exposure intervals of 1, 3, 6 and 9 hr.

Analysis of intracellular uridine pool

Exponentially growing HeLa S_3 cells in 30-ml suspension cultures were incubated at 37° with 20 μCi of [5-^{3}H]uridine (23 Ci/mmole, Schwarz/Mann) for 15 min. At the end of the incubation period the cultures were cooled rapidly by placing them in an ice–water bath. The cells were pelleted by centrifugation at 900 *g* for 5 min at 4°; the pellets were washed twice in 20 vol. of cold Earle's balanced salt solution and centrifuged at 900 *g* for 5 min. The pellets were extracted with 2 ml of ice-cold 0.25 N perchloric acid ($HClO_4$) by vortexing for 15 min. The remainder of the procedure was carried out at 4°, except where indicated. The extracts were centrifuged at 900 *g* for 5 min and the supernatant fractions were saved. The supernatant fluid was adjusted to pH 8 with KOH and the potassium perchlorate was removed by centrifugation at 900 *g* for 5 min. The supernatant fractions were extracted twice with 2 ml of ether (anhydrous) for 10 min each time, at room temperature. The aqueous layers were separated by centrifugation at 900 *g* for 10 min and then evaporated to dryness. Each residue was dissolved in 1 ml of 0.5 M NH_4HCO_3, pH 8.0, and 50 μg of *Escherichia coli* alkaline phosphatase (Worthington Biochemical Corp., Freehold, NJ) was added. The mixtures were incubated at 37° for 3 hr. The extent of dephosphorylation was determined by the conversion of known amounts of uridine-5′-triphosphate (UTP) and uridine-5′-monophosphate (UMP) to uridine (Urd) (Sigma Chemical Co., St. Louis, MO) and was monitored by thin-layer chromatography (t.l.c.). After 3 hr dephosphorylation was complete. The reaction mixtures were evaporated to dryness under vacuum, and the residues were dissolved in 0.5 ml of double glass-distilled water and were used to determine intracellular uridine-pool sizes and specific activities.

Uridine was separated by two-dimensional thin-layer chromatography on MN 300 cellulose precoated plastic sheets with solvent I (methanol–HCl [sp. g. 1.18]–water [70:20:10, by vol.]) and solvent II (*n*-butanol–methanol–water–ammonia [sp. g. 0.90] [60:20:20:1, by vol.]) [18]. The uridine pool size of each sample was determined from the extinction coefficient of uridine (Urd $\varepsilon_{max} = 10.1 \times 10^3$) [19, 20].

Isolation of chromatin

The procedure for chromatin isolation [14, 21] was carried out at 4°. The chromatin of HeLa S_3 cells isolated in this manner has been found to be composed of histones, nonhistone proteins, DNA and RNA in approximate ratios of 1.0:0.8:1.0:0.09 [21–24].

In vitro *chromatin transcription*

Exponentially growing HeLa S_3 cells were exposed to micromolar concentrations of cannabinoids in the

culture medium for 10 hr. One control culture was treated only with vehicle (95% ethanol); a second control culture was untreated. Chromatin was isolated and then transcribed *in vitro* [21] using fraction V *E. coli* RNA polymerase prepared according to the method of Berg *et al.* [25].

In vitro *nuclei transcription*

Exponentially growing HeLa S_3 cells were exposed to various micromolar concentrations of cannabinoids in the culture medium for 10 hr. Nuclei were isolated by a modification of the procedure of Sarma *et al.* [26] as reported by Detke *et al.* [27]. Nuclear transcription was carried out as described by Detke *et al.* [27].

RESULTS AND DISCUSSION

Influence of cannabinoids on cell proliferation

At the start of each experiment the cell density was adjusted to 2.5×10^5 cells/ml to provide an environment for exponential growth. The cannabinoid was present in the culture medium throughout the experiment. Growth was determined by measuring the increase in cell density at specific time intervals during the doubling time of the untreated control culture (approximately 24 hr). Cell viability was more than 98 per cent for the control, vehicle-treated, and all the cannabinoid-treated cultures, at 10 and 40 min after initiation of the growth experiments; thus it is unlikely that the cannabinoids and/or the drug vehicle produced toxic, nonspecific cell destruction that would result in an incorrect, initial, 'viable' cell number.

In four independent experiments, micromolar concentrations caused a dose-dependent depression of the exponential growth of HeLa cells. As shown in Fig. 1(a), Δ^9-THC at concentrations of 5, 10, 15, 20 and 40 μM depressed the exponential growth of HeLa S_3 cells approximately 8, 12, 20, 31 and 55 per cent respectively. A Δ^9-THC concentration of 0.5 μM did not measurably affect growth, compared with the vehicle-treated cultures (data not shown). Δ^8-THC-treated cultures (Fig. 1(b)) exhibited growth depressions of approximately 13, 22 and 37 per cent at respective Δ^8-THC concentrations of 1, 5 and

