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# Site of Inhibition of Leydig Cell Testosterone Synthesis by $\Delta^1$-Tetrahydrocannabinol

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SUMMARY

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The lowering of testosterone levels in both humans and experimental animals is apparently one of the effects caused by exposure to marihuana or certain of its components. This effect could be produced either by an indirect action on the central nervous system or by a direct action on the testes, specifically the Leydig cells. Using isolated murine Leydig cells as a model, a stepwise examination of the steroidogenic pathway was carried out to locate the site of action of $\Delta^1$-tetrahydrocannabinol in this system. The evidence points to the release of "precursor" cholesterol from its ester storage form as the point of inhibition. Generally, the pharmacological effects of marihuana have been difficult to link to a specific biochemical process. The findings reported here provide an example where such a correlation may be possible.

## INTRODUCTION

The mood-altering effect of $\Delta^1$-THC[1] is the best known property of this constituent of *Cannabis sativa*; however, a number of other changes can be brought about in subjects exposed to this drug (1). One which has received considerable attention recently is the reported lowering of testosterone production in both human (2) and experimental animal (3) subjects.

The *in vivo* synthesis of testosterone could be depressed either by direct action on testicular Leydig cells or through some centrally-mediated effect resulting in a lowering of gonadotrophin secretion (4). Dalterio *et al.* (5) have reported data which would allow for either possibility or perhaps a combined mechanism to explain the lowering of plasma testosterone by $\Delta^1$-THC. The findings of List *et al.* (6) that $\Delta^1$-THC reduces rat testicular microsomal androgen production and stimulates the hepatic metabolism of testosterone suggest that the clinical effect may not be centrally mediated. More recently, this group (7) has reported that esterase activity in rat testes was specifically reduced by *in vivo* treatment with $\Delta^1$-THC, but it is not clear from the data whether the affected esterase is involved in steroidogenesis. In support of a centrally mediated effect, Collu *et al.* (8) found that plasma LH was depressed by $\Delta^1$-THC in prepubertal male rats.

The present study was undertaken to provide a more detailed understanding of how $\Delta^1$-THC could inhibit testosterone synthesis by direct action on mouse testes. In a preliminary report, we demonstrated that the isolated Leydig cell can be used as a

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[1] The abbreviations used are: $\Delta^1$-THC, $\Delta^1$-tetrahydrocannabinol; LH, luteinizing hormone; hCG, human chorionic gonadotrophin.



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model for this purpose (9). $\Delta^1$-THC at a dose of 3.2 $\mu$M caused a 60% reduction in testosterone production by LH-stimulated mouse Leydig cells. This dose was close to what has been reported for $\Delta^1$-THC levels measured in canine testes after a single administration of 0.5 mg/kg of $\Delta^1$-THC (10) suggesting that effective doses in our model were realistic in terms of the human use of cannabis.

For convenience, the biosynthesis of testosterone can be thought of as a series of separate stages which are outlined in Fig. 1. Each of these steps can be studied individually under the appropriate experimental conditions. We have done these studies with a view towards determining the effect of $\Delta^1$-THC at each stage and would now like to report our findings.

## MATERIALS AND METHODS

*Preparation of Leydig cells and subcellular fractions.* Leydig cells were prepared from testes of 60–90 day old CD-1 mice and 200–300 g Sprague-Dawley rats by the procedure described previously (9). The Leydig cells were preincubated for 30 min at 32° in Krebs-Ringer bicarbonate (KRB) buffer; 0.1% bovine serum albumin; 2 mM EDTA pH 7.4. Cell viability was greater than 80% by the Trypan blue exclusion test.

Leydig cells, suspended in 0.25 M sucrose: 0.05 M Tris-HCl buffer, pH 7.4, (7 ml/pair testes), were homogenized with a motor-driven Teflon homogenizer and centrifuged in a refrigerated Sorvall RC-2B centrifuge (SS-34 rotor) at 1500 × *g* for 5 min. The pellet of cellular debris was rehomogenized as above and the resulting supernatants combined and centrifuged at 7000 × *g* for 15 min. The mitochondrial fraction was washed once and the pellet suspended in sucrose Tris buffer solution. The 7000 × *g* supernatant was either utilized for one of the cholesterol esterase assays or centrifuged at 105,000 × *g* for 60 min in an IEC preparative centrifuge (B60) to isolate microsomes. The 105,000 × *g* supernatant, which was freed from floating fat, was used for an additional esterase assay. Protein content of subcellular fractions was determined by the Ninhydrin method (11).

