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PLASMA MEMBRANE INHIBITION OF MACROMOLECULAR PRECURSOR TRANSPORT BY THC

BERNARD DESOIZE, COLETTE LÉGER and GABRIEL NAHAS

Faculté de Pharmacie, Laboratoire de Biochimie, 51, rue Cognacq-Jay, 51096 Reims Cedex, INSERM U-26, Laboratoire de Pharmacologie et de Toxicologie Cellulaires, 200, rue du Faubourg Saint-Denis, 75475 Paris Cedex 10-France and Department of Anesthesiology, College of Physicians and Surgeons, Columbia University, New York, N.Y., U.S.A.

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Abstract— 10^{-6} to 10^{-4} M of delta-9-tetrahydrocannabinol (THC) or of other cannabinoids, which all have in common the C ring of olivetol, inhibit in cultured lymphocytes incorporation of [^{3}H]thymidine. The inhibitory effect of olivetol derivatives is related to their octanol–water partition coefficient (liposolubility). Within 15 min of incubation, THC inhibits precursor pool formation and macromolecular incorporation of thymidine, uridine and leucine. THC inhibits also [^{14}C]aminoisobutyric acid uptake into the cell, but does not alter the cellular "leakage" of this amino acid analogue. Using the isotopic dilution technique with L1210 murine lymphoma cells and human lymphocytes, it was observed that THC decreases [^{3}H]thymidine uptake within fifteen seconds after addition of the drug to the culture. Experiments performed at 0° indicate that THC has no action on thymidine binding to the carrier. All of these observations suggest that THC in micromolar concentration inhibits DNA synthesis through a "non-specific" alteration of membrane configuration. This effect, due to the liposolubility of the drug, could induce conformational changes of membrane-bound transport systems which would inhibit their function.

The inhibitory effects of delta-9-tetrahydrocannabinol and other cannabinoids, in 10^{-6} to 10^{-4} M concentration, on cell division and macromolecular synthesis of cultured lymphocytes [1–3] and other eucaryocytic cells [4–8] has been reported by a number of investigators. It was suggested [9] that this effect was related primarily to the liposolubility of these compounds in the double lipid layer of the plasma membrane [10]. Others reported that THC exerted its inhibitory effect on the mitochondrion [11]. However, the 10^{-3} M concentrations of THC used in these latter experiments are lethal to the whole cell culture. Furthermore, the authors have observed that THC has no action on cellular glycolysis and does not alter ATP concentration [12].

The purpose of the present study was to further investigate the possible mediation of the inhibitory effect of THC on macromolecular synthesis through a non-specific action of the drug on the plasma membrane of the cell. In this investigation, the relationship between the octanol–water partition of olivetol derivatives and inhibition of thymidine incorporation was studied first. The effect of THC on cellular pool formation of precursors and on their macromolecular incorporation was investigated next. Finally, the kinetics of precursor transport systems were measured, using the isotopic dilution technique with 15 to 60 sec pulse exposure.

METHODS

Materials. Methyl-[^{3}H]thymidine (2Ci/m-mole and 47.5 Ci/m-mole), 5-[^{3}H]uridine (52 Ci/m-mole), 4-5-[^{3}H]leucine (50 Ci/m-mole), 1-[^{14}C]aminoisobutyric acid (AIBA, 10 mCi/m-mole) were obtained from Amersham. THC was kindly provided by NIDA (National Institute on Drug Abuse). The drug solubilized in alcohol was mixed with the culture medium and added to the cultures so as to reach a final concentration of 10^{-5} to 1.5×10^{-4} M. Similar amounts of alcohol vehicle were added to the control cultures. Purified phytohemaglutinin (PHA) was obtained from Burroughs Welcome Company. Media, biological extracts and salt solutions were obtained from Grand Island Biological Company (GIBCO), New York. Olivetol and its derivatives were obtained from Aldrich Chemicals.

Culture of human lymphocytes. The technique used was that of Hartzman *et al.* [13]. Venous blood was collected under heparin (50 i.u./ml) from healthy male donors. Mononucleated cells were separated according to the technique of Boyum [14] by density gradient centrifugation with Ficoll–Isopaque. After three washes the cells were resuspended at a concentration of 0.8×10^6 cells per ml in RPMI 1640 supplemented with antibiotics, glutamine, 20 per cent of pooled human serum and 4 μg/ml of purified PHA. Cells were cultured under a volume of 0.25 ml, 1.25 ml or 5 ml at 37°, in a 5 per cent CO_2 atmosphere saturated with water vapor.