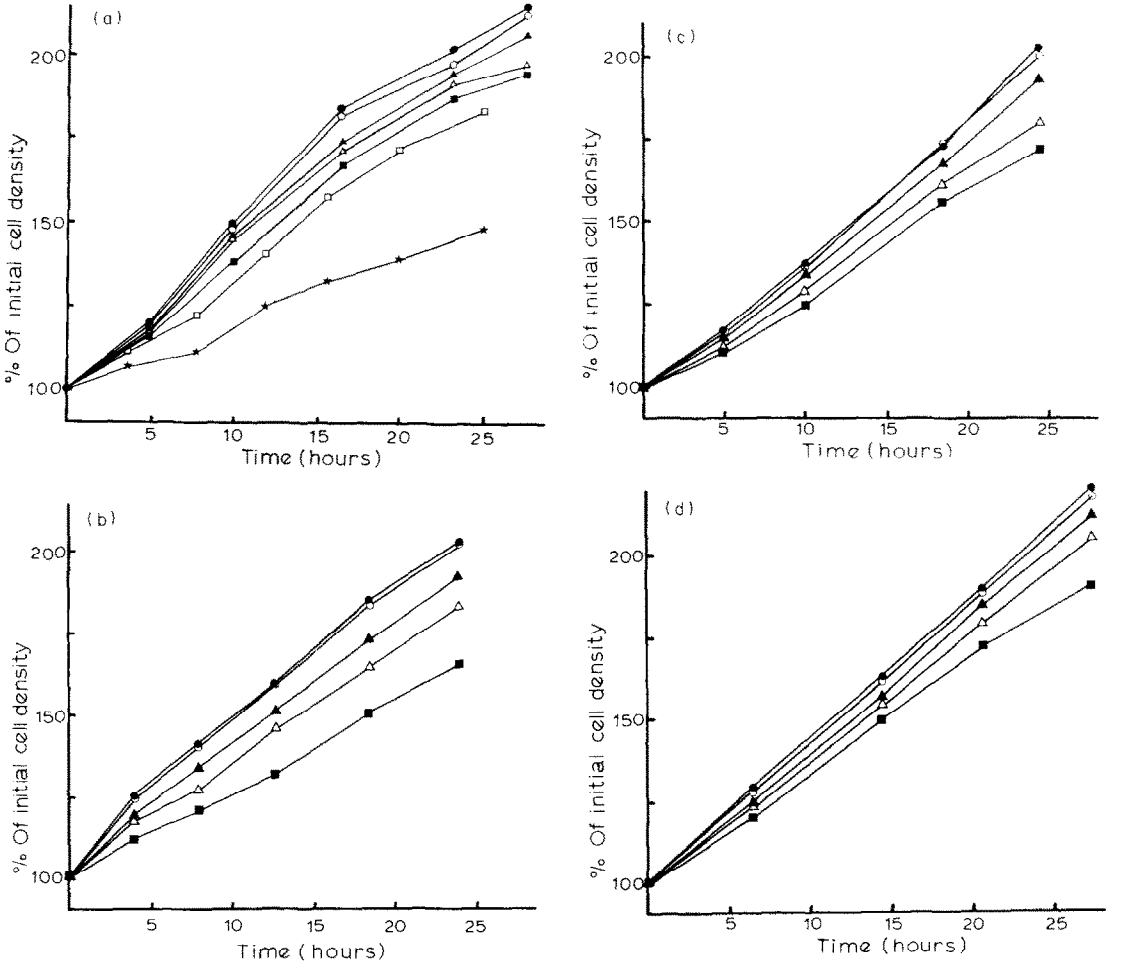

Fig. 1. Effects of various concentrations of cannabinoids on exponentially growing HeLa S_3 cells in suspension cultures. Untreated controls (●); vehicle-treated controls (○). Panel (a): 5 μM Δ^9-THC (▲); 10 μM Δ^9-THC(△); 15 μM Δ^9-THC (■); 20 μM Δ^9-THC (□); and 40 μM Δ^9-THC (★). Panel (b): 1 μM Δ^8-THC (▲); 5 μM Δ^8-THC (△); and 10 μM Δ^8-THC (■). Panel (c): 5 μM 11-OH-Δ^9-THC (▲); 10 μM 11-OH-Δ^9-THC (△); and 15 μM 11-OH-Δ^9-THC (■). Panel (d): 5 μM CBN (▲); 10 μM CBN (△); and 15 μM CBN (■).

10 μM. Figure 1(c) shows that 11-OH-Δ^9-THC at 5, 10 and 15 μM concentrations caused growth depressions of about 8, 20 and 32 per cent respectively. Cannabinol-treated cultures (Fig. 1(d)) exhibited growth depressions of approximately 7, 13 and 30 per cent at cannabinoid concentrations of 5, 10 and 15 μM respectively. The vehicle-treated cultures (Fig. 1 (a)–(d)) grew a little more slowly compared with untreated control cultures.

These growth studies serve as an indication of the relative influence of the four cannabinoids on cell proliferation. At approximately equal drug concentrations, the order of decreasing effects on cell proliferation was Δ^8-THC, 11-OH-Δ^9-THC, CBN, Δ^9-THC. These results suggest that the psychoactivity of a compound is not directly related to its effect on cell proliferation, for the nonpsychoactive cannabinoid (CBN) is not the least effective in depressing cell proliferation. Compared with the other three compounds, however, more Δ^8-THC is associated with the cell nucleus; its apparently higher potency, therefore, may be due to a larger amount of the drug being present at a potentially active site.

The cell growth studies were used to determine the drug concentrations to be employed in subsequent experiments. Those that caused a 30–40 per cent growth depression in continuously dividing HeLa S_3 cell cultures were used: 30 μM for Δ^9-THC, 10 μM for Δ^8-THC, 15 μM for 11-OH-Δ^9-THC, and 15 μM for CBN.

Reversibility of cannabinoid effect on cell proliferation

Because most behavioral effects of cannabinoids have been shown to be reversible, we examined the reversibility of cannabinoid-induced growth inhibition. HeLa S_3 cells were grown for 24 hr in the presence of 30 μM Δ^9-THC (a drug concentration that exerts approximately 35 per cent inhibition of cell growth); growth was observed for another 48 hr after release from Δ^9-THC treatment. During the first 24 hr, while in 30 μM Δ^9-THC, the cells grew 35 per cent less than appropriate controls (Fig. 2a). After release from Δ^9-THC treatment, the cells grew 21 per cent less than controls in the next 24 hr (Fig. 2b), returning to their normal rate of cell proliferation during the following 24 hr (Fig. 2c). Thus, the effect of Δ^9-THC on cell proliferation appears to have been reversible within 48 hr after termination of the exposure of HeLa cells to the drug.

Intracellular concentrations and subcellular distributions of cannabinoids

Two questions relevant to interpretation of these cell growth studies and other data to be described subsequently are: (1) How much cannabinoid enters or is associated with the cells? and (2) What is the subcellular distribution of the drug? Measuring the drug level of various intracellular compartments is important for evaluation of the site, and the mechanism, of action of cannabinoids, although the association of cannabinoids with particular intracellular organelles or macromolecular complexes does not necessarily reflect a functional interaction. Because cannabinoids exhibit high affinities for nonspecific components of serum in the cell culture medium and for cell culture glassware, the concentration of cannabinoid in the growth medium may not be a reliable reflection of the amount of drug available to the cells.

