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EFFECT OF Δ^9-TETRAHYDROCANNABINOL ON MITOCHONDRIAL PROCESSES*

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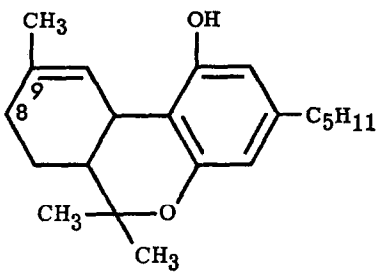
Abstract—The major psychoactive component of marijuana, Δ^9-tetrahydrocannabinol (THC), strongly affected rat liver mitochondria *in vitro*. At concentrations of 15–60 nmoles/mg of mitochondrial protein, THC uncoupled state IV respiration and decreased respiratory control and ADP/O ratios. Energy-linked changes in fluorescence of 8-anilino-1-naphthalene sulfonate were prevented or reversed by THC. THC also produced large amplitude swelling of mitochondria and release of matrix enzymes. These effects were greatly potentiated by Mg^{2+}. Likewise, flocculation of mixed phospholipid micelles by Mg^{2+} was potentiated greatly by low concentrations of THC. Studies with micelles prepared from purified phospholipids suggest that THC may specifically destabilize the cardiolipin in mixed micelles.

OF THE many cannabinoids found in marijuana and its active extracts, the major psychoactive compound is Δ^9-tetrahydrocannabinol (THC‡).[1] Its structure is shown in Fig. 1. Recent isolation and purification of this material[2] permits direct testing of its influence on subcellular organelles. Because of the great insolubility in water and the polycyclic structure of this compound, it was predicted that THC would have rather strong effects upon membrane-dependent processes. The hydrophobic portion



Δ^9-Tetrahydrocannabinol

FIG. 1. Structure of Δ^9-tetrahydrocannabinol (THC).

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‡ Abbreviations used: THC, Δ^9-tetrahydrocannabinol (atoms numbered according to the dibenzopyran system); RLM, rat liver mitochondria; NAD^+, nicotinamide adenine dinucleotide; ADP, adenosine diphosphate; ATP, adenosine triphosphate; P_i, inorganic phosphate; RCR, respiratory control ratio; ADP/O, nanomoles of ADP added per nanoatoms of extra O_2 consumed; ANS, 8-anilino-1-naphthalene sulfonate; MDH, malate dehydrogenase.

of the molecule would be expected to interact with hydrophobic regions in the membrane and perhaps perturb the functioning of the mitochondrial membrane system. This study describes the effects of THC upon respiration, swelling, and energy-linked changes in fluorescence of 8-anilino-1-naphthalene sulfonate (ANS) with rat liver mitochondria (RLM).

MATERIALS AND METHODS

Rat liver mitochondria (RLM) were prepared as described by Johnson and Lardy.[3] Oxygen consumption was measured with an oxygen electrode as described previously.[4] The incubation medium (5 ml) was 0·225 M in sucrose, 10 mM in P_i (pH 7·4), 5 mM in $MgCl_2$, 20 mM in KCl, and 20 mM in triethanolamine. THC was a gift from Dr. Robert B. Forney and was isolated from marijuana and purified to greater than 99 per cent purity by the procedure described by Turk *et al.*[2] Since the light brown oil dissolved readily in 95% ethanol, small volumes (< 0·03 ml) of alcoholic solutions of THC were added to the mitochondrial incubation medium described above. Swelling of the mitochondria was measured by changes in the optical density at 540 nm in 3 ml of incubation medium (as described above) containing 0·5–0·8 mg of RLM protein.

For studies of the release of mitochondrial malate dehydrogenase, $MgCl_2$ was omitted from the incubation medium. To 3 ml of Mg-free medium were added 1 μg of antimycin and 0·5 mg of RLM protein. After swelling had been monitored at 520 nm for a few min at 30°, the contents of the cuvette were transferred to 1·5-ml plastic tubes and centrifuged in an Eppendorf centrifuge for 5 min. The supernatant was removed carefully with a Pasteur pipette and stored on ice for assay of malate dehydrogenase. This assay was done at 38° in a volume of 1 ml of reaction medium containing 90 μmoles of glycine–NaOH buffer (pH 9·9), 100 μmoles of malate, 2·5 μmoles of neutralized NAD^+, and 1 μg of antimycin. The production of NADH was followed at 340 nm. To permit determination of the total malate dehydrogenase activity present in mitochondria before incubation, the RLM were disrupted with deoxycholate.