Culture of L1210 *cells (murine lymphoma).* The cells were cultured at a concentration of 0.2×10^6 cells per ml in Dulbecco's modified Eagle's medium (GIBCO) supplemented with antibiotics, glutamine and 10 per cent of foetal calf serum (GIBCO), under a volume of 30 ml in Roux' flasks (Corning) at 37° in a 5 per cent CO_2 atmosphere saturated with water vapor.

Lipid solubility of olivetol derivatives and inhibition of thymidine uptake. Human lymphocytes were cultured with increasing concentrations of diphenol derivatives during three days. [^{3}H]Thymidine (0.5 μCi) was added at the 66th hour. The cells in triplicate culture were harvested at the 72nd hour with a Multiple Automatic Sample Harvester (MASH, Biological Associates). Viability of the cells was verified at the end of the

culture with trypan Blue or phase contrast microscopy. Results were discarded when a higher than usual ratio (7.5 per cent) of cellular death was found. Cellular radioactivity was measured by scintillation counting. As thymidine uptake varies considerably in different lymphocyte populations, uptake of the precursor into the test cultures is compared with its uptake into parallel control cultures. Results are then expressed in terms of percentage of precursor uptake in the control culture. The concentration of drug which inhibits 50 per cent of the precursor (IC_{50}) is obtained from the dose–response curves. The IC_{50} of these drugs is correlated with their octanol water partition coefficient on log/log coordinates. Partition coefficient was measured as follows: the compounds were dissolved in octanol (0.5 mg/ml). Octanol and buffer were mixed three times an hour during a 30 hr incubation. The drug concentration in octanol and in buffer are measured by u.v. spectrophotometry.

Measurements of the cellular pool and of macromolecular incorporation of [^{3}H]*thymidine, uridine, leucine and of* [^{14}C]*aminoisobutyric acid (AIBA) uptake.* 0.8×10^6 human lymphocytes were cultured in a volume of 1 ml of medium. At the 64th hr, 2 μCi of one of the labeled precursors, thymidine, uridine and leucine, or of [^{14}C] amino isobutyric acid (AIBA), were added to the culture with 2.5×10^{-4} M THC dissolved on 0.05 ml of human pooled serum. It was previously reported that this concentration inhibits by 50 per cent thymidine incorporation in mitogen stimulated lymphocytes [9]. After 15 min the cells were washed three times by centrifugation in a refrigerated centrifuge ($400\ g \times 10$ min). The cell pellet was then resuspended in 5 per cent ice cooled trichloracetic acid, and incubated at 37° for one hour. Radioactivity is evaluated in the supernatant which contains the cellular pool (acid soluble) and in the precipitate which contains the macromolecules (acid insoluble).

Measurement of [^{14}C]*AIBA transport and leakage.* 0.8×10^6 human lymphocytes were cultured in a 1 ml vol. At the 64th hour the cells were centrifuged and resuspended for a second 2 hr incubation in 0.18 ml phosphate buffered saline (PBS, pH = 7.4) and 0.02 ml of RPMI containing 0.4 μCi of [^{14}C]AIBA. After 2 hr, the cells were centrifuged again and resuspended in a radioactive free medium. Following different time intervals up to 45 min, the cells were centrifuged a third time ($150\ g \times 10$ min) and the radioactivity evaluated in the supernatant and in the cells, after resuspension of the pellet in 1 ml of medium and 0.25 ml of serum. Quenching was similar in the supernatant and in the pellet. In other experiments, the cultures, after the second centrifugation, were resuspended for the third incubation in 1 ml of RPMI and 0.25 ml of serum containing different concentrations of THC ($1–2.5 \times 10^{-4}$ M) and reincubated at 37° during 30 min in a 5 per cent CO_2 atmosphere saturated in humidity.