To determine the amounts of the cannabinoids associated with various cellular compartments, the HeLa S_3 cells were incubated with ^{3}H-labeled cannabinoids for 30 min and subcellular fractions were isolated by a detergent procedure routinely used in our laboratory [28]. To compensate for nonspecific sequestering, all experiments were carried out in culture flasks of the same dimensions. The total recovery of the radiolabeled cannabinoids was more than 86 per cent. The percentages of the various cannabinoids associated with the cells were 6.3–10.0 per cent (9.1 ± 0.2 per cent for Δ^9-THC, 9.1 ± 0.3 per cent for Δ^8-THC, 10.0 ± 0.2 per cent for 11-OH-Δ^9-THC, and 6.3 ± 0.1 per cent for CBN) of that added to the culture medium. Of the drug entering the cell, 1.27–10.4 per cent (1.27 ± 0.03 per

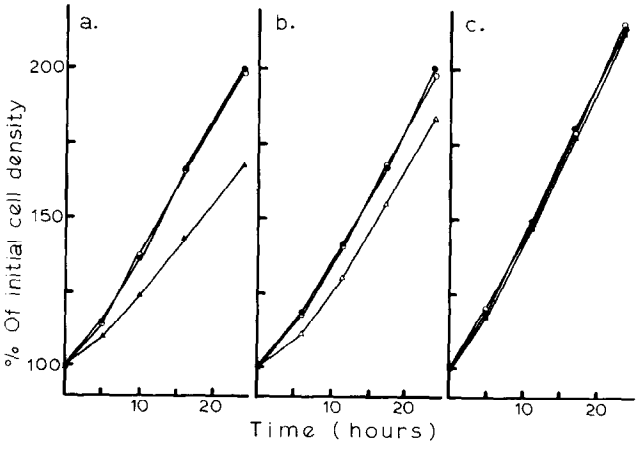

Fig. 2. Reversal of effect of Δ^9-THC on exponentially growing HeLa S_3. Untreated controls (●); vehicle-treated controls (○). Panel a: initial 24-hr treatment of cells with 30 μM Δ^9-THC (▲). Panel b: cell growth of pretreated cells (△) for the 24 hr following the first resuspension in Δ^9-THC-free medium. Panel c: cell growth of pretreated cell (■) for the 24 hr following the second resuspension in Δ^9-THC-free medium.

Table 1. THC (at a culture medium concentration of 30 μM) in the subcellular fractions of HeLa S_3 cells*

Fraction	Δ^9-THC/ 10^3 cells (pg)	Δ^8-THC/ 10^3 cells (pg)	11-OH-Δ^9-THC/ 10^3 cells (pg)	CBN/ 10^3 cells (pg)
Cellular	1436 ± 26	1558 ± 56	1724 ± 21	1306 ± 14
Cytoplasmic	1418 ± 25	1394 ± 36	1708 ± 19	1285 ± 14
Nuclear	18 ± 0.5	162 ± 20	18 ± 0.9	22 ± 0.8
Nucleoplasmic	4 ± 0.2	56 ± 5	3 ± 0.2	3 ± 0.2
Chromatin	14 ± 0.3	107 ± 16	15 ± 0.7	18 ± 0.7

* Exponentially growing HeLa S_3 cells in suspension cultures were exposed for 30 min at 37° to 30 μM [^{3}H]-Δ^9-THC, [^{3}H]-Δ^8-THC, [^{3}H]-11-OH-Δ^9-THC or [^{3}H]-CBN, with a specific activity of 20 μCi/mg. Results were calculated as picograms (pg) of cannabinoid from the amount of [^{3}H]cannabinoid determined from internal standards appropriate for each fraction and each is expressed as the mean ± average deviation of three separate determinations.

cent for Δ^9-THC, 10.4 ± 1.3 per cent for Δ^8-THC, 1.06 ± 0.05 per cent for 11-OH-Δ^9-THC, and 1.65 ± 0.06 per cent for CBN) was isolated in the 'nuclear' fraction and 0.86–6.9 per cent (0.96 ± 0.02 per cent for Δ^9-THC, 6.9 ± 1.0 per cent for Δ^8-THC, 0.86 ± 0.04 per cent for 11-OH-Δ^9-THC, and 1.39 ± 0.05 per cent for CBN) was associated with chromatin (the isolated eukaryotic genome) (Table 1, data expressed as picograms of cannabinoid per 10^3 cells). 11-OH-Δ^9-THC exhibited the largest amount associated with the cellular fraction, while Δ^8-THC exhibited the largest amounts associated with the nuclear and chromatin fractions. However, the influence of detergent during nuclear isolation on cannabinoid binding must be considered. Δ^8-THC also displayed the most pronounced depression of cell growth rates. This apparently higher potency of Δ^8-THC may be related to the larger quantity of the drug associated with the nucleus of the cell.

Our results appear to be inconsistent with those obtained previously from subcellular fractionation procedures in which crude nuclear preparations were made from mouse brain. Dewey *et al.* [29] reported that crude mouse brain nuclei contain 20–25 per cent of total intracellular radiolabeled Δ^9-THC, a percentage of the cellular cannabinoid higher than we found associated with the HeLa cell nuclear fraction. To resolve these apparent differences in the proportions of [^{3}H]-Δ^9-THC in nuclei of mouse brain and HeLa cells, we prepared a crude nuclear fraction from [^{3}H]-Δ^9-THC-treated HeLa cells by the sucrose procedure of DeRobertis *et al.* [16, 17]. In the HeLa nuclei prepared by the sucrose method, 86 per cent of the radiolabeled cannabinoid was associated with the cytoplasmic fraction and 14 per cent with the crude nuclear fraction. The level of Δ^9-THC in the sucrose-prepared HeLa nuclei compares favorably with that in mouse brain nuclei isolated by the same procedure [29]. Since the HeLa nuclei prepared by the sucrose method are associated with significant amounts of cytoplasmic material (observed by phase contrast microscopy) and the HeLa nuclei prepared by our detergent procedures are not, the elevated levels of Δ^9-THC found in sucrose-prepared nuclei from HeLa or mouse brain cells may be the result of drug present in cytoplasmic contaminants. Alternatively, the possibility that *bona fide* nuclear material is lost during preparation of nuclei with detergents must be considered.

