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IN VIVO INCORPORATION OF LABELED PRECURSORS INTO CANNABINOIDS IN SEEDLINGS OF CANNABIS SATIVA L. (CANNABACEAE).

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SUMMARY

This study examined the incorporation of labeled exogenous precursors in cannabinoid biosynthesis by Cannabis. Shoot apices, cut from 72 hr and 20 day seedlings grown in a growth chamber at 25°C under a 20 hr/4 hr light/dark cycle, were immersed at designated intervals into vials of either U^{14}C-sucrose (5-15 mCi/mM) or 1',2'-^{3}H-olivetol (0.04 Ci/mM) as well as control conditions. Extracted cannabinoids were analyzed by chromatographic and scintillation techniques. Seedlings incubated 2 hr in sucrose incorporated label into all carboxylated (acid) and decarboxylated (neutral) cannabinoids and were detected immediately following the pulse with highest specific activity present after a 22 hr chase. Acid and neutral forms of cannabichromene (CBC), cannabigerol (CBG) and Δ^9-tetrahydrocannabinol (THC) were present after all chases. Seedlings incubated 2 hr in olivetol incorporated label into acid and neutral CBC, CBG and THC with highest specific activity at 0 chase period. A lower specific activity was detected for CBC than CBG and THC, although CBC was present in greatest quantity. Seedlings readily incorporated and carboxylated precursors to form cannabinoids. The pattern of incorporation indicated that a bifurcation in the pathway leading to cannabinoid formation was present in the developing plant.

The presence of cannabinoids in Cannabis sativa L. and their interaction with man are topics that only recently have come under intensive investigation. While man has either wanted to enhance or inhibit cannabinoid production in the plant, little has become known about Cannabis biochemistry. Studies of the plant have produced conflicting results, and while cannabinoids can be synthesized chemically, their in vivo biosynthesis is poorly defined.

Our experiments with seedlings show that cannabinoids appear in a specific pattern during seedling development (1). With the use of a precise procedure for monitoring the time of seedling germination and growth, a seedling population of known age can be harvested and analyzed for cannabinoid content. Cannabinoids are not present in the seed, and our results have shown that cannabichromene (CBC) is the first cannabinoid detected at 52-54 hr after germination. No other cannabinoids are detectable until 60-62 hr when both cannabigerol (CBG) and Δ^9-tetrahydrocannabinol (THC) are detected in addition to CBC. As the seedlings age, the ratio of CBC to THC changes dominance of CBC in young seedlings to dominance of THC reflective of the cannabinoid profile in adult plants (2).

In this study we have used the seedling system to investigate in vivo isotope incorporation into cannabinoids. This initial labeling study was designed to examine whether a general substrate, sucrose, as well as a presumed specific cannabinoid precursor, olivetol, would incorporate into cannabinoids. Derived data indicate that cannabinoid biosynthesis may be complex and contribute to an interpretation that a pathway bifurcation may be associated with cannabinoid formation in the developing plant.

METHODS

Seed germination. Cannabis seeds were obtained from a THC strain of Mexican origin grown under greenhouse conditions (3). Approximately 1,000 seeds were used in each experiment. Seeds were dark-germinated in petri dishes each containing 500 seeds in a minimal amount of distilled water. At 17-20 hr after wetting the seeds, they were examined for germination which is defined as the splitting of the seed coat. Any seeds already germinated were discarded, and subsequent checks were made every 2 hr until 29-32 hr after wetting, thus defining germination within a 2 hr interval. Germinated seeds were transferred to covered 16 oz glass jars, each containing 30 seeds on a layer of damp absorbent cotton. Seedlings were maintained under a light/dark (20 hr/4 hr) cycle in a growth chamber at 25°C.

Radioactive compound incorporation. Seedlings 72 hr and 20 days after germination were harvested, cut approximately 3-4 mm below the shoot apex, and cut ends immersed in 0.5 ml of experimental medium consisting of either buffer, labeled, or unlabeled substrates. Labeled substrates included U^{14}C-sucrose (5-15 mCi/mM) and 1',2'-^{3}H-olivetol (0.04 Ci/mM, National Institute on Drug Abuse). This procedure was done every 2 hr as each of 6 batches of seedlings in the glass jars reached 72 hr in age. Each seedling batch remained in the experimental solution, fresh solution for each set, for 2 hr. Seedlings then were rinsed with buffer and, depending on experimental protocol, either harvested or the cut ends were placed in distilled water for a chase period.

Analysis. Seedlings were extracted for cannabinoids as previously described (4). Extracts then were separated into individual cannabinoids by thin layer chromatography (TLC). The individual bands were removed and counted in a Beckman LS-100 scintillation counter to determine the amount of label present. Cannabinoid quantitation was done by gas liquid chromatography (GLC) (4), and cannabinoid confirmation was done both by GLC and high

performance liquid chromatography (HPLC) (4, 5).