Measurement of [^{3}H]*thymidine uptake after 15 to 30* sec *pulse exposures.* The isotopic dilution technique described by Strauss [15] was adapted for the present experiments. L1210 cells (murine lymphoma) were centrifuged ($150\ g \times 10$ min) and resuspended (15×10^6 cells per ml) in PBS supplemented with lg/l of glucose (PBSG); 0.1 ml of this suspension was mixed in a hemolysis tube at time zero with 0.07 ml of PBSG containing 0.5 μCi of tritiated thymidine, uridine or leucine, 0.1 ml of PBSG containing cold thymidine and 0.03 ml of fetal calf serum. The cells were incubated with increasing concentration of cold thymidine (3.5×10^{-8} to 10^{-2} M) but a constant concentration of [^{3}H]thymidine (0.5 μCi per tube). THC in 10^{-5} to 2.5×10^{-4} M concentration was added to the test cultures.

When human lymphocytes, stimulated with PHA, are used after 3 days of culture, the number of cells cannot be accurately measured at the time of the experiment because of the cellular aggregates. In this instance, lymphocytes were counted at the onset of culture, and diluted to 8×10^5 cells/ml; after 3 days of culture we assumed that there were 1×10^6 cells/ml [16]. The cell suspension was shaken in a water bath at 37° during incubations varying from 7 to 60 sec. A 0.2 ml vol. of the suspension was filtered through a fiberglass filter, and washed with isotonic ice cold mannitol (0.3 M). The time interval between the moment the cells were added to the medium prewarmed at 37°, and the moment the cold mannitol was poured on the cells may have been as short as 7 sec. The filter was placed in a counting vial and the cells were dissolved with 0.5 ml of 1 N NAOH by warming one hour at 37°. The solution was neutralized with 0.05 ml of HNO_3 (10 N) and scintillation fluid (Instagel, Packard), then added. The quenching was constant in all experiments. The radioactivity harvested in the filter decreased to a plateau (background radioactivity) with increasing concentration of cold thymidine. Transport was calculated by subtracting background radioactivity retained on the filter. Other experiments were performed in similar fashion after incubating cells at 0° instead of 37°.

RESULTS

Inhibitory effects of olivetol derivatives on thymidine uptake. The IC_{50} of these compounds was significantly correlated with their octanol–water partition coefficient ($r = 0.9467$, $P < 0.001$). The ortho position of the hydroxyl group, as well as the length of the alkyl chain, increases the cytotoxicity of the molecule: the greater its liposolubility, the greater its toxicity.

Effects of THC on precursor uptake and incorporation after 15 min *incubation (Table 1).* After 15 min THC inhibited uptake of precursors into the cellular pool and their incorporation into the macromolecules: THC 2.5×10^{-4} M inhibited by 50 to 60 per cent

Table 1. Inhibition of cellular uptake and of macromolecular incorporation of labeled precursors and of [^{14}C]AIBA by delta-9-tetrahydrocannabinol (THC, 2.5×10^{-4} M)

% Inhibition by THC (2.5×10^{-4} M)	Uptake into cellular pool	Incorporation into macromolecules
[^{3}H]Thymidine	50.5 ± 1	59.0 ± 1
[^{3}H]Uridine	60.2 ± 3	64.6 ± 4
[^{3}H]Leucine	9.5 ± 1	28.0 ± 1
[^{14}C]AIBA	50.0 ± 3	—

The drug was incubated 15 min with PHA stimulated cultured human lymphocytes. The serum concentration of the medium was 25 per cent. Results are expressed in terms of percentage of precursor uptake in a parallel culture. Each figure represents 2 to 5 experiments.

Table 2. Measurements of plasma membrane leakage of ^{14}C-AIBA from human lymphocytes

Second Incubation time * (min)	% of [^{14}C]AIBA in supernatant #1	#2
0	—	44
15	32	54
30	42	65
45	50	66

The cells were incubated for 2 h with [^{14}C]AIBA, centrifuged and resuspended in a new medium. Radioactivity was evaluated in the supernatant and in the cells.

* Not including the 10 min centrifugation required to separate the cells from the supernatant.

Table 3. Effect of THC on plasma membrane "leakage" of [^{14}C]AIBA from human lymphocytes

THC concentration ($\times 10^{-4}$ M)	% [^{14}C]AIBA in supernatant
0	55.0 ± 0.3
0.95	53.0 ± 1.9
1.6	55.0 ± 1.2
2.5	53.0 ± 1.3

The cells are incubated for 2 h with [^{14}C]AIBA, centrifuged and resuspended for 30 min in a new medium containing varying concentrations of THC.

cellular uptake and macromolecular incorporation of [^{3}H]thymidine and [^{3}H]uridine. The incorporation of [^{3}H]leucine into protein, however, was less inhibited (10 per cent) than its cellular uptake (28 per cent). [^{14}C]AIBA cellular uptake was inhibited by THC in a manner comparable to the inhibitory effect of this drug on thymidine and uridine.

