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INFLUENCE OF PSYCHOACTIVE AND NONPSYCHOACTIVE CANNABINOIDS ON CHROMATIN STRUCTURE AND FUNCTION IN HUMAN CELLS

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Abstract—The influences of two naturally occurring psychoactive cannabinoids (Δ^9-tetrahydrocannabinol and Δ^8-tetrahydrocannabinol), of a psychoactive cannabinoid metabolite (11-OH-Δ^9-THC), and of a nonpsychoactive cannabinoid (cannabinol) on the composition, metabolism, and structure of human (HeLa cell) chromatin were examined. The effects of all four cannabinoids on the composition and synthesis of chromosomal proteins were studied by pulse-labeling with [^{3}H]leucine. The relative amounts of both histones and nonhistone chromosomal proteins remained unaffected, but a decrease in [^{3}H]leucine incorporation into both histones and nonhistone proteins was observed. Post-translational acetylation and phosphorylation of chromosomal proteins were studied in cannabinoid-treated cells by pulse-labeling with [^{3}H]acetate and [^{32}P]. Variations were observed in [^{3}H]acetate incorporation into histones and [^{32}P] incorporation into both histones and nonhistone chromosomal proteins. Pancreatic DNase I digestion of chromatin from cannabinoid-treated HeLa cells failed to demonstrate any drug-induced alteration in DNA–protein interactions.

Evidence from several laboratories indicates that cannabinoids influence a broad spectrum of behavioral [1–3], physiological [4–5], and biochemical processes [6–27]. The biological processes that are influenced by cannabinoids are, at least in part, a reflection of drug-induced perturbations at the cellular and molecular levels. Cannabinoids have been shown to affect membranes [28], chromosomes [4, 5, 29, 30], cell proliferation [1, 6, 14, 17], nucleic acid and protein synthesis [1, 6, 14, 17], and hormone activity [31–33]. Because such processes involve complex and interdependent biochemical events requiring modifications of gene readout, it is important to assess the action of cannabinoids on the properties of the genome.

In eukaryotic cells the genome is a nucleoprotein complex, referred to as chromatin, consisting primarily of DNA and two classes of chromosomal proteins—histones and nonhistone chromosomal proteins. The histones have been shown to play a key role in the packaging of DNA into nucleosomes, structures that resemble beads on a string, under the electron microscope. Several lines of evidence also suggest that histones may be responsible for the nonspecific repression of genetic sequences. Although some of the complex and heterogeneous nonhistone chromosomal proteins are probably involved in genome structure and in a broad spectrum of enzymatic activities that occur at the level of the genome, other components of these proteins may determine the availability of genetic sequences for transcription. The structural and functional properties of chromatin have been reviewed extensively [34–39]. We approached the question of cannabinoid-induced effects on genome structure and function by examining drug-induced changes in the composition and metabolism of chromosomal proteins and by assessing the influence of cannabinoids on chromatin structure.

MATERIALS AND METHODS

Cell culture

Exponentially growing (log phase) HeLa S_3 cells, a hypotetraploid line of human cervical carcinoma cells, were grown in suspension culture as described previously [27].

Drug administration

Cannabinoids and their administration are described in the preceding paper [40].

Extraction of histones

Chromatin was isolated as described previously [27, 41, 42]. The chromatin was extracted four times with 2 ml of 0.4 N H_2SO_4 for 30 min, each time at 4° in a glass Dounce homogenizer with a tight fitting pestle. The homogenate was centrifuged in a Beckman JS-13 rotor at 12,000 *g* for 30 min after each extraction. Two volumes of 95% ethanol at 4° were added to the pooled mineral acid supernatant fractions, and the histones were allowed to precipitate for 12 hr, the histones were then collected by centrifugation at 12,000 *g* for 30 min. The supernatant fraction was decanted and the precipitated histones were dried in a 100% N_2 atmosphere.

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Polyacrylamide gel electrophoresis of total chromosomal proteins

For the electrophoretic fractionation of total chromosomal proteins, 8.75% sodium dodecylsulfate (SDS)-polyacrylamide slab gels were prepared by the method of Laemmli [43]. A Bio-Rad Laboratories (Richmond, CA) slab gel electrophoresis apparatus was used.

Samples were electrophoresed for approximately 6.5 hr at 30 milliamps per slab using an electrophoresis buffer containing 0.192 M glycine–0.1% (w/v) SDS–0.025 M Tris base (pH 8.2–8.3). After electrophoresis, the slab gel was fixed in 12.5% (w/v) trichloroacetic acid (TCA)–40% (v/v) ethanol–7% (v/v) acetic acid at room temperature for 12 hr. The slab gel was then stained in 0.1% (w/v) Coomassie Brilliant Blue–40% (v/v) ethanol–7% (v/v) acetic acid for 5 hr (a linear relation exists between the protein concentration and the amount of Coomassie Blue dye bound to the gel). The slab gel was then diffusion destained in 20% (v/v) ethanol–7% acetic acid and scanned in a Beckman Acta CII spectrophotometer at 600 nm. The areas under the optical density profiles were integrated with a compensating polar planimeter (Kueffel & Esser Co., Morristown, NJ) to determine the amounts of protein in discrete molecular weight regions of the gels.

For analysis of radioactive isotope incorporation by the various polypeptide bands, the gels were frozen on dry ice and sliced into 2 mm fractions with a Hoeffer (San Francisco, CA) gel slicer. The slices were solubilized in 400 μl of 35% hydrogen peroxide overnight at 37°, Triton X-100–toluene scintillation fluid was added, and the radioactivity was assayed in a Beckman liquid scintillation counter. The specific activity of a particular molecular weight region of a gel was obtained by dividing the total counts per minute (cpm) of the region into the area of that region in the optical density profile.

Polyacrylamide gel electrophoresis of histones

Histones were fractionated electrophoretically with respect to charge and molecular weight on 15% polyacrylamide acetic acid–urea slab gels according to the method of Panyim and Chalkley [44]. The stacking gel was polymerized with 0.0063% (w/v) ascorbic acid, 0.0002% (w/v) $FeSO_4$, and 0.0019% (v/v) hydrogen peroxide as catalysts. A Bio-Rad Laboratories slab gel electrophoresis apparatus was used.

After electrophoresis, the slab gels were fixed and stained simultaneously in 0.1% (w/v) amido black in 40% (v/v) ethanol–7% (v/v) acetic acid for 12 hr. The slab gels were diffusion destained in 35% (v/v) ethanol–7% acetic acid. The gels were also spectrophotometrically scanned at 600 nm. The areas under the optical density profiles were integrated with a compensating polar planimeter to determine the amounts of protein corresponding to each histone region of the gels.

For analysis of radioisotope incorporation into the various histone bands, the gels were frozen on dry ice and sliced into 2 mm fractions. The slices were solubilized in 400 μl of 35% hydrogen peroxide overnight at 37°, Triton X-100–toluene scintillation fluid was added, and the radioactivity was assayed in a Beckman liquid scintillation counter. The specific activity of each histone band was expressed as cpm per unit area (as reflected by optical density).

Composition of chromosomal proteins

Exponentially growing HeLa S_3 cells were exposed to one of several micromolar concentrations of a cannabinoid in the culture medium for 10 hr. An untreated control and a vehicle (95% ethanol)-treated control were used. The cells were pelleted by centrifugation at 500 *g* for 5 min, resuspended at a 15-fold higher cell density in Earle's balanced salt solution containing 15 μCi/ml of L-leucine-[4,5-^{3}H] (62 Ci/mole, Schwarz/Mann, Orangeburg, NY) and 2% fetal calf serum, and then pulse-labeled for 15 min at 37°. Nuclei and chromatin were isolated as described previously. An aliquot of the chromatin sample was used for the electrophoretic fractionation of total chromosomal proteins on 8.75% polyacrylamide slab gels according to the method of Laemmli [43]. Histones were extracted from the remainder of the chromatin and fractionated by electrophoresis on 15% polyacrylamide slab gels according to the method of Panyim and Chalkley [44]. Optical density scans and radioactivity profiles were obtained and used to calculate specific activities.