Fluorescence was measured with an Aminco Bowman Spectrophotofluorometer with a front-face attachment. Excitation was at 365 nm and fluorescence was followed at 480 nm. The reaction medium was 0·225 M in sucrose, 20 mM in KCl, 20 mM in triethanolamine, and also contained, in a total volume of 3 ml, 0·3 μmoles of ANS, 5 μg of rotenone, and 3 mg of RLM protein.

Phospholipids were purified from heavy beef heart mitochondria by the method of Rouser and Fleischer.[5] Purified cardiolipin was purchased from Sylvanna Chemical Company, Orange, N.J. Micelles were prepared by pipetting the desired quantity of phospholipid in chloroform into a small beaker (10–30 ml), evaporating the chloroform with a stream of nitrogen, and adding a medium 0·25 M in sucrose, and 10 mM in tris Cl, pH 7·4. The mixture was sonicated with a Bronwill Biosonik Model BPI sonicator on ice for three 1-min bursts alternating with 1-min cooling intervals. Triton micelles were prepared from Triton X-100 (Calbiochem, B grade) by the same procedure. The suspension was transferred to a test tube and allowed to equilibrate in a 30° water bath for 1 hr. Micelles of mixed mitochondrial phospholipids or Triton X-100 were made at a concentration of 10 mg (dry weight)/ml, and 0·1 ml was added to the incubation cuvette to give a final concentration of 1 mg in 3 ml. Phosphatidylcholine, cardiolipin, and a mixture of the two were sonicated at a lipid concentration

of 4 mg in 12 ml and used without dilution. Flocculation was monitored by following the optical density at 520 nm.

RESULTS

THC increased state IV respiration at concentrations of 15–60 nmoles/mg of RLM protein with succinate and NAD-linked substrates. The respiratory control ratio (state III/state IV) was significantly decreased in all cases with these levels of THC, whereas the ADP/O ratio was decreased at concentrations greater than 30 nmoles/mg of protein. At still higher concentrations, THC inhibited respiration with NAD-linked substrates. Addition of similar volumes of ethanol to control incubations produced none of these changes. Typical results are shown in Fig. 2.

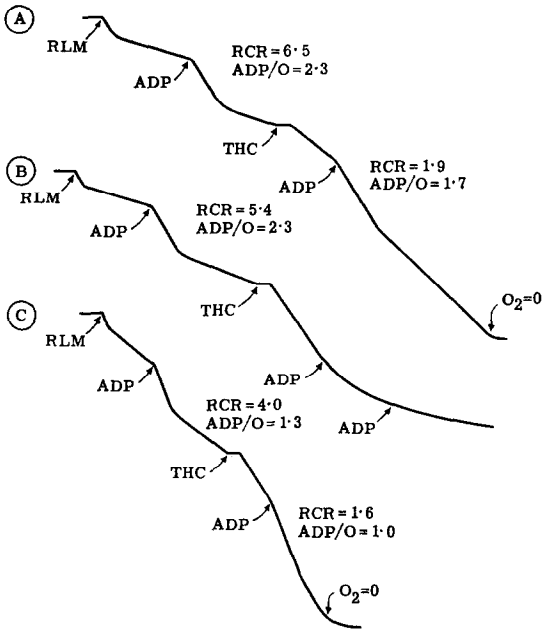

FIG. 2. Effect of THC on oxygen consumption by RLM. RLM (2 mg protein/ml) were incubated in 5 ml of the medium described in the text. Further additions were: (A) 10 μmoles of glutamate and 2·5 μmoles of malate; 895 nmoles ADP/addition and 36 nmoles THC/mg protein were added where indicated on the trace. (B) As in A, except 72 nmoles THC/mg protein were used. (C) 10 μmoles of succinate and 5 μg of rotenone; 537 nmoles of ADP/addition and 36 nmoles of THC/mg protein were added where indicated on the trace. The respiratory control ratio (RCR) and ADP/O ratios are indicated on the trace for each addition of ADP.