Effect of THC on [^{14}C]amino isobutyric acid (AIBA) transport (Tables 2 and 3). Appearance of radioactivity in the supernatant following centrifugation is an indication of the plasma membrane reverse transport or "leakage" of [^{14}C]AIBA. In the first experiment (Table 2) after the cells had been resuspended, the cellular leakage of [^{14}C]AIBA was proportional to the time of incubation; the concentration of label in the supernatant was already measurable after 10 min of centrifugation (corresponding to time zero in this experiment) which was required to separate cells from supernatant. In the second experiment (Table 3) when THC, 1 to 2.5 $\times$ 10^{-4} M was incubated with the cells for 30 min, there was no change in the cellular "leakage" of AIBA.

Effect of THC on [^{3}H]thymidine uptake after 15 to 60 sec (Figs. 1–6). Transport of the precursor was measured by subtracting the radioactivity retained by the filter after an incubation of the cells with a high concentration of thymidine, from the radioactivity retrieved after an incubation with a low concentration of the precursor (with a *constant* concentration of labeled precursor). As cold thymidine concentration increased, radioactivity harvested on the filter decreased to a plateau which measures background radioactivity (corresponding to mechanical trapping, adsorption and passive diffusion of the labeled molecule) (Fig. 1). In this system, thymidine transport was linear with time over 60 sec and extrapolated to zero, indicating unidirectional flow (Fig. 2). Inhibition of thymidine transport by THC was observed within 15 sec after addition of the drug to L1210 lymphoma cells and was significant after the 30th second. This inhibition was dose related (Fig. 3): After a 30 sec pulse, thymidine uptake is linearly correlated to the logarithm of THC concentration ($P < 0.001$). The concentration of THC required to inhibit 50 per cent uptake of thymidine (IC_{50}) in this model was 3.4 $\times$ 10^{-4} M.

In human PHA-stimulated lymphocytes, a significant inhibition by THC of thymidine transport was observed within 15 sec (Fig. 4). When the cells were incubated at 0°, addition of THC to the cultures did not change uptake of [^{3}H]thymidine (Fig. 5).

Uptake of uridine and leucine were also significantly inhibited by THC: within 15 sec for leucine and after

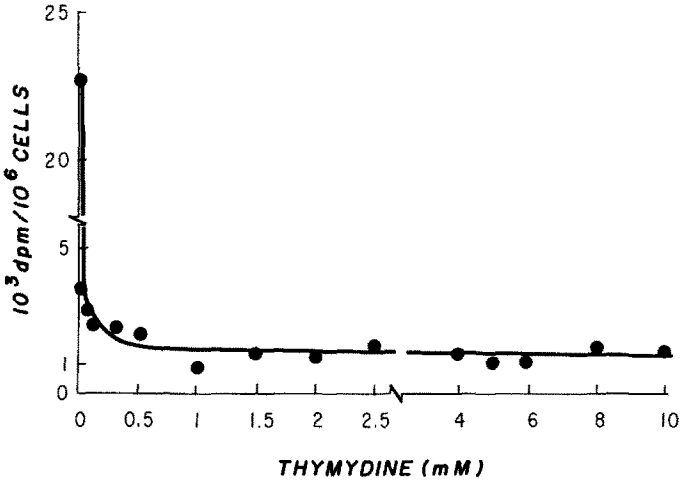

Fig. 1. Radioactivity harvested after 30 sec incubation of L1210 cells in the presence of increasing doses of cold thymidine and the same amount of [^{3}H]thymidine (3.2 $\times$ 10^{-8} M).

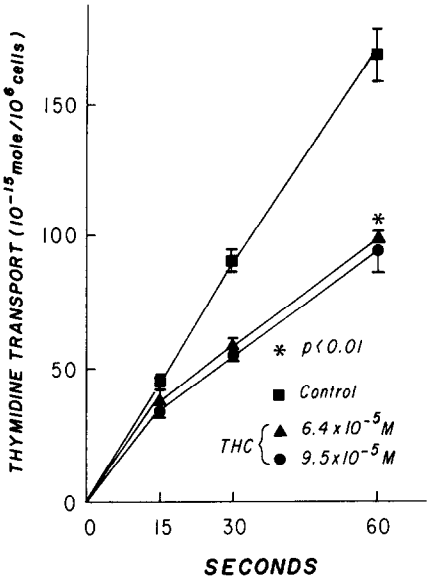

Fig. 2. Inhibition of thymidine transport by THC (6.4 and 9.5 x 10^{-4} M) in L1210 lymphocytes incubated at 37°. Each point represents triplicate measurements.