Influence of cannabinoids on nucleic acid and protein synthesis in intact cells

Cell proliferation involves a complex, interdependent series of biochemical events requiring differential gene expression, and such modifications in gene expression, including RNA and protein synthesis, are prerequisites for DNA replication and mitotic division. We examined, therefore, whether treatment of human cells with cannabinoids results in perturbations of macromolecular biosynthetic events essential for cell proliferation. We assayed the influence of cannabinoids (at concentrations that affect cell proliferation) on the biosynthesis of protein, DNA, and RNA in continuously dividing HeLa S_3 cells by pulse-labeling the cells with [^{3}H]leucine, [^{3}H]thymidine or [^{3}H]uridine, respectively, and determining the incorporation of these radioactive protein and nucleic acid precursors into acid-insoluble material. The effects of the cannabinoids on cellular uptake of the radiolabeled acid-soluble precursors were also monitored because a decrease of radiolabeled precursors in the acid-soluble pool would limit incorporation into the acid-insoluble fractions; any fluctuations in precursor uptake would yield misleading data as to the actual direct effect of cannabinoids on macromolecular synthesis.

Effects of drug vehicle on incorporation of radiolabeled nucleic acid and protein precursors. Exponentially growing HeLa S_3 cells were exposed for 1 hr to 24, 48, 72 and 96 mM concentrations of ethanol. The cells were then pulse-labeled for 15 min with [^{3}H]leucine, [^{3}H]thymidine and [^{3}H]uridine, and the incorporation of radioactivity into the acid-insoluble and acid-soluble fractions was determined (Fig. 3). After 1 hr of exposure the vehicle-treated cells displayed a marked, dose-dependent decrease in labeled precursor incorporation into DNA and RNA. The vehicle effect on the incorporation of [^{3}H]leucine into protein was not significant. The vehicle did not affect the size of the intracellular radiolabeled pools and thus appeared to have no effect on the cellular uptake of the radiolabeled acid-soluble precursors.

To determine whether the vehicle effect was temporary, the same pulse-labeling studies were carried out with HeLa S_3 cells treated with 0.15% ethanol for 1, 3, 6 and 9 hr prior to pulse-labeling with the appropriate radioactive precursors (Fig. 4). Again,

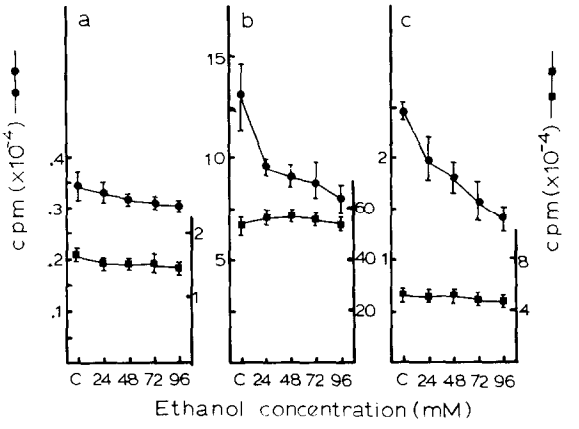

Fig. 3. Effects of drug vehicle (ethanol) on the incorporation of (a) [^{3}H]leucine, (b) [^{3}H]thymidine, and (c) [^{3}H]uridine into protein, DNA, and RNA respectively. Exponentially growing HeLa S_3 cells in suspension culture (containing 0.15% ethanol) were pulse-labeled for 15 min with the appropriate precursor. The incorporation of radioactivity into the acid-insoluble (●) and acid-soluble (■) fractions was determined for cells pretreated for 10 hr with 24, 48, 72 and 96 mM concentrations of drug vehicle (ethanol). C = untreated control.

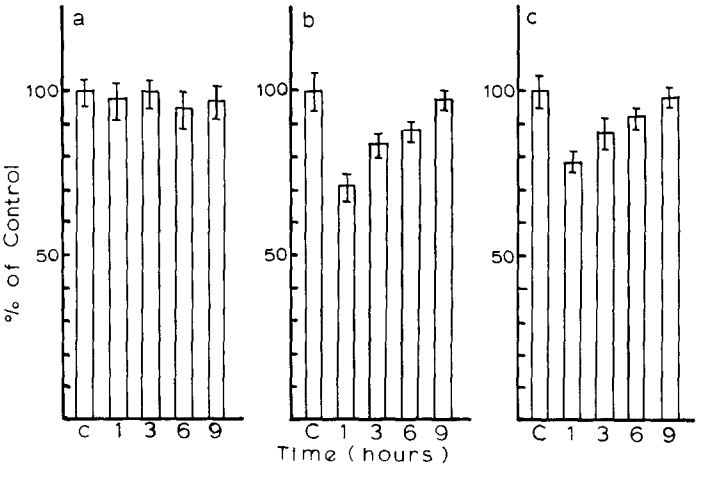

Fig. 4. Reversal effect of drug vehicle (ethanol) on the incorporation of (a) [^{3}H]leucine, (b) [^{3}H]thymidine, and (c) [^{3}H]uridine into protein, DNA, and RNA respectively. Exponentially growing HeLa S_3 cells in suspension cultures were pulse-labeled for 15 min with the appropriate radioactive precursor. The incorporation of radioactivity into the acid-insoluble fractions was determined for cells pretreated with 71 μl of 95% ethanol/50 ml of culture medium for 1, 3, 6 and 9 hr. Appropriate controls were obtained for each radioactive precursor at each time interval. Results are plotted as percent incorporation of control for each radioactive precursor at 1, 3, 6 and 9 hr of preincubation with drug vehicle.

no significant effect of the vehicle was observed on incorporation of [^{3}H]leucine into protein; the initial vehicular depression of the incorporation of [^{3}H]thymidine and [^{3}H]uridine into DNA and RNA, respectively, had reversed and returned to normal 9 hr after introduction of the vehicle into the culture medium. Therefore, in subsequent drug and vehicle studies, the cells were treated for at least 10 hr to avoid vehicular depression of radiolabeled precursor incorporation into DNA and RNA.