*Measurement of testosterone production in Leydig cells.* Incubations containing 2

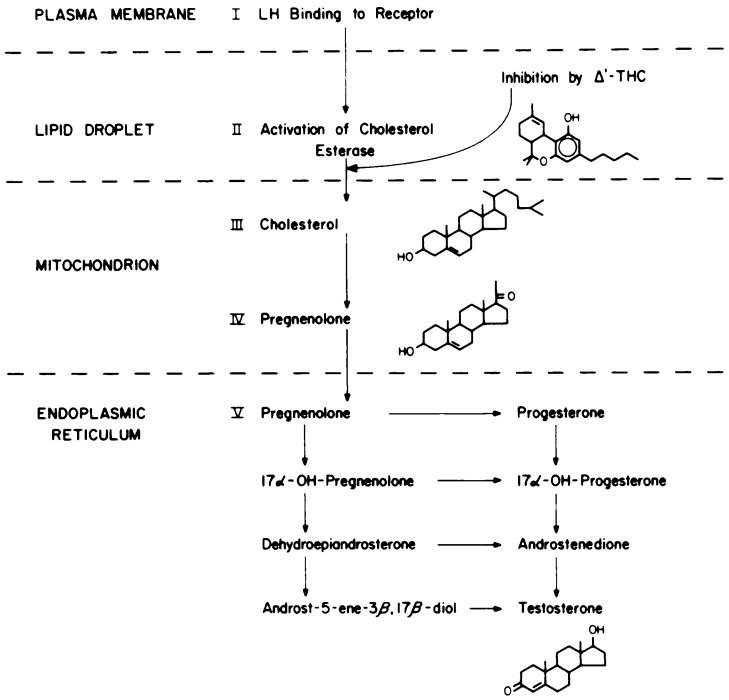


FIG. 1. *Events leading to Leydig cell testosterone synthesis*

ml Leydig cells ($10^6$ cells/ml) and 1 μg/ml LH (Sigma; equine pituitary) were carried out with $\Delta^1$-THC (NIDA) at various doses contained in 20 μl ethanol for 2 hr at 32° in an atmosphere of 95% $O_2$:5% $CO_2$ with gentle agitation. The suspensions were analyzed for testosterone using the NEN Corp. testosterone "RIA pak" as described previously (9). The addition of $\Delta^1$-THC at the highest levels used in these experiments caused no interference with the measurement of testosterone. Viability of the cells was not changed by exposure to $\Delta^1$-THC at the doses used when measured by Trypan blue exclusion.

*Determination of cholesterol esterase activity.* Labelled [1,2-$^3$H] cholesteryl palmitate was prepared by the procedures described by Moyle *et al.* (12). Cholesterol esterase [EC 3.1.1.13] activity was measured by following the conversion of ester into free cholesterol using the cell fractions described above. Reaction conditions are described in the table legend (Table 1). Lipids were extracted with the addition of 10 volumes of chloroform and 0.4 ml 0.154 M NaCl to yield greater than 90% recoveries. Cholesterol (20 μg) and cholesteryl palmitate (20 μg) were added to the dried extracts prior to thin layer chromatography on silica gel G (0.25 mm thick) with hexane: diethyl ether:acetic acid (15:10:1, v/v). Zones corresponding to cholesterol and cholesteryl palmitate were removed and assayed in a Packard Tri-Carb Liquid Scintillation Counter. No other major zones of radioactivity were found on the chromatograms.

*Determination of cholesterol desmolase activity.* Cholesterol side-chain cleavage activity was measured by the conversion of $^{14}$C-cholesterol to pregnenolone and progesterone by the 7000 × *g* mitochondrial fraction (12). Experimental details are described in the table legend (Table 2). The products were extracted and isolated by two dimensional tlc (9).