Post-translational modifications of chromosomal proteins

Exponentially growing HeLa S_3 cells were treated with various micromolar concentrations of cannabinoids in the culture medium for 10 hr. An untreated control and a vehicle (95% ethanol)-treated control were used. The cells were pelleted by centrifugation at 500 *g* for 5 min. To study acetylation of chromosomal proteins, the cells were resuspended at a 15-fold higher cell density in Earle's balanced salt solution containing 30 μCi/ml of sodium acetate[^{3}H] (0.74 Ci/mmole, Schwarz/Mann) and 2% fetal calf serum and, then were pulse-labeled for 30 min at 37°. To study phosphorylation of chromosomal proteins, the cells were initially grown for 10 hr in the presence of cannabinoids in supplemented Joklik-modified Eagle's Minimal Essential Medium containing one-tenth the normal amount of phosphate and, then, resuspended (at a higher cell density) in the same medium and pulse-labeled with 200 μCi/ml of carrier-free [^{32}P] in water (50 mCi/ml, New England Nuclear Corp., Boston, MA) for 30 min at 38°. Nuclei and chromatin were isolated as described previously. An aliquot of the chromatin sample was used for the electrophoretic fractionation of total chromosomal proteins on 8.75% polyacrylamide slab gels according to the method of Laemmli [43]. Histones were extracted from the remainder of the chromatin and were fractionated by electrophoresis on 15% polyacrylamide slab gels according to the method of Panyim and Chalkley [44]. Optical density scans and radioactivity profiles were obtained and used to calculate specific activities.

In the phosphorylation studies, some of the Laemmli gels were treated with TCA according to the method of Bhorjee and Pederson [45] in order to hydrolyze [^{32}P]labeled nucleic acids that might have coelectrophoresed with the chromosomal pro-

teins. The gels were incubated in 5% (w/v) TCA at 90° for 30 min, and the were washed twice for 3 hr in 1 liter of 5% (w/v) TCA at 23° to remove the hydrolysis products. The gels were then washed for 12 hr in 7.5% (v/v) acetic acid before proceeding with the procedures as described above.

Digestion of chromatin with deoxyribonuclease I

Pancreatic deoxyribonuclease I (DNase I, Sigma Chemical Co., St. Louis, MO) was used as a probe to study DNA–protein interactions in chromatin samples isolated from exponentially growing HeLa S_3 cells exposed to 30 μM Δ^9-THC in the culture medium. Both untreated and vehicle (95% ethanol)-treated controls were used. Chromatin samples were resuspended in double distilled water to 250–300 μg DNA/ml (determined by measurements of the absorbance at 260 mm); in a given set of assays all of the chromatin samples contained equal amounts of DNA per ml. To 100 μl of chromatin was added 10 μl of buffer [0.01 M Tris–HCl (pH 7.4)–0.01 M NaCl–3 mM $MgCl_2$] containing eleven times the desired final concentration of DNase I. The samples were then incubated at 37° for 3–4 hr. At the desired times, digestion was stopped by the addition of 900 μl of cold 8.4% (v/v) perchloric acid (PCA), and the samples were centrifuged at 1,400 g for 10 min to deposit the acid-precipitated, undigested chromatin. The supernatant fractions were aspirated and the precipates were dissolved in 1 ml of 0.1 N NaOH. The amounts of DNA hydrolyzed in the supernatant fractions and the amounts of undigested DNA in the resuspended precipitates were determined by measurement of the absorbance at 260 nm.

Two types of digest studies were performed. In one study the DNase I concentration was kept constant at 50 μg/ml and individual digestions carried for various time periods up to 3–4 hr, the results being plotted as per cent DNA digested versus digestion time. In the other, the DNase I concentration was varied from $10^{-2.3}$ to 10^3 μg/ml and all the digestions contrived for 90 min; the results were plotted as percent DNA digested versus $\log_{10}$ of the DNase I concentration in μg/ml.

RESULTS AND DISCUSSION

Nonhistone chromosomal proteins

We employed two methods for fractionating chromosomal proteins. Total chromosomal proteins were fractionated according to molecular weight by electrophoresis in the high resolution, one-dimensional, SDS–8.75% polyacrylamide gel system of Laemmli [43] using a Tris–glycine buffer. The rela-

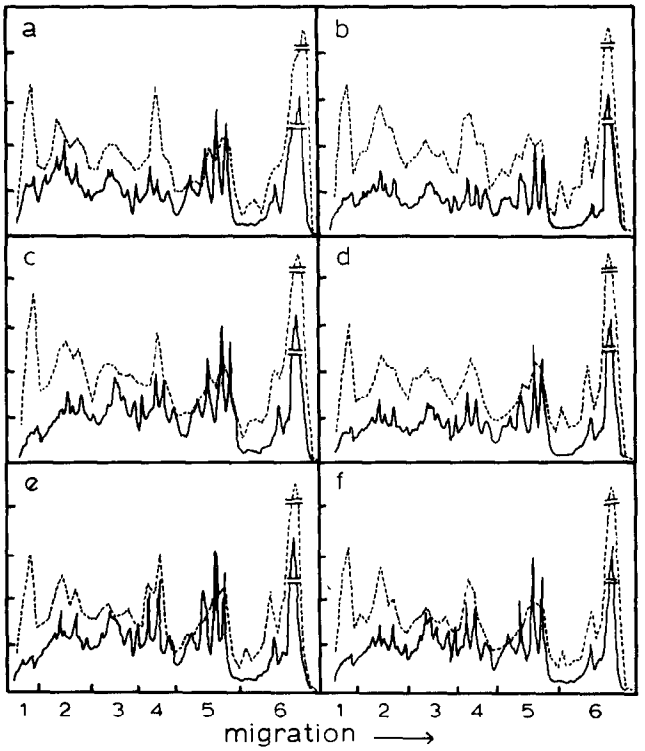


Fig. 1. Absorbance (A_{600}——) and radioactivity (– – –) profiles of polyacrylamide gel electrophoresis of total chromosomal proteins extracted from HeLa S_3 cells pulse-labeled with 15 μCi/ml of [^{3}H]leucine for 15 min after treatment with cannabinoids for 10 hr. The following cannabinoid concentrations were used: 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), 15 μM CBN (f). Control (a) and vehicle-treated (b) samples did not contain cannabinoids. The abscissa is divided into six molecular weight groups: (1) 200,000–150,000; (2) 150,000–100,000; (3) 100,000–70,000; (4) 70,000–50,000; (5) 50,000–35,000; (6) 35,000–17,000. Each mark on the ordinate represents 1 per cent of total gel radioactivity and corresponds to 245 cpm in (a), 209 cpm in (b), 192 cpm in (c), 204 cpm in (d), 213 cpm in (e), and 213 cpm in (f). Approximately 40 μg protein was loaded for each sample.