Effects of cannabinoids on incorporation of radiolabeled precursors into protein, DNA and RNA. The effects of various concentrations of cannabinoids on incorporation of radiolabeled precursors into total cellular protein, DNA, and RNA (acid-insoluble radioactivity) and into intracellular precursor pools (acid-soluble radioactivity) were examined (Fig. 5). In general, dose-dependent depression of the apparent rates of DNA and RNA synthesis in HeLa cells occurred after 10 hr of exposure to a cannabinoid

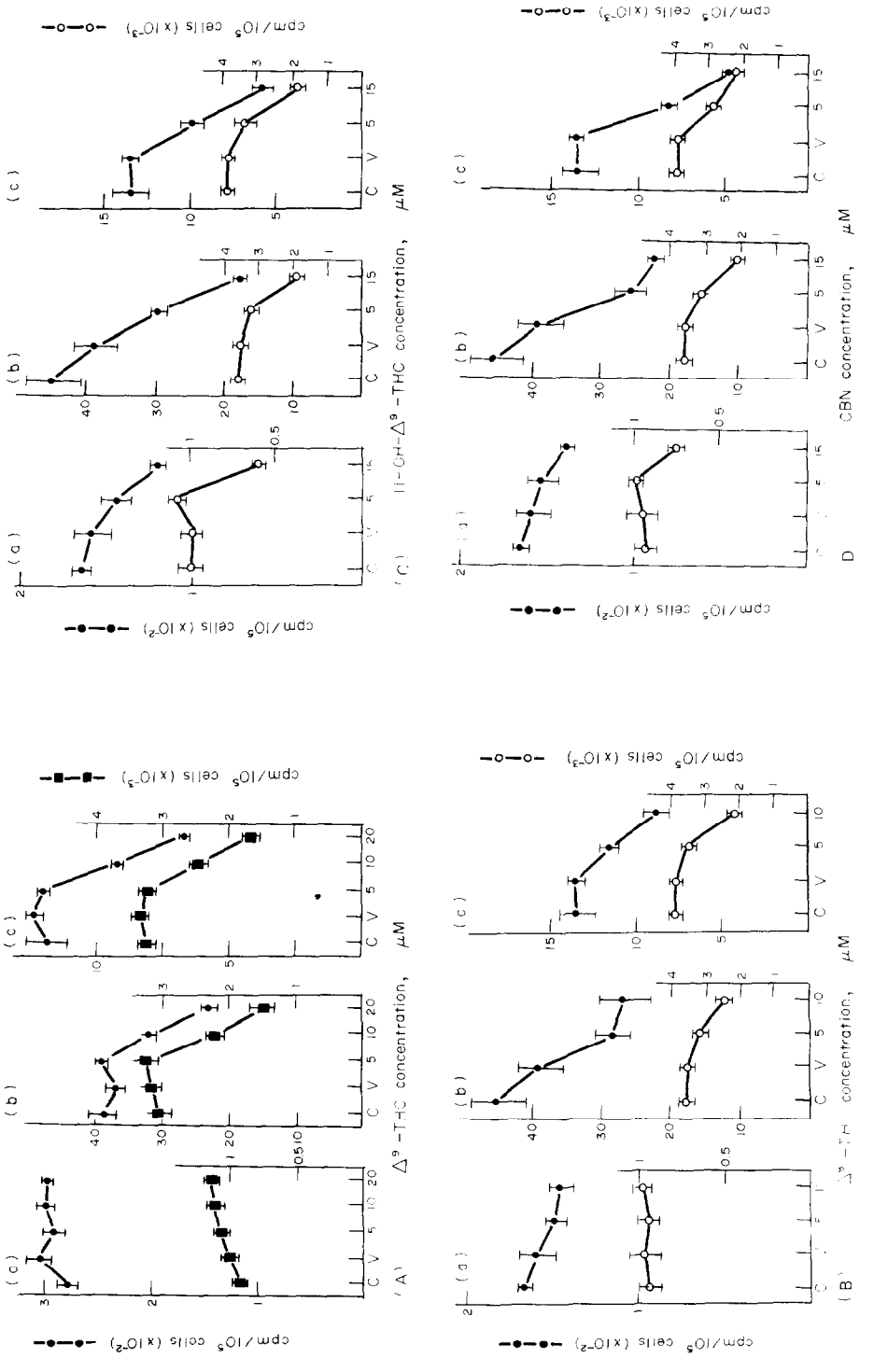

Fig. 5. Effect of cannabinoids on the incorporation of (a) [^{3}H]leucine, (b) [^{3}H]thymidine, and (c) [^{3}H]uridine into protein, DNA, and RNA respectively. Exponentially growing HeLa S_3 cells in suspension culture were pulse-labeled for 15 min with the appropriate radioactive precursor. The incorporation of radioactivity into the acid-insoluble (●) and the acid-soluble (■ or ○) fractions was determined for cells pretreated for 10 hr with the indicated micromolar concentrations of cannabinoids or for control (C) or vehicle-treated (V) cells. A = Δ^9-THC; B = Δ^9-THC; C = 11-OH-Δ^9-THC; and D = CBN.

concentration of 5 μM or higher. The inhibitory effect of cannabinoid on the apparent rate of protein synthesis was not as marked and, in the case of Δ^9-THC, required a concentration of at least 30 μM before an effect was observed.

A dose-dependent depression (17–44 per cent for DNA, 19–35 per cent for RNA) of the apparent rates of DNA and RNA synthesis was observed after 10 hr of exposure to 10–20 μM concentrations of Δ^9-THC (Fig. 5A). The apparent rate of protein synthesis was depressed (23–25 per cent) in 30 and 40 μM Δ^9-THC (data not shown). A 5 μM concentration of Δ^9-THC did not affect the apparent rates of DNA or RNA synthesis, and exposure to 5, 10 or 20 μM Δ^9-THC did not significantly affect the apparent rate of protein synthesis (Fig. 5A).