Under conditions of the assay for respiration, the lower concentrations of THC that caused uncoupling did not appear to cause swelling of the RLM in media containing 5 mM $MgCl_2$. At the upper range of uncoupling concentrations and at still higher concentrations (> 60 nmoles/mg protein), THC led to rapid and extensive large amplitude swelling in the presence of Mg^{2+}. Traces of optical density changes which demonstrate the magnitude of the swelling are presented in Fig. 3. Failure of rotenone, antimycin, and oligomycin to inhibit swelling induced by THC suggests that energy is not required for this action. The presence of an oxidizable substrate was not required.

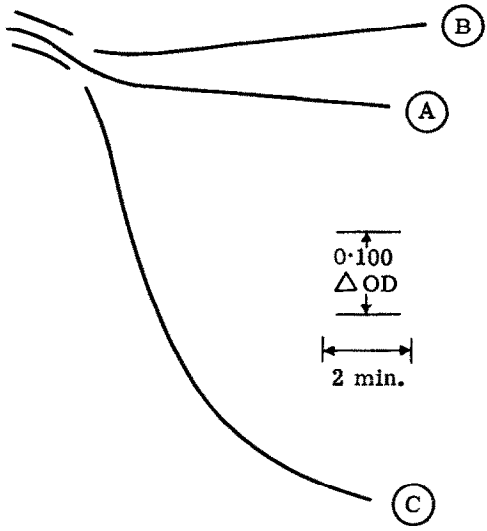

FIG. 3. THC-induced swelling of RLM. The incubation medium (3 ml) contained 10 μmoles of glutamate, 2·5 μmoles of malate, 1 μmole of ADP, and 0·8 mg of RLM protein. Curve A, control (no THC). Curve B, 30 nmoles THC/mg protein were added at the break in the curve. Curve C, 60 nmoles THC/mg protein were added.

The swelling of mitochondria induced by THC was greatly facilitated by Mg^{2+}, as seen in Fig. 4. The large amplitude swelling in the presence of THC and Mg^{2+} was accompanied by release of most of the mitochondrial malic dehydrogenase, a marker enzyme for the matrix space.

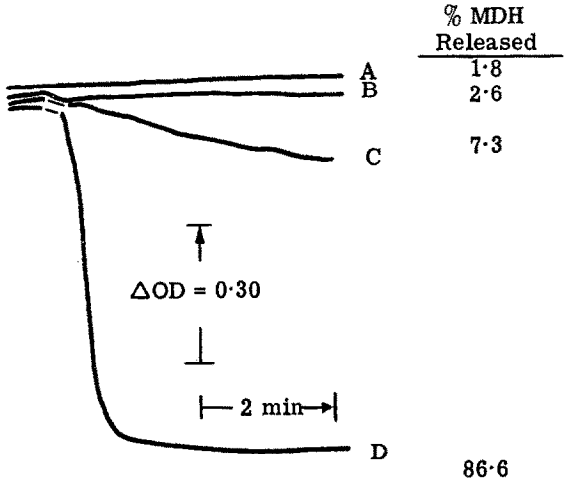

FIG. 4. Effect of THC and Mg^{2+} on swelling and release of malate dehydrogenase (MDH). The incubation medium (3 ml) contained 1 μg antimycin and 0·5 mg RLM protein. (A) Control without $MgCl_2$. (B) Control with 5 mM $MgCl_2$. (C) No $MgCl_2$. THC, 60 nmoles/mg protein, was added at the break in the curve. (D) In the presence of 5 mM $MgCl_2$, THC, 60 nmoles/mg protein, was added at the break in the curve. The release of MDH into the supernatant medium is indicated for each sample.