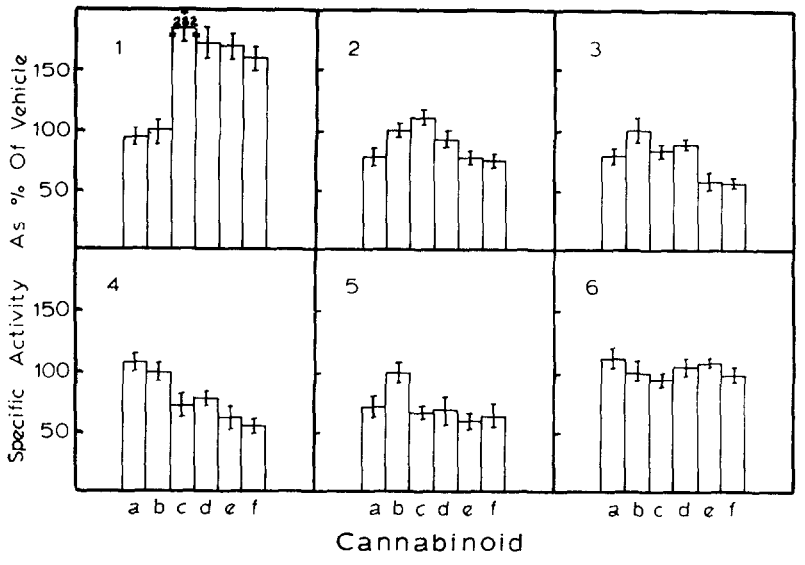


Fig. 2. Specific activities (as percent of vehicle-treated control) of the different molecular weight groups of total chromosomal protein extracted from HeLa S_3 cells pulse-labeled for 15 min with 15 μCi/ml of [^{3}H]leucine after 10 hr exposure to the individual cannabinoids. The following concentrations were used: none (control) (a), none (vehicle-treated control) (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in 8.75% polyacrylamide slab gels, and the absorbance and radioactivity profiles were used to calculate the specific activity of each of six molecular weight groups: (1) 200,000–150,000; (2) 150,000–100,000; (3) 100,000–70,000; (4) 70,000–50,000; (5) 50,000–35,000; and (6) 35,000–17,000. Results are plotted as the mean ± average deviation for two independent determinations. Each sample contained approximately 40 μg protein.

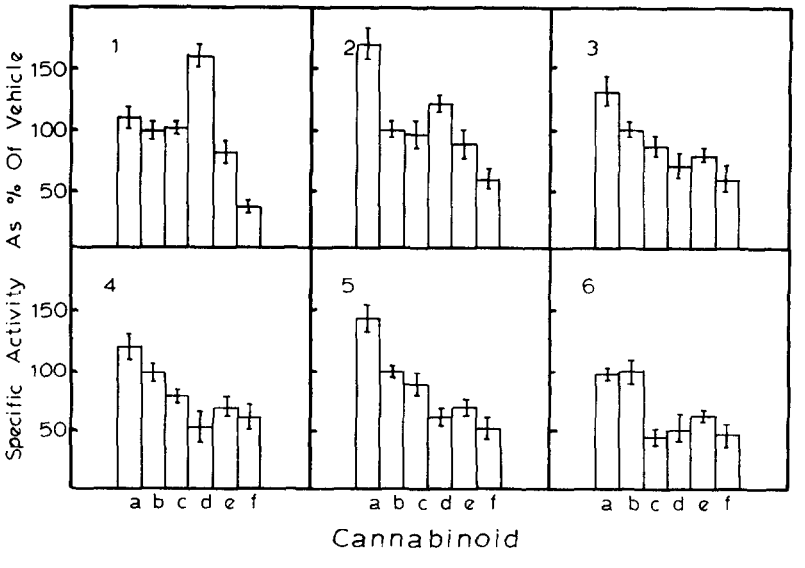


Fig. 3. Specific activities (as percent of vehicle-treated control) of different molecular weight groups of total chromosomal proteins extracted from HeLa S_3 cells exposed for 10 hr to cannabinoids and then pulse-labeled in the presence of the same cannabinoid concentrations with 15 μCi/ml of [^{3}H]leucine. The following concentrations of cannabinoids were used: none (control) (a), none (vehicle-treated control) (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in 8.75% polyacrylamide slab gels, and the absorbance and radioactivity profiles were used (as described in Materials and Methods) to calculate the specific activity of each of six molecular weight groups: (1) 200,000–150,000; (2) 150,000–100,000; (3) 100,000–70,000; (4) 70,000–50,000; (5) 50,000–35,000; (6) 35,000–17,000. Results are plotted as the mean ± average deviation for two independent determinations. Each sample contained approximately 40 μg protein.

tive mobilities of polypeptides in this gel system are directly proportional to the logarithms of their molecular weights. The SDS–8.75% polyacrylamide Laemmli gel system fractionates nonhistone chromosomal proteins into a variety of bands ranging from approximately 200,000 to 17,000 in molecular weight, but histones are resolved into only three bands—two bands corresponding to H_1 and one band (which moves with the solvent front) containing the four remaining histones (H_3, H_2b, H_2a, H_4). For this reason, histones were fractionated with respect to charge and molecular weight on 15% polyacrylamide–acetic acid–urea gels according to the method of Panyim and Chalkley [44]. Acetic acid–urea gels, 28 cm in length, can resolve each of the five histones.

To assess the effects of various cannabinoids on chromosomal protein synthesis, the specific activities of the proteins were measured. HeLa S_3 cells were exposed for 10 hr to the following cannabinoid concentrations: 30 μM Δ^8-THC, 10 μM Δ^9-THC, 15 μM 11-OH-Δ^9-THC, or 15 μM CBN. We found that these concentrations depressed the proliferative capacity of HeLa S_3 cells by 30–40 per cent. The cells were then resuspended in Earle's balanced salts solution and pulse-labeled with 15 μCi/ml of [^{3}H]leucine for 15 min. Total chromosomal protein was extracted from drug-treated cells and from untreated and vehicle-treated cells and fractionated in SDS–polyacrylamide slab gels. Figure 1 shows radioactivity and absorbance profiles of the stained gels. To study and compare the specific activities, the total chromosomal protein was divided into six molecular weight groups: (1) 200,000–150,000; (2) 150,000–100,000; (3) 100,000–70,000; (4) 70,000–50,000; (5) 50,000–35,000; and (6) 35,000–17,000. There are various peaks within each molecular weight group and each peak may contain many different proteins of similar molecular weight. Therefore, caution is required in the interpretation of any change observed, because a charge reflects the net change of many protein species within a group rather than the alteration of individual proteins. Composition of the total chromosomal protein with respect to the six molecular weight groups in cannabinoid-treated samples and in untreated and vehicle-treated controls was similar. As shown in Fig. 2, however, the specific activity of each of the six molecular weight groups varied widely when expressed as a percentage of the specific activity of the protein extracted from the vehicle-treated cells (72–282 per cent for Δ^8-THC, 75–175 per cent for Δ^9-THC, 62–173 per cent for 11-OH-Δ^9THC, and 55–165 per cent for CBN). The highest molecular weight group (1) markedly increased in specific activity (165–282 per cent of vehicle) in all drug-treated samples, while the lower molecular weight groups (2–5) displayed generally varied decreases in their specific activities. These results may reflect changes in the rate of synthesis or turnover of total chromosomal proteins. The lack of a change in the relative composition of the proteins, however, suggests a change in turnover rather than a relative increase or decrease in the accumulation of any one particular molecular weight group of chromosomal proteins.

The same experiment was done with cells pretreated with cannabinoids for 10 hr. None of the cannabinoids caused significant changes in the relative composition of the six molecular weight groups of chromosomal protein. There were significant changes in specific activities, however, and these changes differed somewhat from those previously obtained with cannabinoid-pretreated cells pulse-labeled in the absence of cannabinoids. These differences may be attributable to a loss of cannabinoids prior to pulse-labeling in the former experiment, or to an acute effect of the vehicle and/or drug (superimposed on 10 hr of pretreatment) during the pulse-labeling in the latter procedure. Figure 3 shows that there was a generalized cannabinoid-induced decrease in the specific activities of all molecular weight groups when expressed as a percentage of the specific activity of the chromosomal protein extracted from vehicle-treated cells. Only Δ^9-THC caused a significant increase in specific activitiy of the proteins of molecular weight groups 1 and 2 (165 and 125 per cent of vehicle respectively). These results, as in the previous studies, indicate a cannabinoid-induced change (generally a decrease) in the net turnover of the various molecular weight groups of chromosomal proteins. The wide variation in specific activities of the various molecular weight groups of chromosomal proteins treated with the same cannabinoid suggest that this effect was not due to a generalized cannabinoid-induced decrease in the amount of [^{3}H]leucine made available to the cell.