Δ^8-THC depressed (35–40 per cent for DNA and 9–30 per cent for RNA) the apparent rates of DNA and RNA synthesis at 5 and 10 μM concentrations (Fig. 5B); very slight inhibition (9–12 per cent) of the apparent rate of protein synthesis was observed at 5 and 10 μM concentrations of Δ^8-THC.

11-OH-Δ^9-THC (Fig. 5C) decreased (34–60 per cent for DNA and 27–59 per cent for RNA) the apparent rates of DNA and RNA synthesis at 5 and 15 μM concentrations; 13–27 per cent depression in the apparent rate of protein synthesis was observed at 5 and 15 μM concentrations of 11-OH-Δ^9-THC.

Cannabinol depressed (42–51 per cent for DNA and 40–66 per cent for RNA) the apparent rates of DNA and RNA synthesis at 5 and 15 μM concentrations (Fig. 5D); the apparent rate of protein synthesis was depressed 12–16 per cent in 5 and 15 μM cannabinol.

Effect of cannabinoids on levels of intracellular, acid-soluble nucleic acid and protein precursors. The data in Fig. 5(A–D) also clearly indicate that the observed decreases in the incorporation rates of radiolabeled precursors into DNA, RNA, and protein were accompanied by decreased levels of radiolabeled precursors in the intracellular, acid-soluble precursor pools. In most cases, the decrease in radiolabeled precursor uptake was comparable to the decrease in macromolecular incorporation; this was true of all four cannabinoids studied. It is important therefore to consider the possibility that the observed cannabinoid-induced reduction of radiolabeled precursor incorporation into cellular macromolecules reflected a decreased availability of intracellular radiolabeled precursor. Such cannabinoid-induced changes in nucleotide and amino acid precursor pools could be caused by a decreased precursor uptake resulting from an inhibition of permeability or transport across the cellular membrane. Another possibility is a cannabinoid-induced increase of the endogenous precursor pools of the cell, thus impeding or slowing down exogenous precursor uptake.

To investigate further the cannabinoid effect on the intracellular precursor pools for RNA synthesis, we used two-dimensional thin-layer chromatography to analyze both the size and the specific activity of the intracellular uridine precursor pools of cells treated with Δ^9-THC. Exponentially growing HeLa S_3 cells were maintained for 10 hr in the presence of a 30 μM concentration of Δ^9-THC and then pulse-labeled for 15 min with [^{3}H]uridine. The acid-soluble

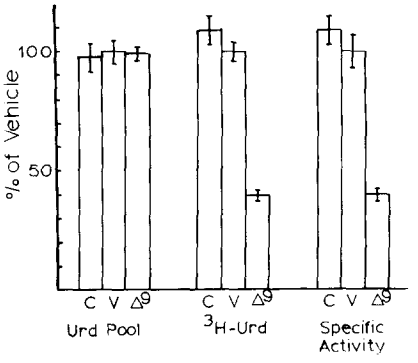

Fig. 6. Intracellular uridine pool, [^{3}H]uridine, and uridine pool specific activity of HeLa S_3 cells pulse-labeled with [^{3}H]uridine after 10 hr of exposure to 30 μM Δ^9-THC. Exponentially growing HeLa cells (30 ml) were grown for 10 hr in the presence of 30 μM Δ^9-THC and then pulse-labeled for 15 min at 37° with 20 μCi of [^{3}H]uridine (23 Ci/mmole). The acid-soluble intracellular pool was isolated and digested with *E. coli* alkaline phosphatase. The uridine pool was isolated by two-dimensional chromatography and measured spectrophotometrically (100 per cent = 15 nmoles Urd). Radioactivity was measured in a scintillation counter (100 per cent = 4723 cpm of [^{3}H]uridine). The specific activity was calculated as the ratio of cpm [^{3}H]uridine/mmole uridine. Results are plotted as percent of vehicle; each value is the mean ± average deviation of three separate samples. Key: C = control; V = vehicle-treated control; and Δ^9 = Δ^9-THC-treated sample.

intracellular material was isolated, extracted with anhydrous ether, and digested with *E. coli* alkaline phosphatase [30–32]; thus, the uridine pool measurements reflect the total uridine present as nucleoside and nucleotide precursors. The size of the uridine pool was measured spectrophotometrically, and the radioactivity of [^{3}H]uridine was assayed in a liquid scintillation spectrometer. The specific activity of the uridine pool was calculated as a ratio of cpm [^{3}H]uridine/nmoles uridine. Figure 6 shows the results plotted as percentages of the uridine pools of vehicle-treated cells (100 per cent vehicle uridine pool = 15 nmoles of uridine; 100 per cent vehicle [^{3}H]uridine = 4723 cpm of [^{3}H]uridine). This procedure recovered more than 93 per cent (with respect to uridine) determined with known amounts of UTP, UMP and uridine subjected to the treatment given to the actual cellular samples.

The size of the uridine pool of Δ^9-THC-treated cells was the same as that of untreated and vehicle-treated cells. The amount of [^{3}H]uridine taken up by the Δ^9-THC-treated cells, however, was approximately 40 per cent of that taken up by the untreated and vehicle-treated cells, and this decrease was also reflected in the specific activity of the drug-treated cells calculated from the former measurements of pool size and radioactivity. Thus, the observed decrease of precursor uptake by the cells can be attributed to a cannabinoid-induced decrease in permeability or transport across the cell membrane and not to a change in the size of the endogenous pools of the cells.

Effects of cannabinoids on in vitro *RNA synthesis.* To assess further the influence of cannabinoids on

RNA synthesis and to circumvent the problem of cannabinoid-induced modifications in the acid-soluble, intracellular nucleotide precursor pool, we assayed RNA synthesis in two *in vitro* systems—isolated nuclei and chromatin. *In vitro* chromatin transcription was done using exogenously added *E. coli* RNA polymerase prepared according to the method of Berg *et al.* [25]. *E. coli* RNA polymerase has been successfully used for transcription of mRNA sequences coding for globin [33, 34], ovalbumin [35] and histones [36–39]. Though it is probable that transcription with *E. coli* RNA polymerase is not identical to that with eukaryotic RNA polymerase, the *E. coli* polymerase has been found to preferentially transcribe certain specific genes (histone, globin and ovalbumin).