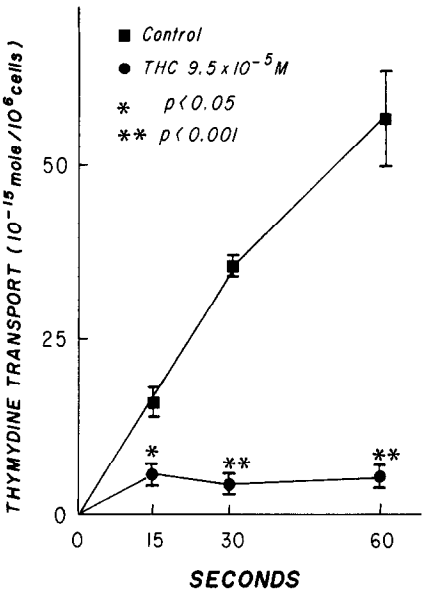

Fig. 4. Inhibition of thymidine transport by THC (9.5 x 10^{-5} M) in PHA stimulated human lymphocytes. Each point represents triplicate measurements.

60 sec for uridine. Uptake curves of these precursors measured during 60 sec did not extrapolate through zero (Fig. 6).

DISCUSSION

All cannabinoids have in common the C ring of olivetol, and inhibit to the same extent macromolecular synthesis [9]. Olivetol also exerts an inhibitory effect on thymidine uptake similar to that produced by THC [17]. When the liposolubility of olivetol is increased through lengthening of its aliphatic chain or changing the position and number of hydroxyl groups, the cytotoxicity of the resulting compounds is increased. Inhibition of thymidine uptake by these compounds is significantly correlated with their octanol–water partition coefficient.

In the course of incubation periods with THC, which do not exceed 15 minutes, the amount of precursor uptake corresponds to the difference between the amount entering into the cell, and the amount leaving the cell. This latter is referred to as "plasma membrane leakage". Blevin and Regan [5] suggested that THC increases plasma leakage, thereby decreasing cellular pool formation of precursor. To investigate plasma leakage, [^{14}C]AIBA was used. This amino acid derivative is transported into the cell like other amino acids, but is not incorporated into the macromolecule. In the present experiments THC (1 to 2.5 x 10^{-4} M) did not influence amino isobutyric acid cellular "leakage". The inhibition of precursor incorporation during 15 min incubation cannot be explained by an increase "leakage" of the membrane, as suggested by Blevin and Regan [5].

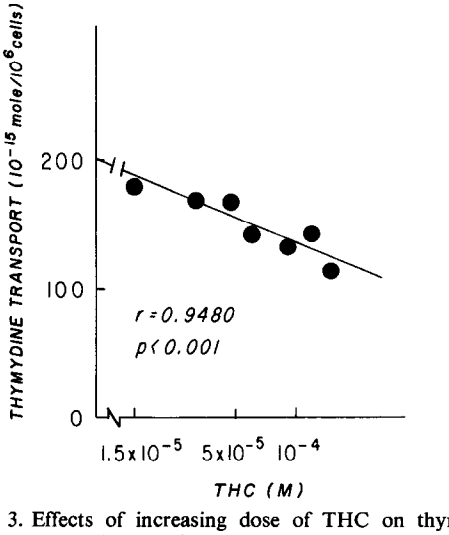

Fig. 3. Effects of increasing dose of THC on thymidine transport in L1210 cells after 30 sec incubation.

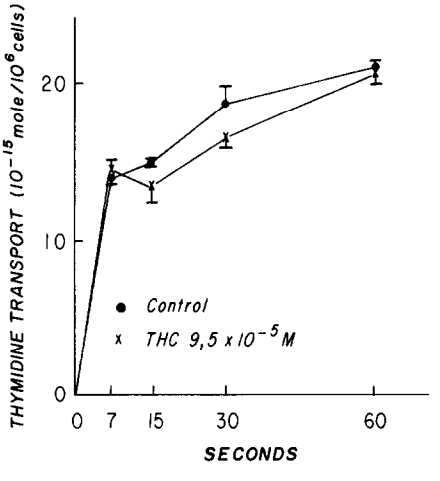

Fig. 5. Effect of THC on thymidine uptake of L1210 cells incubated at 0°.