These cells have been exposed to cannabinoids for 10 hr but have been pulse-labeled for only 15 min. Therefore, the effects of cannabinoids on the relative amounts of chromosomal proteins must be referable to the entire time during which the cells were treated with the drug. The incorporation of [^{3}H]leucine into chromosomal proteins, however, must be referable only to the effects observed during the 15 min of pulse-labeling. From the area of the optical density scans we can, thus, estimate the relative composition (not the absolute amounts) of the chromosomal proteins affected by 10 hr of treatment with cannabinoids. Changes in the specific activity, on the other hand, give the relation between the newly synthesized chromosomal proteins (pulse-labeled for 15 min) and the chromosomal protein already present (affected by 10 hr of cannabinoid treatment). A decrease in specific activity, therefore, could result from any one of several possible cannabinoid-induced effects, among which are decreased synthesis of chromosomal protein, increased breakdown of newly synthesized chromosomal protein, and decreased transport into the nucleus of the cell, the converse would apply to an increase in specific activity. These considerations apply to the pulse-labeling experiments of nonhistone chromosomal proteins just described and to the [^{3}H]leucine pulse-labeling experiments of histone proteins that follow.

Histones

The effects of cannabinoids on the composition and metabolism of histones were studied as follows. Exponentially growing HeLa S_3 cells were pretreated for 10 hr with cannabinoid concentrations of 10 μM Δ^8-THC, 30 μM Δ^9-THC, 15 μM 11-OH-Δ^9-THC or 15 μM CBN, concentrations that bring about approx-

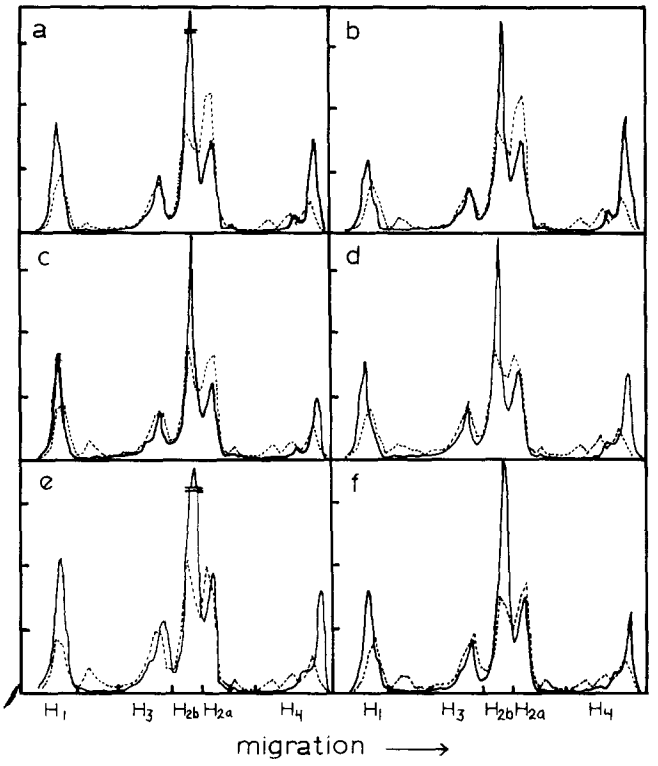


Fig. 4. Absorbance (A_{600} ——) and radioactivity (– – –) profiles of acetic acid–urea–15% polyacrylamide gel electrophoresis of histone proteins (H_1, H_3, H_2b, H_2a, H_4) extracted from HeLa S_3 cells pulse-labeled with 15 μCi/ml of [^{3}H]leucine for 15 min after treatment with cannabinoids for 10 h. The following cannabinoid concentrations were used: 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Each mark on the ordinate represents 5 per cent of total gel radioactivity and corresponds to 996 cpm in (a), 1026 cpm in (b), 739 cpm in (c), 718 cpm in (d), 1038 cpm in (e), and 874 cpm in (f). Approximately 120 μg protein was loaded for each sample.

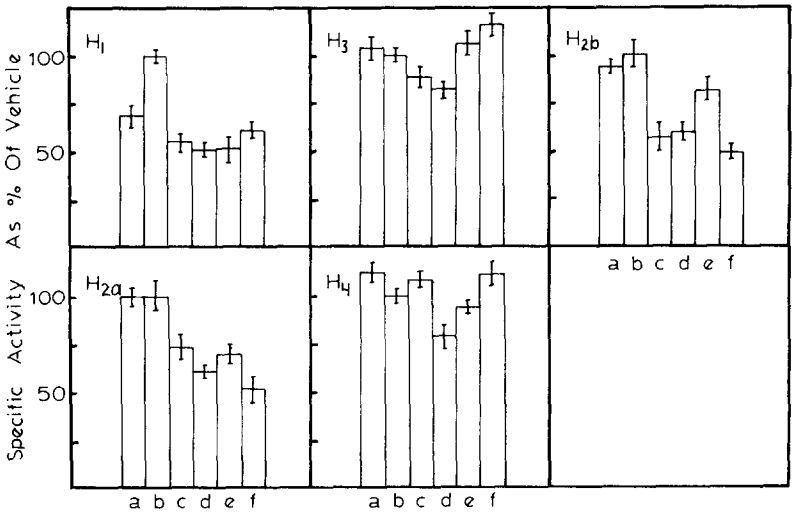


Fig. 5. Specific activities (as percent of vehicle-treated control) of histone proteins (H_1, H_3, H_2b, H_2a, H_4) extracted from HeLa S_3 cells pulse-labeled for 15 min with 15 μCi/ml [^{3}H]leucine for 15 min. The following concentrations of cannabinoids were used: none (control) (a), none (vehicle-treated control) (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in acetic acid–urea–15% polyacrylamide slab gels, and the absorbance and radioactivity profiles were used to calculate the specific activity of each histone protein. Results are plotted as the mean ± average deviation of two independent determinations. Each sample contained approximately 120 μg protein.

imately the same decrease in the proliferative capacity of HeLa S_3 cells. The cells were washed, resuspended in Earle's balanced salt solution, and pulse-labeled with 15 μCi/ml of [^{3}H]leucine for 15 min. Histones were extracted from drug-treated cells and from untreated and vehicle-treated cells, and fractionated by electrophoresis in acetic acid–urea–15% polyacrylamide slab gels. The radioactivity and absorbance profiles of the amido black-stained gels (Fig. 4) were divided into five major histone classes: H_1, H_3, H_2b, H_2a, and H_4 which were used to calculate the percentages of total histone and the specific activity for each class of histones. None of the four cannabinoids caused significant changes in the relative compositions of the five histones. As shown in Fig. 5, however, the specific activities of the histones varied for the different histone classes when expressed as percent of the specific activity of histones extracted from vehicle-treated cells (56–105 per cent for Δ^8-THC, 52–83 per cent for Δ^9-THC, 53–94 per cent for 11-OH-Δ^9-THC, 50–107 per cent for CBN). In general, a decrease in specific activity was brought about by most of the cannabinoids; histones H_1, H_2b and H_2a exhibited the most marked decrease. The lack of a change in the relative composition of the histones, along with the decreases in specific activity, suggests a cannabinoid-induced change (generally a decrease) in the synthesis of histones.