*Conversion of $^3$H-pregnenolone to testosterone.* The conversion of $^3$H-pregnenolone to testosterone was measured by incubating 2 ml of mouse Leydig cell microsomal suspension containing NADPH. Experimental details are described in the table legend (Table 3). The steroid products were extracted and analyzed by tlc as described

TABLE 1

*Effect on cholesterol esterase activity*[a]

| Cell fraction | $\Delta^1$-THC | Conversion ± SD | Inhibition |
|---|---|---|---|
| | (μM) | (%) | (%) |
| 7000 × *g* supernate | 0 | 15.9 ± 1.1 | — |
| 7000 × *g* supernate | 3.2 | 3.9 ± 0.3 | 75.5 |
| 7000 × *g* supernate | 16 | 1.5 ± 0.2 | 90.6 |
| 105,000 × *g* supernate | 0 | 16.0 ± 1.2 | — |
| 105,000 × *g* supernate | 16 | 0.7 ± 0.2 | 95.6 |

[a] $^3$H-cholesteryl palmitate (670,000 dpm; 21.5 Ci/mmole) was added in 20 μl ethanol to 2 ml of supernate. The $\Delta^1$ THC was added in 20 μl of ethanol and the mixture incubated for 1 hr at 37°. Protein concentration for the 7000 × *g* supernate was 0.38 mg/ml and for the 105,000 × *g*, 0.47 mg/ml.

TABLE 2

*Effect on the conversion of cholesterol to pregnenolone and progesterone by Leydig cell mitochondrial desmolase*[a]

| $\Delta^1$-THC | $\%C_{21}$ products[b] ± SD |
|---|---|
| (μM) | |
| 0 | 12.8 ± 0.5 |
| 3.2 | 13.0 ± 0.8 |
| 16 | 15.4 ± 1.7 |
| 32 | 15.4 ± 0.5 |

[a] $^{14}$C-Cholesterol (50,000 dpm; 50–60 mCi/mmole) was incubated for 90 min at 32° with 2 ml of mitochondrial suspension containing 0.25 M sucrose: 0.05 M Tris buffer, pH 7.4: 0.01 M isocitrate and 2 mg of NADPH. Protein concentration was 0.32 mg/ml.

[b] These values are the combined yields of radiolabelled pregnenolone and progesterone isolated by tlc.

TABLE 3

*Effect on the microsomal conversion of pregnenolone to testosterone in mouse Leydig cells*[a]

| $\Delta^1$-THC | Testosterone ± SD |
|---|---|
| (μM) | (ng/hr) |
| 0 | 47.1 ± 5.9 |
| 3.2 | 57.2 ± 10.9 |
| 16 | 39.9 ± 7.7 |

[a] 2 ml microsomal suspension containing $^3$H-pregnenolone (2 μg, 210,000 dpm) was incubated at 37° for 1 hr in sucrose-tris (pH 7.4) with NADPH (1 mg/ml). Protein concentration was 0.32 mg/ml. Absolute conversion was determined using $^{14}$C-testosterone as a recovery marker.

previously (9).

*Uptake of $^{14}$C-cholesterol by mouse Leydig cell mitochondria.* $^{14}$C-cholesterol (60,000 dpm) was incubated in a Dubnoff incubator with 2 ml of mitochondrial suspension (0.2 mg protein/ml) in polypropylene tubes for 60 min at 32° containing 10 μg $\Delta^1$-THC/10 μl ethanol. Control incubations were carried out with 10 μl ethanol/ml incubation medium. The suspension was centrifuged at 7000 × *g* for 15 min at 4°. The resulting supernatant was decanted and an 0.5 ml aliquot assayed for radioactivity. The pellet was suspended in 2 × 1 ml tissue solubilizer (NCS, Amersham-Searle) and assayed in a liquid scintillation counter with 10 ml of Aquasol (NEN). The percent incorporation into mitochondria was calculated as mitochondrial dpm/total dpm recovered × 100.