Changes in fluorescence of ANS have been shown to correlate well with the energy state of the mitochondrion.[6] The effects of THC on the fluorescence of ANS in the presence of rat liver mitochondria are shown in Fig. 5. In the presence of Mg^{2+}, 67 nmoles of THC/mg of protein increased the fluorescence of ANS in mitochondrial

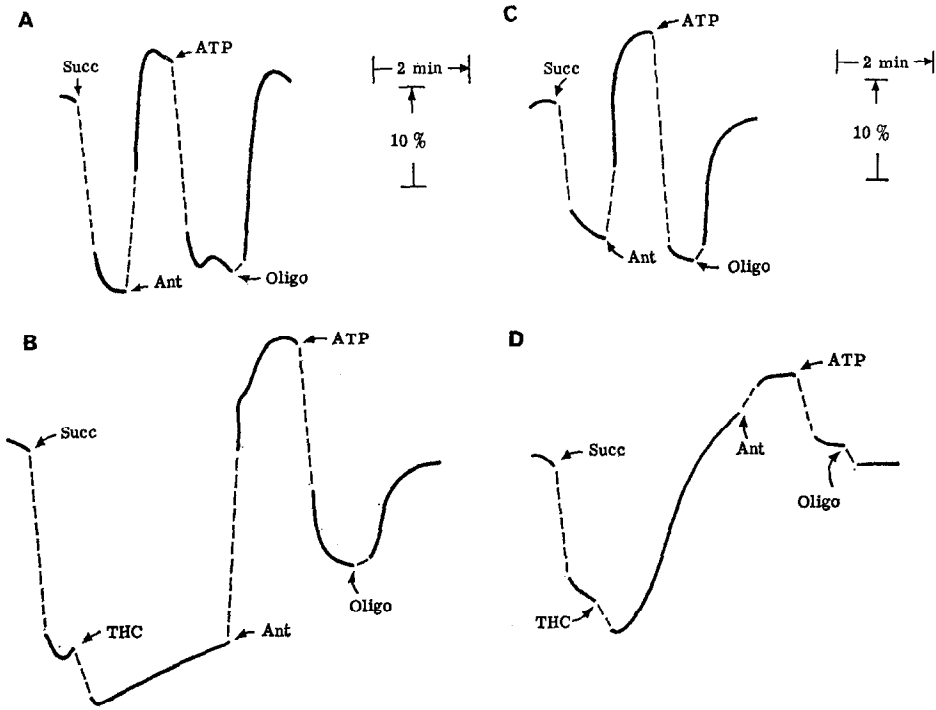

FIG. 5. Effect of THC and Mg^{2+} on energy-linked changes in ANS fluorescence. To the medium described in the text were added as indicated on the trace: 5 μmoles succinate (succ), 0·6 μg antimycin (ant), 5 μmoles ATP, 15 μg oligomycin (oligo), and 200 nmoles THC (67 nmoles/mg protein). Upward deflection represents an increase in fluorescence as indicated by the arrow. (A) Control without $MgCl_2$. (B) Effect of THC without $MgCl_2$. (C) Control with 5 mM $MgCl_2$. (D) Effect of THC with 5 mM $MgCl_2$.

suspensions oxidizing succinate and prevented decreases caused by the addition of ATP. This concentration of THC was not effective in the absence of Mg^{2+}. Other studies also showed that THC at 67 nmoles/mg protein in the presence of Mg^{2+} (but not in the absence) would prevent the change induced by succinate oxidation and would reverse the change caused by ATP. Higher concentrations of THC reversed and prevented energy-linked changes with or without Mg^{2+}. Either the higher concentrations of THC caused more deleterious changes or the amount of Mg^{2+} normally associated with the mitochondria was sufficient for the action of these higher doses.