Similar studies were carried out with cells pretreated with cannabinoids for 10 hr but with the pretreatment concentrations of cannabinoids present in the pulse-labeling medium. The cannabinoids did not produce significant changes in the relative composition of the five histone classes. But, as was also observed in the chronic cannabinoid treatment experiments just described, the cannabinoids produced a generalized decrease in the specific activities of the different histone classes (Fig. 6) when expressed as percent of the specific activity of the corresponding histones extracted from vehicle-treated cells (50–82 per cent for Δ^8-THC, 61–80 per cent for Δ^9-THC, 65–84 per cent for 11-OH-Δ^9-THC, and 75–99 per cent for CBN). Only 11-OH-Δ^9-THC caused a significant specific activity increase in H_1 (144 per cent of vehicle). Thus, the results of this latter study also suggest a cannabinoid-induced change (generally a decrease) in the synthesis of histones.

In evaluating the histone pulse-labeling experiments, one must consider the possibility of a cannabinoid-induced alteration in the binding of newly synthesized, radiolabeled histones to DNA and chromatin, which would lead to differences in their extractability and, therefore, to variations in specific activity. Such changes in the preferential release or restriction of histones may, in part, reflect subtle cannabinoid-induced variations in post-translational histone modifications.

The composition and metabolism studies just described suggest that cannabinoids do not alter the relative composition of either histones or nonhistone chromosomal proteins associated with the genome. All four cannabinoids, however, bring about various changes (mostly decreases) in the apparent rates of synthesis and/or turnover of both histones and nonhistone chromosomal proteins. Since chromosomal proteins have been implicated in the packaging,

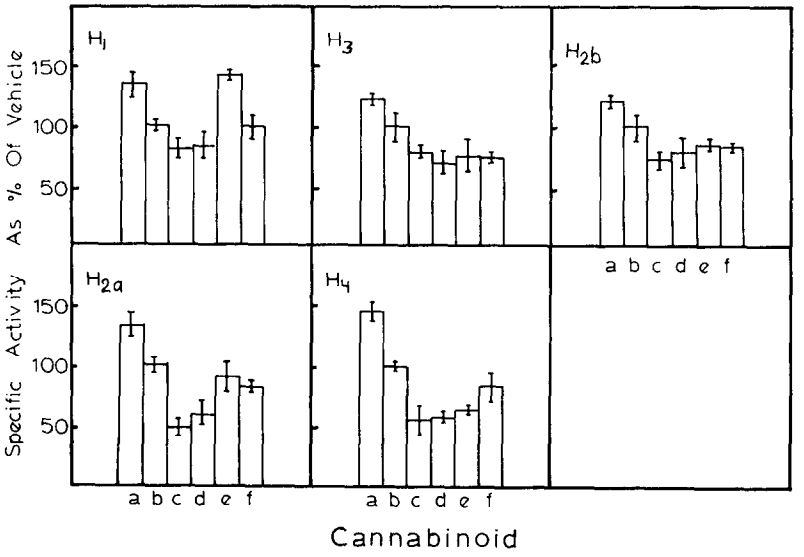


Fig. 6. Specific activities (as percent of vehicle-treated control) of histone proteins (H_1, H_3, H_2b, H_2a, H_4) extracted from HeLa S_3 cells exposed for 10 hr to cannabinoids and then pulse-labeled in the presence of the same cannabinoid concentrations with 15 μCi/ml of [^{3}H]leucine for 15 min. The following concentrations of cannabinoids were used: none (control) (a), none (vehicle-treated control) (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in acetic acid-urea-15% polyacrylamide slab gels, and the absorbance and radioactivity profiles were used to calculate the specific activity of each histone protein. Results are plotted as the mean ± average deviation of two independent determinations. Each sample contained approximately 120 μg protein.

structure, and functional integrity of the eukaryotic genome, alterations of this type can affect the structural and functional properties of the genome and, thereby, impair the normal patterns of gene expression essential, in a restricted sense, for regulation of cell proliferation and, in a general sense, for biological viability. Again, as with the cell growth studies, the psychoactive index of the cannabinoids does not appear to be directly related to their effects.

Post-translational modifications of chromosomal proteins

Acetylation. We have also examined the influence of cannabinoids on two post-translational modifications of chromosomal proteins–acetylation of histones and phosphorylation of histones and nonhistone chromosomal proteins. These reversible, enzyme-catalyzed modifications of chromosomal proteins have been implicated in mediating chromosomal protein–DNA as well as chromosomal protein–chromosomal protein interactions that may, in part, be responsible for determining the availability of genetic sequences for transcription. Hence, chromosomal protein acetylation and phosphorylation may play a central role in structural as well as functional properties of the genome (reviewed in Refs. 46 and 47).

To study the effects of cannabinoids on histone acetylation, exponentially growing HeLa S_3 cells were pretreated for 10 hr with cannabinoid concentrations of 10 μM Δ^8-THC, 30 μM Δ^9-THC, 15 μM 11-OH-Δ^9-THC, or 15 μM CBN. The cells were washed, resuspended in Earle's balanced salt solution, and then pulse-labeled with 30 μCi/ml of [^{3}H]sodium acetate for 30 min. Histones were extracted from drug-treated, untreated, and vehicle-treated cells, and were fractionated by electrophoresis in acetic acid–urea–15% polyacrylamide gels. Figure 7 shows the radioactivity and absorbance profiles of the stained gels. These profiles were used to calculate the specific activity for each class of acetylated histones–H_3, H_2b, H_2a, and H_4. Figure 8 shows the specific activities of the acetylated histones expressed as percent of the specific activity of acetylated histones extracted from vehicle-treated cells.

There was a generalized cannabinoid-induced decrease in the specific activities of the acetylated histones when expressed as a percentage of those from vehicle-treated cells [48–79 per cent for Δ^8-THC, 51–72 per cent for Δ^9THC, 70–96 per cent for 11-OH-Δ^9-THC, and 57–75 per cent for CBN). These changes varied significantly among all four classes of acetylated histones extracted from cells treated with the same cannabinoid and, therefore, cannot be caused by a decrease in the [^{3}H]acetate made available to the cell. If the latter were true,

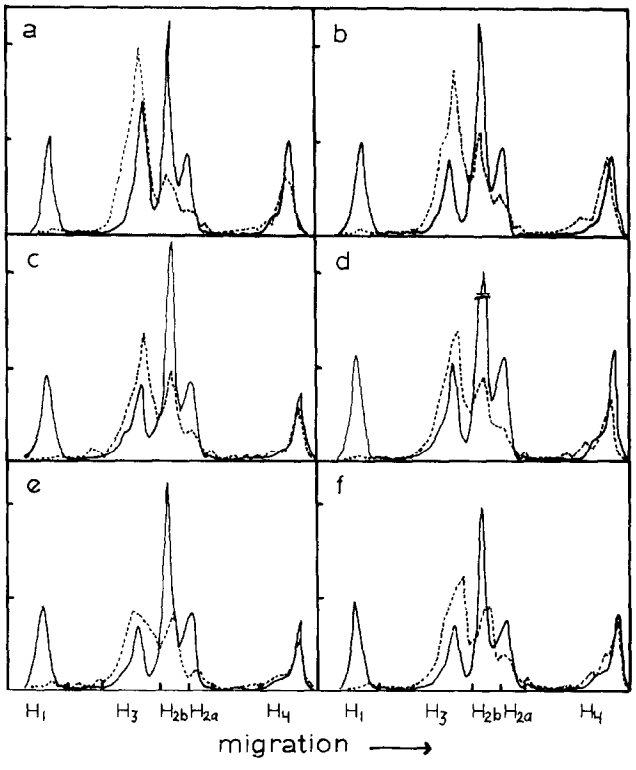


Fig. 7. Absorbance (A_{600} —) and radioactivity (– – –) profiles of acetic acid–urea–15% polyacrylamide gel electrophoresis of histone proteins (H_1, H_3, H_2b, H_2a, H_4) extracted from HeLa S_3 cells pulse-labeled with 30 μCi/ml of sodium acetate[^{3}H] for 30 min after treatment with cannabinoids for 10 hr. The following cannabinoid concentrations were used: 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9 (e), and 15 μM CBN (f). Each mark on the ordinate represents 5 per cent of total gel radioactivity and corresponds to 226 cpm in (a), 211 cpm in (b), 174 cpm in (c), 181 cpm in (d), 195 cpm in (e), and 175 cpm in (f). Approximately 120 μg protein was loaded for each sample.