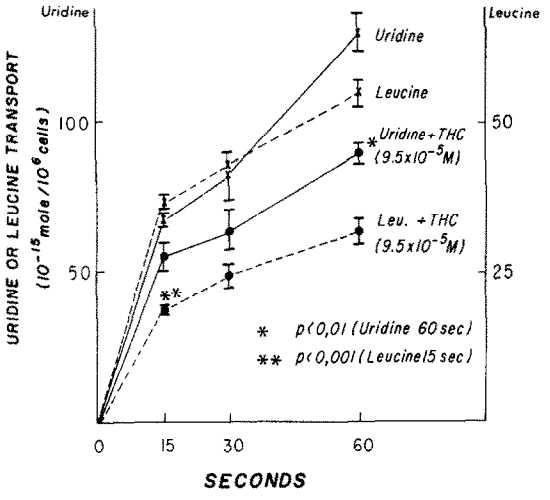

Fig. 6. Inhibition of uridine and leucine uptake by THC. Each point represents triplicate measurements.

Our last series of experiments indicate that THC interferes with the membrane transport system of thymidine: the velocity of thymidine transport is constant during at least one min. We have observed in other experiments that this transport rate remains constant for as long as 4 min. Within 15 seconds after addition of THC to the L1210 cells, a decrease in precursor transport is observable and within 30 seconds it is significant. This inhibition is of a greater magnitude in normal human lymphocytes than in lymphoma murine cells.

The uptake curves of uridine and leucine, unlike that of thymidine, do not extrapolate through zero indicating that one is not measuring a simple, unidirectional transport. Nevertheless, THC produced a very rapid inhibition of the cellular uptake of these precursors.

At 0° enzymatic activity is very slow, while the velocity of association of the substrate (thymidine) with the "enzyme" is little affected. During the first seven seconds of incubation, one may assume that thymidine uptake represents thymidine bound to the receptor sites of the carrier. By extrapolation we calculated that if 1×10^{-14} moles of thymidine were bound to a million cells (Fig. 5), there should be approximately 6,000 thymidine binding sites per cell. The very small increment of thymidine uptake during the remaining 53 sec of incubation reflects the weak activity of the transport system at 0°. At that temperature THC has no measurable effect on thymidine uptake, an indication that the drug does not act on the binding of thymidine to the carrier but rather on the transport function of the carrier. This mechanism could account for the non-competitive inhibitory effect of THC on thymidine uptake we reported earlier [9].

The precise molecular mechanism of action of the drug on the membrane cannot be determined by the present experiments. However, the concentration required to produce the observed inhibition indicates that the effect of THC on the membrane is "non-specific" and related to its liposolubility [18]. Similar inhibition of macromolecular synthesis in cultured lymphocytes has been observed with other psychotropic drugs in 10^{-6} to 10^{-3} M concentrations [19]. This inhibitory effect was correlated to the liposolubility of these drugs: the higher their octanol water partition coefficient, the greater their cytotoxicity [20].

Like other lipophiles and psychotropic drugs, THC expands erythrocyte membranes and increases their resistance to hemolysis [21, 22]. The concentration of THC required to decrease *in vitro* hemolysis by 50 per cent (AH_{50}) is similar (10^{-5} M) to the concentration of this drug which will inhibit by 50 per cent (IC_{50}) thymidine incorporation in cultured human lymphocytes [19].

Membrane expansion by THC could, as a result, produce conformational changes of the phospholipid and protein components and membrane bound carriers might be inhibited [18, 23]. An inhibition of the transport system of the precursors required for macromolecular synthesis would produce a decrease of their intracellular pool. As a secondary consequence, there would be a decrement in macromolecular synthesis by lack of precursors.

As THC also penetrates into the cell it might also interact with the nuclear membrane as suggested by Stein *et al.* [25] and by Carchman *et al.* [26].

Such a nonspecific effect of THC is exerted with micromolar concentrations which might be reached *in vivo* only in heavy chronic consumption. By contrast, the acute psychotropic effects of this drug are exerted with nanomolar concentrations as a result of stereospecific interaction with receptor sites located in the central nervous system [24].

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