Studies of the effect of THC on isolated phospholipids of mitochondria are shown in Fig. 6. THC had little effect on micelles of mixed mitochondrial phospholipids in the absence of Mg^{2+}, but subsequent addition of Mg^{2+} led to rapid flocculation of the micelles. Other studies showed that addition of this concentration of Mg^{2+} first did not lead to flocculation until THC was added. This was not a general behavior of all material in micellar dispersement, as shown in Fig. 7. THC produced little effect

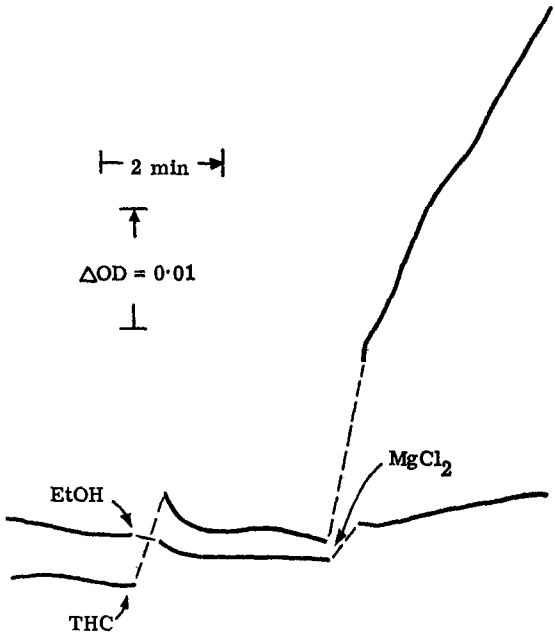

FIG. 6. Flocculation of mixed mitochondrial phospholipids by THC. The optical density of a suspension of mixed mitochondrial phospholipids, prepared as described in the text and containing 1 mg phospholipid (dry weight) in 1 ml, was followed for 2 min. To one sample 120 nmoles THC in 0·03 ml 95% ethanol was added, and 0·03 ml 95% ethanol was added to the other. The optical density was followed for about 2 more minutes. Then $MgCl_2$ was added to each sample to a final concentration of 5 mM. An increase in O.D. represents an increase in turbidity of the suspension because of flocculation of the micelles.

on micelles of the water soluble detergent, Triton X-100, in the presence or absence of Mg^{2+} (Fig. 7A). Figure 7B shows that THC, with or without Mg^{2+}, had no effect on micelles of purified phosphatidylcholine prepared from the mitochondrial phospholipids used in Fig. 6. On the other hand, micelles of cardiolipin were flocculated immediately by Mg^{2+} whether THC was present or not as seen in Fig. 7C. This was a very rapid and extensive change, the full scale deflection in Fig. 7C representing ten times the deflection of Figs. 6–7B. After examination of two cases of purified phospholipids where THC and Mg^{2+} had no effect on the stability of their micelles and one case where THC was not needed for a large effect of Mg^{2+}, it was found that mixed micelles of phosphatidylcholine plus cardiolipin paralleled the results obtained with micelles of mixed mitochondrial phospholipids. An example of these results is seen in Fig. 8. Addition of THC without Mg^{2+} (or Mg^{2+} without THC) had little effect on mixed micelles of phosphatidylcholine–cardiolipin 2:1 (w/w), but subsequent addition of Mg^{2+} caused extensive flocculation of THC-sensitized micelles.

DISCUSSION

These studies demonstrate that THC is a fairly active reagent with regard to altering the structure and function of the rat liver mitochondrial system. In this respect, THC is not as powerful at uncoupling mitochondria as a number of other lipophilic compounds. Under the conditions of the assays reported here, respiratory control was