one would expect to observe the same amount of change in the acetylation of all four histone classes extracted from cells treated with the same cannabinoid. Our results indicate interference (a decrease) with the histone acetylation process brought about by cannabinoid treatment of the cells. Cannabinoid-induced changes in the structure of the histones may modify the availability of acetylation sites and, therefore, alter the gain or loss of acetate groups. Alternatively, the cannabinoids may be affecting the histone acetylases or deacetylases.

The same experiment was repeated with cells pretreated with cannabinoids for 10 hr but with the pretreatment concentrations of cannabinoids present in the pulse-labeling medium. The specific activities of histones extracted from cannabinoid-treated cells displayed a generalized decrease when expressed as percent of specific activity of acetylated histones extracted from vehicle-treated cells (47–95 per cent for Δ^8-THC, 58–94 per cent for Δ^9-THC, 75–80 per cent for 11-OH-Δ^9-THC, and 79–98 per cent for CBN). It should be emphasized that, in acetylation studies, changes in histone acetylation may influence the extractability of histones [48–50] and, thus, alter their apparent specific activities.

Phosphorylation. We initially addressed the possible influence of cannabinoids on phosphorylation of chromosomal proteins as follows. Exponentially growing HeLa S_3 cells were grown in Joklik-modified Eagle's Minimal Essential Medium containing one-tenth the normal amount of phosphate. The cells were exposed for 10 hr to cannabinoid concentrations of 10 μM Δ^8-THC, 30 μM Δ^9-THC, 15 μM 11-OH-Δ^9-THC, or 15 μM CBN. The cells were resuspended at a 15-fold higher cell density in the same cannabinoid-containing medium and then pulse-labeled with 200 μCi/ml of carrier-free [^{32}P]phosphate for 30 min. Total chromosomal proteins were extracted from drug-treated, untreated, and vehicle-treated cells and were fractionated in SDS-polyacrylamide slab gels. Figure 9 shows the radioactivity and absorbance profiles of Coomassie Blue-stained gels. These profiles were utilized to calculate the specific activity for each molecular weight group of proteins. Figure 10 shows that the specific activities of these proteins varied widely among the different molecular weight groups when expressed as per cent of the specific activity of the proteins extracted from vehicle-treated cells (95–125 per cent for Δ^8-THC, 34–57 per cent for Δ^9-THC, 75–110 per cent for 11-OH-Δ^9-THC, and 51–83 per cent for CBN). There was no general pattern to these changes, except that Δ^9-THC produced the greatest depression in the specific activities of most molecular weight groups. Although the highest molecular weight group (1) exhibited the largest cannabinoid-induced increases in specific activities (up to 826 per cent of vehicle for Δ^9-THC and 286 per cent of vehicle for Δ^8-THC), these extremely large increases could be due to the presence of [^{32}P]-labeled nucleic acids that might have co-electrophoresed with chromosomal proteins [51]. The radioactivity and absorbance profiles of Fig. 9 support this possibility by showing high levels of radioactivity (perhaps due to the presence of [^{32}P]-labeled nucleic acid) and low amounts of protein in the high molecular weight group (1). An alternative explanation would be the magnification of experimental errors in calculating the cpm per unit area ratio for such large cpm values associated with a very small

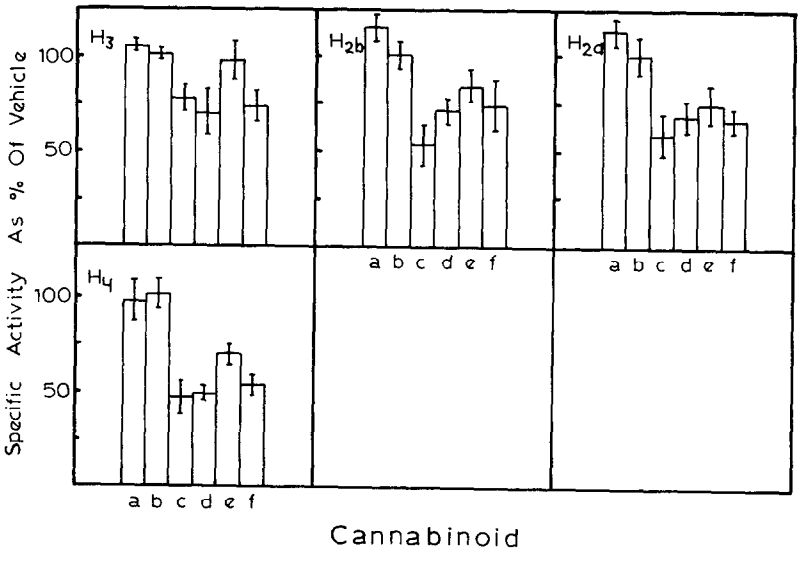


Fig. 8. Specific activities (as percent of vehicle-treated control) of acetylated histone proteins (H_3, H_2b, H_2a, H_4) extracted from HeLa S_3 cells pulse-labeled for 30 min with 30 μCi/ml of [^{3}H]sodium acetate after 10 h of exposure to cannabinoids. The following concentrations of cannabinoids were used: none (control) (a), none (vehicle-treated control) (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in acetic acid–urea–15% polyacrylamide slab gels, and the absorbance and radioactivity profiles were used to calculate the specific activity of each histone protein. Results are plotted as the mean ± average deviation of two independent determinations. Each sample contained approximately 120 μg protein.

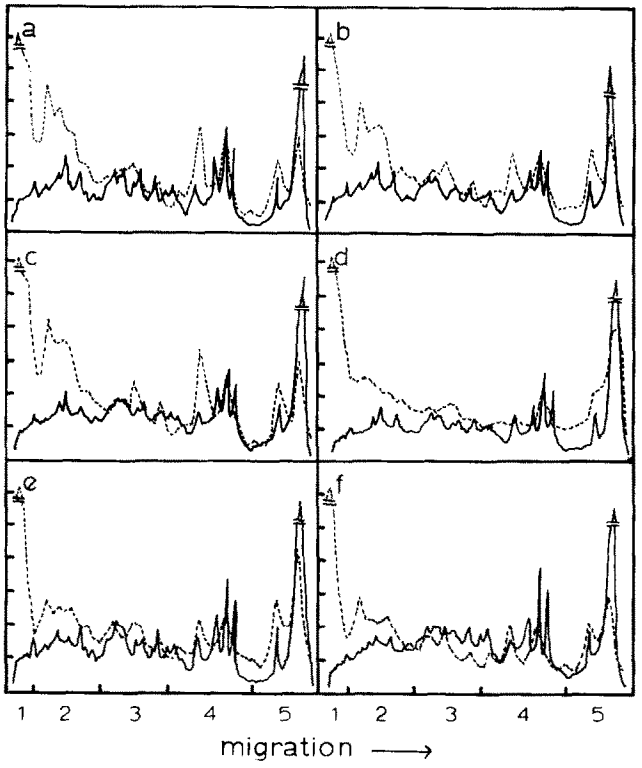


Fig. 9. Absorbance (A_{600} ——) and radioactivity(– – –) profiles of polyacrylamide gel electrophoresis of total chromosomal proteins extracted from HeLa S_3 cells exposed for 10 hr to cannabinoids and then pulse-labeled in the presence of the same cannabinoid concentrations with 200 Ci/ml of ^{32}P for 30 min. The following cannabinoid concentrations were used: none (control) (a), none (vehicle-treated control) (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). The abscissa is divided into five molecular weight groups: (1) 200,000–150,000; (2) 150,000–90,000; (3) 90,000–55,000; (4) 55,000–30,000; and (5) 30,000–17,000. Each mark on the ordinate represents 1 per cent of total gel radioactivity and corresponds to 150 cpm in (a), 135 cpm in (b), 127 cpm in (c), 56 cpm in (d), 117 cpm in (e), and 126 cpm in (f). Approximately 80 μg protein was loaded for each sample.