Exponentially growing HeLa S_3 cells were treated for 10 hr with 30 μM Δ^9-THC or 15 μM CBN; untreated and vehicle-treated control cultures were used for comparison. Chromatin was isolated as described previously [20] and then transcribed with *E. coli* RNA polymerase. Transcription was measured with various amounts of DNA as chromatin (ranging from 1.5 to 65 μg) for 10 min at 37° in the presence of 0.4 μCi of [^{14}C]-ATP as reported previously [40]. The cpm of [^{14}C]-ATP incorporated into acid-insoluble material versus micrograms of DNA in chromatin, a measure of template activity, was not altered in cannabinoid-treated cells compared with untreated and vehicle-treated cells (Fig. 7). The total template activity of chromatin reflects the percentage of the genome available for transcription

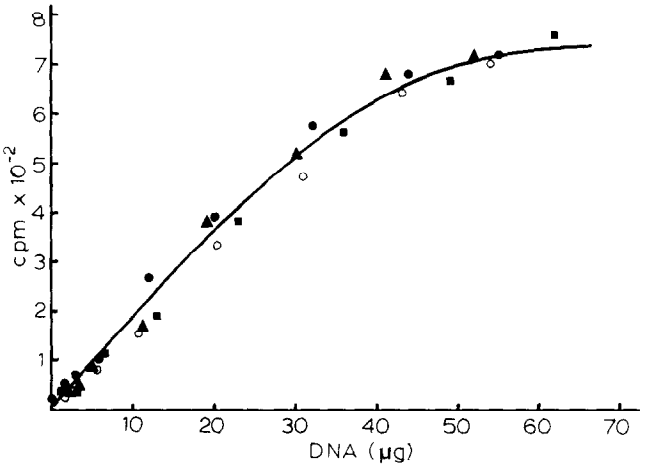

Fig. 7. Effects of Δ^9-THC and cannabinol (CBN) on *in vitro* transcription of chromatin isolated from exponentially growing HeLa S_3 cells pretreated for 10 hr with 30 μM Δ^9-THC (▲) and 15 μM CBN (■) in suspension culture. Control (●) and vehicle-treated (○) samples were also studied. *In vitro* transcription of isolated chromatin was carried out using *E. coli* RNA polymerase in the presence of [^{14}C]-ATP. Results are plotted as cpm of incorporated [^{14}C]-ATP versus μg of DNA in chromatin.

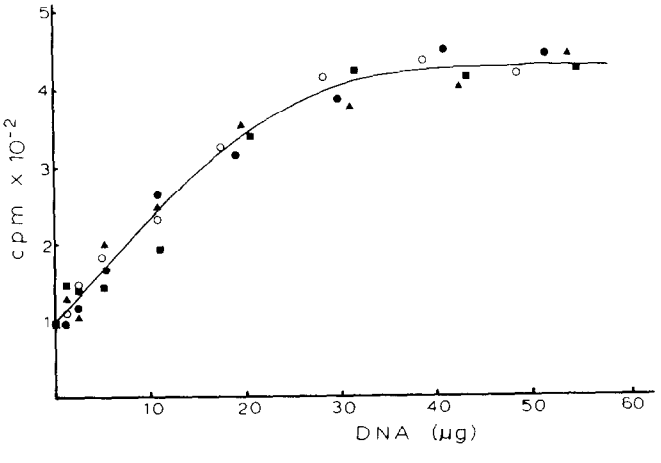

Fig. 8. Effects of Δ^9-THC and cannabinol (CBN) on the *in vitro* transcription of chromatin isolated from exponentially growing HeLa S_3 cells pretreated for 10 hr with 30 μM Δ^9-THC (▲) and 15 μM CBN (■) in suspension culture and transcribed *in vitro* with the same cannabinoid concentrations present in the transcription assay. *In vitro* transcription of isolated chromatin was carried out using *E. coli* RNA polymerase in the presence of [^{14}C]-ATP. Results are plotted as cpm of incorporated [^{14}C]-ATP versus μg of DNA in chromatin. Control (●) and vehicle-treated (○) samples were also studied.

and does not give qualitative information about possible changes in the expression of individual genes.

In the process of isolating chromatin, the amount of bound cannabinoid may have fallen to such a low level that it could not influence the transcription process. Therefore, the same transcription assays were carried out as described above but with 30 μM Δ^9-THC or 15 μM cannabinol present in the transcription assay. The presence of Δ^9-THC or cannabinol in the transcription assay itself did not appear to alter (compared with untreated and vehicle-treated controls) the template activity of chromatin isolated from cannabinoid-treated cells (Fig. 8).

In the preceding transcription studies, an exogenous, prokaryotic RNA polymerase was used. It is possible that cannabinoids do not affect the transcriptional activity of the *E. coli* RNA polymerase but that they affect that of the eukaryotic RNA polymerase. *In vitro* transcription in nuclei has the advantage of using endogenous RNA polymerase *in situ* to ascertain the template activity of the genome. Nuclear isolation and *in vitro* transcription in nuclei were carried out by a modification of the procedure of Sarma *et al.* [26] as described by Detke *et al.* [27]. This procedure has been characterized in our laboratory and has been shown by Detke *et al.* [27] to yield: (1) 70–80 per cent nuclei, without intact cells observed in the nuclear suspension, when examined by phase contrast microscopy; (2) nuclei that exhibit linear incorporation of [^{3}H]-UTP for 45–60 min; and (3) nuclei that retain activity representative of all three classes of eukaryotic, DNA-dependent RNA polymerases.