*Binding of $^{125}$I-hCG to Leydig cell receptors.* The procedures were based on the methods described by Catt, Dufau and Tsuruhara (13, 14). Briefly, approximately 37,000 cpm $^{125}$I hCG (65 μCi/μg, New England Nuclear) in 0.4 ml phosphate buffered alsine (PBS) containing 1 mg/ml bovine serum albumin, pH 7.4, was incubated with 0.5 ml of 1500 × *g* binding fraction. Human chorionic gonadotrophin (Calbiochem; 3810 IU/mg; 100 μg) was added to the appropriate samples in order to determine nonspecific binding. The samples were incubated at 24° for 16 hr; 2 ml PBS was added followed by centrifugation at 1500 × *g* for 15 min. The supernatant was removed, 2 ml PBS added and the samples were again centrifuged at 1500 × *g* for 15 min. The supernatant was again removed and the pellets were assayed in an automatic gamma counter (70% efficiency).

## RESULTS

*Binding of gonadotrophins.* Using $^{125}$I labeled hCG, the extent of binding in a 1500 × *g* fraction of testes could be readily measured as seen in Table 4. Approximately 73% of the radioactivity could be displaced by the addition of excess of hormone, the remainder being due to non-specific binding (14). The addition of $\Delta^1$-THC at doses up to 16 μM caused no significant change in the amounts of bound hormone. At this dose level, testosterone production was reduced by 75% in other samples of Leydig cells (Table 5).

TABLE 4

*Effect upon binding of $^{125}$I-hCG to the 1500 × g fraction of rat testes*[a]

| $\Delta^1$-THC (μM) | hCG (μg/ml) | Bound $^{125}$I ± SD (cpm) |
|---|---|---|
| 0 | 0 | 3848 ± 148 |
| 0 | 100 | 1045 ± 53 |
| 3.2 | 0 | 3864 ± 69 |
| 16 | 0 | 4114 ± 66 |

[a] For conditions, see MATERIALS AND METHODS.

*Basal testosterone production by Leydig cells.* The effect of $\Delta^1$-THC on Leydig cell testosterone synthesis without added LH was studied to determine whether the drug was interfering with LH stimulation. The data obtained are given in Table 6 and show a 44% inhibition at 3.2 μM and a 63% at 16 μM; effects on LH-stimulated cells were comparable (Table 5).

*Cholesterol ester cleavage.* The effect of 3.2 and 16 μM $\Delta^1$-THC on the cleavage of cholesteryl palmitate by the 7000 × *g* supernatant of mouse testes is shown in Table 1. At the higher dose, a 90.6% decrease in the extent of cleavage was observed leading to almost complete inhibition. The lower dose gave a somewhat diminished response, although the inhibition was still very appreciable. The esterase activity in the high speed supernate was also inhibited by the $\Delta^1$-THC (Table 1). Experiments are presently under way in our laboratory to further describe the kinetics of the esterase inhibition. We can say with certainty though, that the cholesteryl palmitate level used here is well below saturation.

*Mitochondrial uptake of $^{14}$C-cholesterol.* When mitochondria prepared from isolated Leydig cells were incubated with $^{14}$C-cholesterol, greater than 80% of the radioactivity was taken up by the organelle (Table 7). When this was done in the presence of 16 μM $\Delta^1$-THC, only about a 10% reduction in uptake was observed in a total of five different experiments.

*Conversion of $^{14}$C-cholesterol to pregnenolone and progesterone.* Cholesterol side-chain cleavage was monitored using a preparation of desmolase obtained from

TABLE 5

*Comparison of the in vitro effects of $\Delta^1$-THC on testicular steroidogenesis*[a]

| Experiment | $\Delta^1$-THC (μM) | | |
|---|---|---|---|
| | 0.32 | 3.2 | 16 |
| LH binding (cells) | — | 0 | 0 |
| LH binding (1500 × *g* fraction) | — | 0 | 0 |
| Esterase activity (7000 × *g* sup.) | — | 75.5 | 90.6 |
| Esterase activity (105,000 × *g* sup.) | — | — | 95.6 |
| Desmolase activity | — | 0 | 0 |
| Cholesterol uptake by mitochondria | — | — | 9.7 |
| Pregnenolone to testosterone conversion | — | 0 | 0 |
| LH stimulated steroidogenesis (cells) | 21 | 44 | 75 |
| Basal steroidogenesis (cells) | — | 43.6 | 63.4 |

[a] Values given represent percent inhibition relative to controls. Individual experiments with additional details are shown in the other tables.