area (small amount of protein) of the absorbance profile. The overall changes in specific activities of drug-treated cells suggest a cannabinoid-induced alteration in the phosphorylation and perhaps dephosphorylation processes of total chromosomal proteins. These changes in phosphorylation varied significantly among all five molecular weight groups of chromosomal proteins extracted from cells treated with the same cannabinoid and, therefore, cannot be attributed to a generalized decrease in the amount of ^{32}P made available to the cell.

To assess further the possibility that ^{32}P-labeled nucleic acid might have co-electrophoresed with the chromosomal proteins, the same amounts of chromosomal proteins from the experiments just described were fractionated in SDS–polyacrylamide gels, but the gels were treated with 5% TCA in order to hydrolyze ^{32}P-labeled nucleic acids. There was a significant decrease in the amount of radioactivity present in the high molecular weight group of chromosomal polypeptides (group 1), indicating that there may have been ^{32}P-labeled nucleic acid present in this group in the previous experiment. Acid treatment, however, did not alter the amount of radioactivity present in the gel regions corresponding to groups 2–5 chromosomal polypeptides. It appears, therefore, that cannabinoids influence phosphorylation of several molecular weight classes of chromosomal polypeptides (groups 2–5) and that, although there also appear to be some cannabinoid-induced alterations in the phosphorylation of the higher molecular weight chromosomal proteins (class 1), the presence of nucleic acid in this higher molecular weight region of our acrylamide gels precludes a quantitative assessment of the phenomenon.

To investigate the effects of cannabinoids on histone phosphorylation, exponentially growing HeLa S_3 cells were grown in Joklik-modified Eagle's Minimal Essential Medium containing one-tenth the normal amount of phosphate. The cells were exposed for 10 hr to cannabinoid concentrations of 10 μM Δ^8-THC, 30 μM Δ^9-THC, 15 μM 11-OH-Δ^9-THC, or 15 μM CBN. They were then resuspended at a 15-fold higher cell density in the same cannabinoid-containing medium and then pulse-labeled with 200 μCi/ml of carrier-free [^{32}P]phosphate for 30 min. Histones were extracted with dilute mineral acid from drug-treated, untreated, and vehicle-treated cells as described previously [49] and were fractionated in acetic acid–urea–15% polyacrylamide slab gels. As expected, only histones H_1 and H_2b were phosphorylated to a significant extent. Figure 11

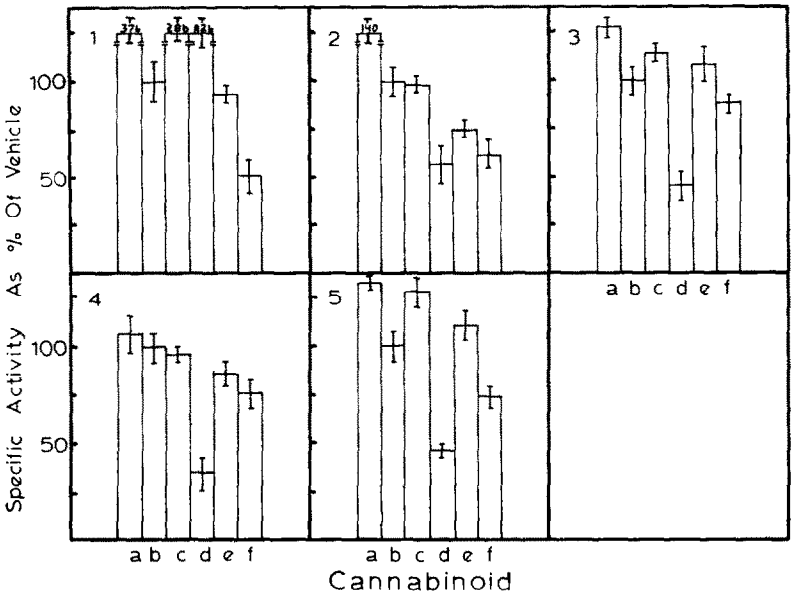


Fig. 10. Specific activities (as percent of vehicle-treated control) of different molecular weight groups of total chromosomal proteins extracted from HeLa S_3 cells exposed for 10 hr to cannabinoids and then pulse-labeled in the presence of the same cannabinoid concentrations with 200 μCi/ml of ^{32}P. The following concentrations of cannabinoids were used: none (control) (a), none (vehicle-treated control) (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in 8.75% polyacrylamide slab gels, and the absorbance and radioactivity profiles were used to calculate the specific activity of each of five molecular weight groups: (1) 200,000–150,000; (2) 150,000–90,000; (d) 90,000–55,000; (4) 55,000–30,000; and (5) 30,000–17,000. Results are plotted as the mean ± average deviation of two independent determinations. Each sample contained approximately 80 μg protein.

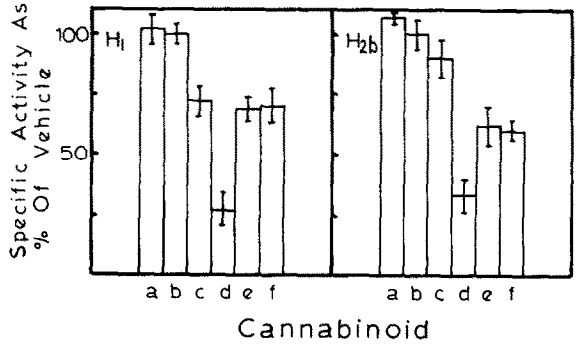
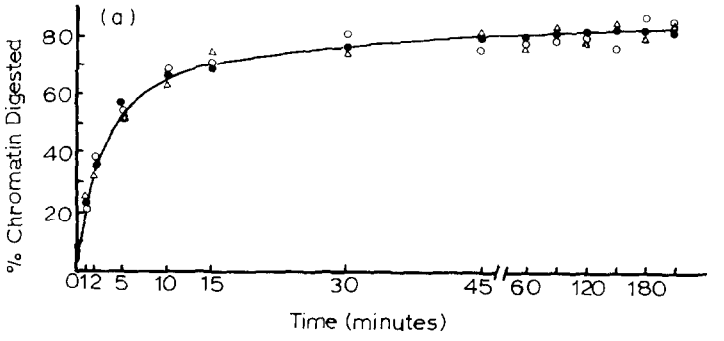


Fig. 11. Specific activities (as per cent of vehicle-treated control) of phosphorylated histones (H_1, H_2b) extracted from HeLa S_3 cells exposed to cannabinoids for 10 hr and then pulse-labeled in the presence of the same cannabinoid concentrations with 200 μCi/ml of ^{32}P for 30 min. The following cannabinoid concentrations were used: none (control) (a), none (vehicle-treated control) (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in 15% acetic acid–urea polyacrylamide slab gels, and the absorbance and radioactivity profiles were used to calculate the specific activity of each histone protein. Results are plotted as the mean ± average deviation of two independent determinations. Each sample contained approximately 135 μg protein.