In our experiments, nuclei were isolated from exponentially growing HeLa S_3 exposed for 10 hr to the following cannabinoid concentrations: 30 μM

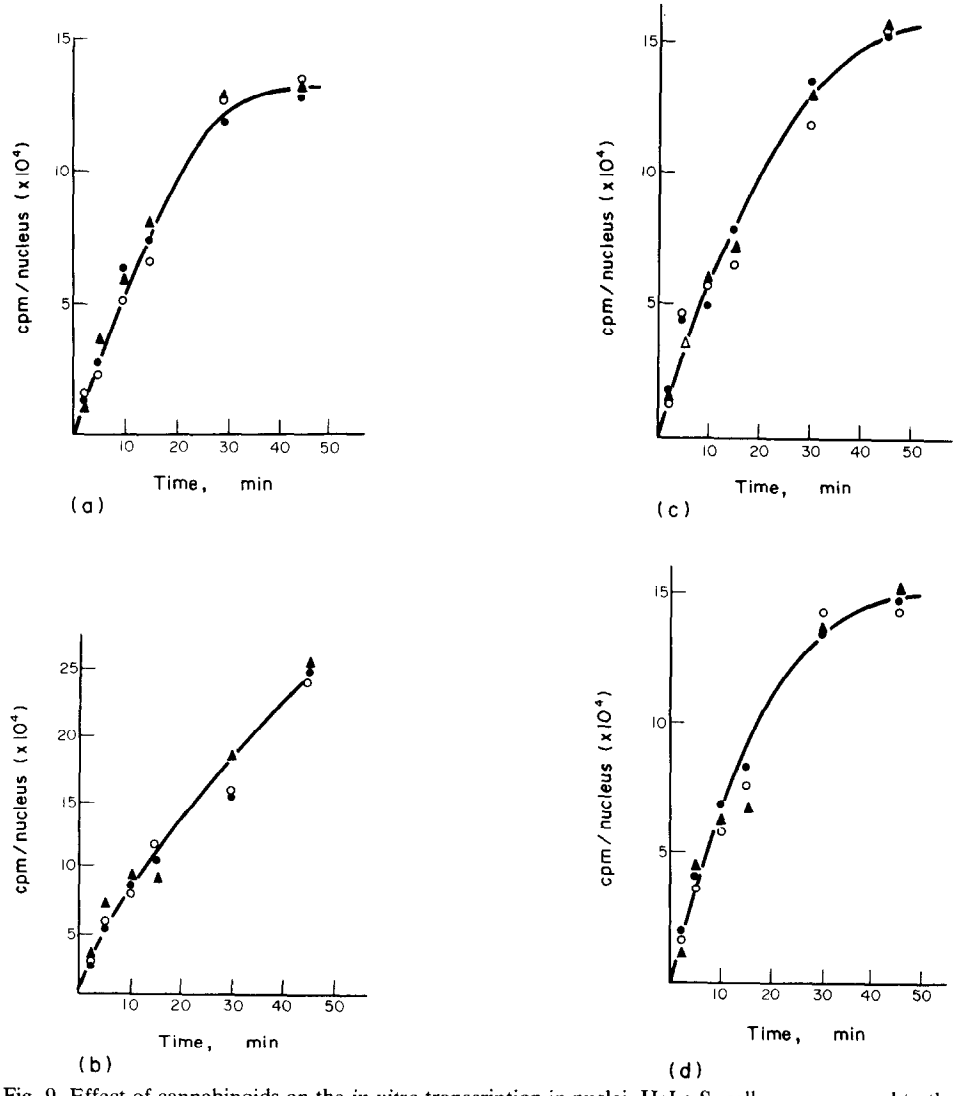

Fig. 9. Effect of cannabinoids on the *in vitro* transcription in nuclei. HeLa S_3 cells were exposed to the cannabinoid for 10 hr prior to isolation of nuclei. Transcription assays were carried out at 25° in a total volume of 100 μl containing 25 μCi of [^{3}H]-UTP (in 0.05 mM UTP) and 1–5 × 10^7 nuclei/ml. Results are plotted as cpm of [^{3}H]-UTP incorporated/nucleus versus time of incubation. Control (●), vehicle-treated (○), and cannabinoid-treated (▲) samples were studied. Key: (a) = 30 μM Δ^9-THC; (b) = 10 μM Δ^8-THC; (c) = 15 μM 11-OH-Δ^9-THC; and (d) = 15 μM CBN.

Δ^9-THC, 10 μM Δ^8-THC, 15 μM 11-OH-Δ^9-THC, or 15 μM CBN. Transcription assays were carried out at 25° in a total volume of 100 μl containing 25 μCi of [^{3}H]-UTP and 1–5 × 10^7 nuclei/ml. The time of incubation was plotted against the cpm of [^{3}H]-UTP incorporated per nucleus. Two types of experiments were performed: (1) nuclear transcriptional activity without cannabinoid added to the transcription medium [Fig 9 (a–d)]; and (2) nuclear transcriptional activity with cannabinoid added to the transcription medium (data not shown). The latter experiment was performed because it is possible that the cannabinoid was extracted from the nuclei during the isolation procedure. The cannabinoids were added to the nuclear transcription assay medium to achieve concentrations of 1.14 mM Δ^9-THC, 1.24 mM Δ^8-THC, 1.30 mM 11-OH-Δ^9-THC, or 1.06 mM CBN. These concentrations were calculated from the previously obtained values of subcellular concentrations of cannabinoids. Figure 9 (a–d) indicates that none of the cannabinoids tested caused any alterations (when compared to untreated and vehicle-treated controls) in the transcriptional activity of nuclei.

Together, the results from our radiolabeled nucleic acid and the protein precursor studies suggest that neither psychoactive nor nonpsychoactive cannabinoids bring about a quantitative change in RNA or protein synthesis. The changes in the apparent rates of precursor incorporation that have been observed in our laboratory and by others [1–5, 9, 41–47] may be cannabinoid-induced modifications at the level of the cell membrane—a possibility that is consistent with reports from several laboratories dealing with cannabinoid-induced changes in nucleic acid precursor transport and in the activities of membrane-associated enzymes. In evaluating the influence of cannabinoids on macromolecular biosynthesis, however, the key question is whether there is a drug-induced effect on expression of specific genes, i.e. changes in the transcription of defined mRNA sequences and/or in the translation of specific polypeptides. Using nucleic acid hybridization analysis with probes for specific genetic sequences and *in vitro* translation of isolated mRNAs from various intracellular compartments, we are presently examining cannabinoid-induced alterations in expression of defined genetic sequences at the transcriptional and at several post-transcriptional levels. Cannabinoid-induced variations in synthesis of specific molecular weight classes of chromosomal proteins are described in the following article [48].

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