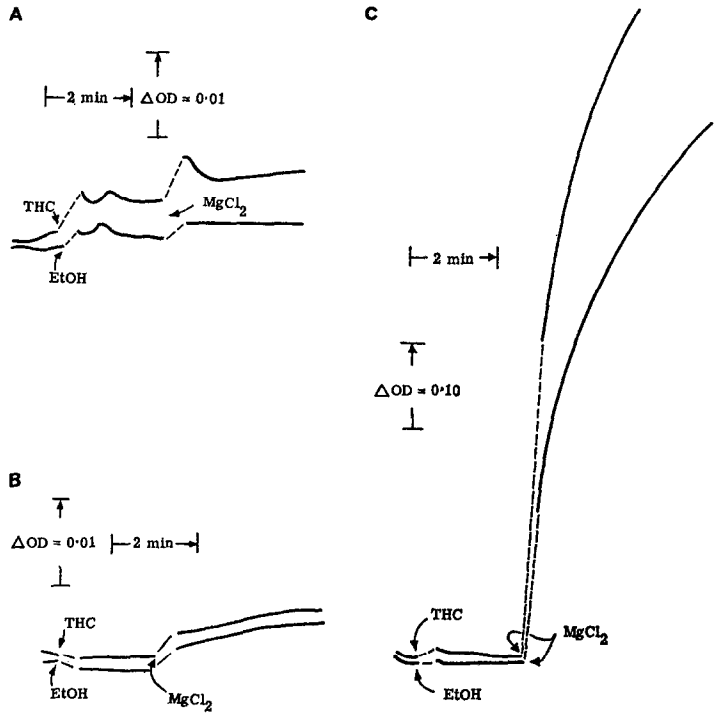

FIG. 7. Effect of THC on micelles of other substances. The clear micelles were prepared as described in the text, and additions were as in Fig. 6. (A) The solution contained 1 mg Triton X-100 in 3 ml. (B) The solution contained 1 mg purified phosphatidylcholine in 3 ml. (C) The solution contained 1 mg purified cardiolipin in 3 ml.

significantly decreased by THC at concentrations of 3×10^{-5} M (15 nmoles/mg of mitochondrial protein), whereas a decrease in the ADP/O ratio was observed only with concentrations in excess of 6×10^{-5} M (30 nmoles/mg of mitochondrial protein). A number of "uncouplers" of mitochondrial processes, e.g. the derivatives of carbonyl cyanide phenylhydrazone, uncouple at much lower concentrations than THC. For example, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone exhibits uncoupling effects at concentrations as low as 10^{-8} M[7]. Since THC appears qualitatively to be more hydrophobic than those compounds which have greater potency as uncouplers, the metabolites of THC which are more polar may show greater activity upon mitochondrial processes. THC has been shown to be metabolized to a number of more polar compounds,[8] including 11-hydroxy THC. Although a minor metabolite, the latter is active psychomimetically[8,9] and has not yet been tested upon mitochondrial processes.

This study indicates that THC induces swelling and lysis of mitochondria in the presence of Mg^{2+}. Byington *et al.*[10] and Smoly *et al.*[11] also found that other relatively hydrophobic compounds caused swelling with Mg^{2+}. In agreement with this study, they found that the swelling was accompanied by release of the matrix enzymes, malate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. Diethylstilbestrol and steroid hormones such as progesterone and 4-chloro-17-methyl-

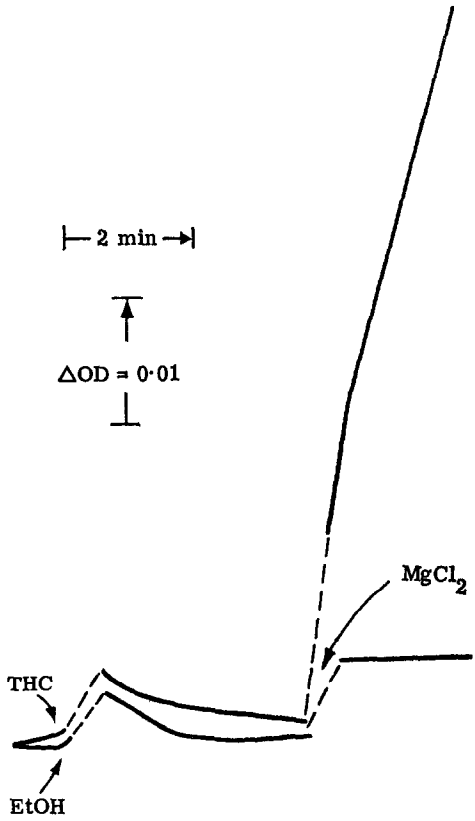

FIG. 8. Effect of THC on mixed micelles of phosphatidylcholine and cardiolipin. A micellar suspension containing 0·67 mg purified phosphatidylcholine and 0·33 mg purified cardiolipin in 3 ml was prepared as described in the text. Additions were as in Fig. 6.