TABLE 6

*Effect on basal testosterone levels in Leydig cells*[a]

| $\Delta^1$-THC | Testosterone produced ± SD | % Inhibition |
|---|---|---|
| (μM) | (*ng/ml*) | |
| 0 | 0.172 ± 0.016 | — |
| 3.2 | 0.097 ± 0.014 | 43.6 |
| 16 | 0.063 ± 0.024 | 63.4 |

[a] For conditions, see MATERIALS AND METHODS.

TABLE 7

*Effect on $^{14}$C-cholesterol uptake in Leydig cell mitochondria*[a]

| $\Delta^1$-THC | %$^{14}$C-cholesterol uptake ± SD | % Inhibition |
|---|---|---|
| (μM) | | |
| 0 | 81.6 ± 0.81 | — |
| 16 | 73.7 ± 0.86 | 9.7 |

[a] Protein concentration was 0.2 mg/ml. See MATERIALS AND METHODS for conditions.

Leydig cell mitochondria as described in the literature (12). The products were isolated by two-dimensional thin-layer chromatography and the results are shown in Table 2. The addition of $\Delta^1$-THC at three dose levels showed no significant effect on this stage of testosterone biosynthesis.

*Conversion of $^3$H-pregnenolone to testosterone.* A microsomal fraction from mouse Leydig cells was prepared according to published procedures (15) and incubated with $^3$H-pregnenolone. In the absence of drug, a 47% conversion to testosterone was attained which was not significantly altered by $\Delta^1$-THC at a level of 16 μM (Table 3).

## DISCUSSION

The initiating step in the Leydig cell synthesis of testosterone is the attachment of trophic hormone to a specific receptor site believed to be located on the plasma membrane. Clearly, a drug which diminishes the extent of this interaction would have an inhibitory effect on testosterone production (Fig. 1). Using procedures which have been thoroughly discussed in the literature (16), we tested the possibility that $\Delta^1$-THC was interfering with the binding of hCG to a subcellular fraction known to contain the receptors. We found that the levels of total binding were comparable to those reported as were the levels of specific binding as shown by the data in the first two lines of Table 4 (13, 14). $\Delta^1$-THC, at levels which cause dramatic decreases in testosterone synthesis (Table 5), had no significant effect on hCG binding indicating that the drug must be acting at some later stage in the biosynthesis.

Additional evidence that the inhibition by $\Delta^1$-THC does not involve hCG directly comes from our findings on basal levels of testosterone in whole cells. Table 6 contains the data which show that the drug brings about a substantial decrease in steroid synthesis in the absence of hCG. An earlier report (17) which showed that in whole testes there was no effect of $\Delta^1$-THC on basal testosterone is in disagreement with the isolated cell results reported here. It may be that the dose used (80 μM) with the whole testes was insufficient to affect basal synthesis in that system.

The next step in the biosynthetic pathway is believed to be the liberation of free cholesterol from cytoplasmic storage pools where it is present as an ester with certain long-chain fatty acids such as palmitic, stearic, oleic and higher polyunsaturated fatty acids (18). The precise mechanism whereby cholesterol esterase is activated in testes is not known at this time; however, mediators such as cAMP (19) and prostaglandins (20) may play a role.

Conditions for monitoring esterase activity have been reported (12) using cholesteryl palmitate in which the cholesterol is labeled with tritium. The esterase activity is contained in the 7000 × *g* supernatant fraction, but this preparation also contains much of the endogenous cholesteryl ester pool which is present as lipid droplets. Despite the possibility of dilution of the radiolabeled substrate by lipid droplets, we were able to observe a very substantial inhibition of esterase activity by $\Delta^1$-THC (Table 1). The experiment was also carried out with a 105,000 × *g* supernate in which endogenous esters are depleted and similar results were obtained (Table 1). These findings raise the strong possibility that inhibition of cholesterol esterase may be responsible for the reduction of testosterone synthesis in intact Leydig cells and perhaps in more complete systems as well.