shows the specific activities of the phosphorylated histones, expressed as per cent of the specific activity of phosphorylated histones extracted from vehicle-treated cells. We observed a generalized cannabinoid-induced decrease in the specific activities of the phosphorylated histones when expressed as percent of those from vehicle-treated cells (73–90 per cent for Δ^8-THC, 27–35 per cent for Δ^9-THC, 61–71 per cent for 11-OH-Δ^9-THC, and 58–71 per cent for CBN). As was the case with total chromosomal protein phosphorylation, Δ^9-THC caused the largest decreases in the specific activities of the phosphorylated histones. The changes in specific activities varied significantly between both classes of phosphorylated histones treated by the same cannabinoid and cannot be caused by a generalized decrease in the ^{32}P made available to the cell. Therefore, our results indicate an interference (decrease) with histone phosphorylation brought about by cannabinoid treatment of the cells. It is possible that the cannabinoids, and especially Δ^9-THC, cause decreases in phosphorylation by interfering with the nuclear protein kinases responsible for phosphorylation [52–54] or by increasing the activity of the dephosphorylating enzymes. Alternatively, the cannabinoids may divert utilization of [^{32}P]phosphate into other levels of cellular metabolism, such as into the synthesis of cyclic AMP.



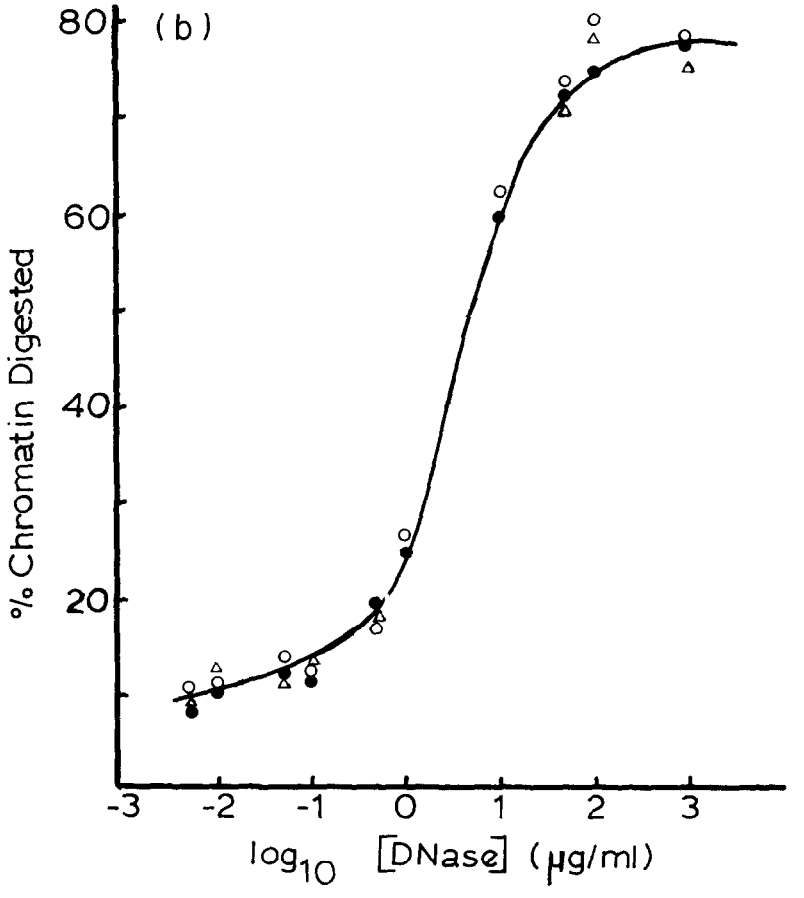


Fig. 12. DNase I digestion [at constant (panel a) and at various (panel b) DNase I concentrations] of chromatin isolated from HeLa S_3 cells exposed to 30 μM Δ^9-THC. Panel a: Chromatin samples containing 25–30 μg DNA were incubated at 37° with 50 μg/ml of DNase I for various time intervals up to 3.5 hr. The percentage of chromatin digested was calculated from the amount of DNA hydrolyzed and the amount of nondigested DNA, as determined by spectrophotometric measurements at 260 nm. Results are plotted as the per cent chromatin digested at various time intervals. Each point is the mean of duplicate digestions. Control (●), vehicle-treated (○), and Δ^9-THC-treated (△) samples were used. Panel b: Chromatin samples containing 25–30 μg DNA were incubated for 90 min at 37° with various concentrations of DNase I ranging from $10^{-2.3}$ to 10^3 μg/ml. The percentage of chromatin digested was calculated from the amount of DNA hydrolyzed and the amount of nondigested DNA, as determined by spectrophotometric measurements at 260 nm. Results are plotted as per cent chromatin digested at various DNase I concentrations. Each point is the mean of duplicate digestions. Control (●), vehicle-treated (○), and Δ^9-THC-treated (△) samples were used.

Since cannabinoids (decrease) phosphorylation of histones and nonhistone chromosomal proteins, it is reasonable to assume that cannabinoid-induced post-translational modifications of chromosomal proteins may, at least in part, be involved with the alterations in genome structure and function. With the data presently available, however, it would be presumptuous to postulate the primary level at which the drugs are acting. Yet it is interesting to note that, as with other parameters we have assayed, there appears to be no correlation between the psychoactive effects of the cannabinoids and their influences on cellular and molecular processes.

Influence of cannabinoids on chromatin structure

Cannabinoid-induced alterations in acetylation and phosphorylation of chromosomal proteins are compatible with drug-induced modifications in the genome. We used nucelases as probes to study the relative interactions between DNA and chromosomal proteins.

The rate of digestion of chromatin-DNA, by DNase I, is dependent on the degree of binding of proteins to DNA. It is possible therefore to study the relative rates of digestion of DNA in chromatin as a means of distinguishing relative differences in chromatin structure that may be related to cannabinoid treatment. Chromatin was isolated from exponentially growing HeLa S_3 cells exposed to 30 μM concentrations of Δ^9-THC and from untreated and vehicle-treated control cultures. Chromatin samples containing 25–30 μg/ml of DNA were incubated at 37° with 50 μg/ml DNase I for various time intervals up to 3.5 hr. The percentage of chromatin digested was calculated from the amount of DNA hydrolyzed and from the amount of non digested DNA measured by spectrophotometric measurements at 260 nm. Figure 12(a) shows the results plotted as percent chromatin digested versus time of incubation. There appeared to be no differences in either the rate or total amount of digestion of chromatin (80 per cent) among the drug-treated, untreated, and vehicle-treated samples.

To confirm these findings, the chromatin digestion was repeated with various DNase I concentrations (from $10^{-2.3}$ to 10^3 μg/ml) and all digestions were carried out for 90 min. Figure 12(b) shows the results plotted as percentage of chromatin digestion versus the log to the base 10 of DNase I concentration in μg/ml. Again, there appeared to be no differences in the amount of chromatin digested by various DNase I concentrations among the drug-treated, untreated, and vehicle-treated samples.

These results suggest that Δ^9-THC (at a 30 μM concentration) does not alter the DNA–protein interaction in chromatin assayed by chromatin digestion with pancreatic DNase I. The crucial question, however, is of a qualitative rather than of a quantitative nature—are specific genetic sequences preferentially nuclease sensitive following treatment with psychoactive and nonpsychoactive cannabinoids? This question is readily approached by analyzing nuclease-sensitive and insensitive DNA sequences, utilizing probes for specific genetic sequences—complementary DNA and cloned genes.

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