19-nortestosterone had similar fragmenting capability. Lipophilic ionophoretic antibiotics such as valinomycin and gramicidin D would produce the same effects at high concentrations. Compounds too polar, e.g. hydrocorticosterone, or too apolar to be very soluble in ethanol, e.g. cholesterol, were shown to be inactive. Likewise, agents with significant water solubility (water-soluble polypeptide antibiotics such as bacitracin, colistin, ristocetin, polymixin, and vancomycin) were without activity over a wide range of concentrations.[10] THC is typical of the class of active hydrophobic materials.

Many studies have established that cations interact with phospholipids (e.g. Shah and Schulman).[12] Many years ago, H. G. Bungenberg de Jong[13] studied the effects of ions on egg lecithin suspensions. He found that clear sols of egg lecithin would not precipitate, or would precipitate only slowly, upon the addition of a number of monovalent or divalent cations. He also found that the sols could be sensitized to precipitation by adding a number of organic non-electrolytes such as cholesterol or triolein.[13] His results are somewhat similar to the ones reported in this study, where THC sensitized phosphatidylcholine-cardiolipin micelles to flocculation by Mg^{2+}.

In a micelle of phospholipid, the fatty tails are oriented inward, away from the water, where they stabilize the micelle by hydrophobic interactions. The surface of the micelle

consists of the partially, or fully ionized groups of the polar portion of the molecules, surrounded by counterions from the suspending medium and their water of hydration.[14] If the micelles carry an effective net charge, mutual repulsion of the particles helps prevent them from coalescing. Hydration of the ionized groups on the surface of the micelle also stabilizes the structure. Ions with a large capability for hydration, like Mg^{2+}, may remove some of the hydration water that stabilized the micelle, and thus lead to cross-linking of micelles, coagulation, and precipitation.[14] Mg^{2+}, in counterbalancing two negative charges on one cardiolipin molecule, may perturb the conformation of the molecule so that the micelle is destabilized. Charge repulsion must play such an important role in stabilizing cardiolipin micelles that Mg^{2+} will flocculate them even without the added destabilizing influence of THC. In mixed phospholipid micelles, on the other hand, the stabilizing effects of both water of hydration and charge repulsion are balanced so that the added destabilizing effect of THC is needed for precipitation.

Chari-Bitron and Bino[15] have studied the effect of Δ^8-tetrahydrocannabinol on the ATPase activity of rat liver mitochondria. They also noticed a great stimulation by Mg^{2+} of the effects of Δ^8-tetrahydrocannabinol. Their conclusion that Δ^8-tetrahydrocannabinol must cause some disorganization of the mitochondria is corroborated by this study. Although they used the Δ^8 isomer and we used the Δ^9, the effects are quite similar and both are psychoactive.[1] THC reacts with the membrane in such a way as to cause the mitochondrion to swell and fragment to the extent that matrix enzymes are released. The mechanism is not completely clear but phospholipids and particularly cardiolipin are implicated by our studies. The mitochondrial inner membrane bears a net negative charge[16] because of the preponderance of negatively charged phospholipids, and probably is stabilized by appreciable water of hydration. THC may destabilize the membrane so that the components are more dependent on mutual repulsion between charged groups for stability. Adsorption of Mg^{2+} would neutralize the negative charges and destabilize the structure as with phospholipid micelles. Additional studies with this type of reagent may help further to define the forces involved in stabilizing biological membranes.

Whether the effects of THC noted in this study are related in any way to the action of THC *in vivo* is unknown. The levels which were necessary to show appreciable effects upon mitochondrial processes were rather high. In addition, extensive damage to the membrane (probably irreversible) was observed at the higher concentrations used. Perhaps the most significant observation of this study with respect to the mechanism of action of THC *in vivo* is the demonstration of the relationship between THC and divalent cations. The latter are known to be important in proper nerve function and this study suggests their interaction with the membrane may be greatly influenced by THC.

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