Some evidence that esterase inhibition can occur *in vivo* as well has been reported by Dalterio *et al.* (17). A small but significant increase in testicular cholesteryl esters was observed four hours after mice were given 50 mg/kg of $\Delta^1$-THC orally. Surprisingly, a dose of 100 mg/kg caused no change; this may have been due to an effect on ester synthesis which is manifest at the higher dose. Additional support for our findings on the isolated cell have been reported by Goldstein *et al.* (7). They have examined testicular esterase isozymes from control and $\Delta^1$-THC-treated rats by gel electrophoresis. A difference in the pattern of bands showing esterase activity was noted between control and drug-treated animals which suggested a decrease in the latter. While these findings were not of a quantitative nature, they do correlate very well with our data using the radioassay method (Table 1) and show that the *in vivo* administration of modest doses of $\Delta^1$-THC (2 mg/kg) can lead to a lowering of testicular esterase activity.

Free cholesterol is taken up by the mitochondria of steroidogenic cells where it is converted to pregnenolone. Since $\Delta^1$-THC has been shown to affect mitochondrial membranes and functions (21, 22), we investigated the possibility that the drug was acting at this point. Neither the uptake nor the side-chain cleavage of cholesterol were appreciably affected by $\Delta^1$-THC (Tables 2 and 7). The slight effect on uptake was observed in a total of five experiments and is not surprising in view of previous reports (21, 22). However, the small amount of uptake inhibition, when compared with the large reduction in esterase caused by $\Delta^1$-THC, indicates that this is probably not an important site of action of the drug.

We have already reported that the final network of reactions leading from pregnenolone to testosterone was unaffected by the presence of $\Delta^1$-THC (9). This was demonstrated using isolated intact, LH-stimulated Leydig cells with both $^3$H-pregnenolone and $^3$H-progesterone as precursors. A recent report, however, gave evidence that $\Delta^1$-THC given *in vivo* could decrease the rates of this microsomal mediated series of reactions (6). To further examine this question, we tested the direct effect of $\Delta^1$-THC on a Leydig cell microsomal preparation and found no significant changes over control (Table 3). These differences are puzzling and one can only speculate at this point on what their causes are. A possibility is that a metabolite is formed *in vivo* which shows a different spectrum of activity from that of the parent $\Delta^1$-THC.

An apparently specific effect of $\Delta^1$-THC on steroid synthesis in an adrenal tumor cell line (Y-1) has been reported (23). Several similarities exist between our observations with Leydig cells and the Y-1 adrenal cells which suggest a similar mechanism of action. The effective dose levels were in the same range for both cell types and in both cases cell viability seemed unaffected by the drug. The site of inhibition in the adrenal cells seemed to be earlier in the pathway than the conversion of pregnenolone

analogous to the case in the Leydig cells. Other cannabinoids such as cannabinol and cannabidiol were effective in the adrenal cells and preliminary experiments by us with Leydig cells gave similar results (unpublished data).

The fact that these similarities in effects were found in cells of different origin suggests that a basic process is involved. It seems reasonable to expect that adrenal cholesterol esterase action is affected in the same way as we have observed for the testes. An interesting and perhaps relevant observation reported for the adrenal cells was that cAMP stimulation of steroidogenesis was blocked by $\Delta^1$-THC (23). In view of our previous findings on the effects of $\Delta^1$-THC on prostaglandin synthesis (24) and those of Kelly and Butcher (25) on cAMP metabolism, it is tempting to speculate that one or perhaps both of these mediators are involved in the steroidogenic effects of cannabinoids.

Our present findings are summarized in Table 5 which shows the effects of $\Delta^1$-THC on each step in the steroidogenic pathway in the Leydig cell. The conclusion that can be made from a comparison of the results is that $\Delta^1$-THC acts almost exclusively on the esterase which is responsible for the liberation of "precursor" cholesterol. Not only is this virtually the only step inhibited, but the dose response closely parallels that which is effective in the intact cell. The implications of this finding for the mode of action of cannabinoids at other sites is not known at this time, however, it may provide a starting point for investigation into other facets of cannabis biochemistry.

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