RESULTS

As reported previously (1), the cannabinoid content of seedlings was observed to undergo quantitative changes during plant development (table 1). In young seedlings between 72-96 hr, CBC occurred in greater quantities than THC and CBG, and composed nearly 50% of the total cannabinoids in tissues. THC occurred in lower quantities than either CBC or CBG. Each cannabinoid increased with plant development showing a several-fold increase from 72-96 hr, but the ratio of cannabinoids remained similar during this period. Between 96 hr and 20 days quantitative changes occurred in the profile in which THC became more abundant than CBC, while CBG was recorded in lowest quantities. These data, typical of cannabinoid composition in seedlings, were derived by GLC for the decarboxylated (neutral) forms of cannabinoids. Carboxylated (acid) forms, the predominant form in the plant, can be detected by HPLC (2, 3, 4, 5) and TLC, although in the absence of carboxylated forms for standards, it was not possible to quantitate cannabinoid acids.

In the isotope incorporation studies the extracted acid and neutral cannabinoids were detected using TLC, HPLC and scintillation. Neutral cannabinoids from tissues were isolated by HPLC or TLC and then quantitated using GLC. Acid cannabinoids were identified only as being present because it is as yet unclear whether heating acid cannabinoids efficiently decarboxylated them. Therefore, specific activities were calculated for cannabinoids as their neutral form.

When labeled sucrose was presented to 72 hr seedlings, label accumulated in each examined cannabinoid (table 2). The label was detected immediately after the 2 hr pulse and continued to accumulate in cannabinoids during the 22 hr chase period. Although the ratio of specific activities of THC/CBG paralleled the ratio of cannabinoid content (approximately 1:2), the specific activity of CBC was relatively low considering CBC was the predominant cannabinoid in the seedlings. Labeled sucrose was detected in TLC bands at the R_f positions of cannabinoid acids, and in HPLC peaks identified as cannabinoid acids. When an extract was heated and then chromatographed, the specific activity of each neutral cannabinoid increased significantly due to being supplemented with decarboxylated acid forms. At the same time, the now barely discernible TLC bands at the R_f positions of cannabinoid acids were found to contain no label. Total quantities of cannabinoids were normal for seedlings of this age, and cannabinoid quantities were the same in both unheated and heated seedling extracts.

In pulse-chase experiments with ^{3}H-olivetol presented to 72 hr seedlings, each cannabinoid was labeled as both carboxylated and decarboxylated compounds (table 2). A two-fold higher level of specific activity was found for THC and CBG as compared to CBC at 0 chase, although CBC was the most abundant cannabinoid in the seedling. During the chase periods the specific activities decreased significantly for both CBC and THC, but somewhat less so for CBG. As observed in the sucrose experiments, unheated extracts produced TLC bands at the R_f positions for cannabinoid acids while heated extracts did not, but heated extracts did result in higher specific activities for neutral cannabinoids. Analyses of other R_f regions from the TLC plates at each successive chase period did not yield other detectable label. The labeled olivetol fed to the seedlings was the neutral (or decarboxylated) form, yet both neutral and acid cannabinoids were accumulated in the plant (table 2). These results were confirmed by TLC of heated and unheated extracts, GLC confirmation of TLC bands, HPLC, and GLC confirmation of HPLC peaks.

Table 1. Cannabinoid Content of Typical *Cannabis* Seedlings Corresponding to Time of Label Incorporation.

Seedling Age	µg Cannabinoid/100 mg Tissue DW			
	CBC	THC	CBG	Total
72 hour	144.9	55.6	100.7	300.2
78 hour	193.5	61.1	145.8	400.4
96 hour	621.0	181.8	319.5	1,122.3
20 day	968.0	2,074.0	85.0	3,127.0

Table 2. Label Incorporation into Cannabinoids in 72 hr *Cannabis* Seedlings.

Time	Specific activity, CPM/µM Cannabinoid			
	CBC	THC	CBG	Acids
2 hr pulse sucrose +				
0 hr chase	435	732	1,964	+
4 hr chase	3,155	3,589	6,504	+
22 hr chase	9,237	6,484	12,791	+
2 hr pulse olivetol +				
0 hr chase	8,138	16,238	17,039	+
4 hr chase	1,799	3,141	6,249	+
22 hr chase	1,580	3,651	13,600	+

+ present but not quantitated.

Comparable experiments incorporating ^{14}C-sucrose into shoots and leaves of 20 day seedlings showed a lower level of incorporation into cannabinoids than determined for 72 hr seedlings, but cannabinoid synthesis did occur (table 3). CBG, although present in the lowest concentration (85 mg/100 µg tissue DW) was found to have the highest specific activity at the end of the pulse period. Both CBG and THC, present in amounts of 2,074 mg/100 µg tissue DW, showed an initial increase in specific activity followed by a decrease during the 22 hr chase period. CBC, 968 mg/100 µg tissue DW, was found to have specific activities lower than CBG and THC with a trend of gradual increase through the 22 hr chase period. While these results were based on neutral cannabinoids, acid cannabinoids also were present as labeled but were not quantitated. Cannabinoid concentrations on a dry weight basis remained constant over the experimental period for all detected cannabinoids.

Table 3. Labeled Sucrose Incorporation into Cannabinoids in 20-Day *Cannabis* Seedlings.

Time	Specific activity, CPM/µM Cannabinoid			
	CBC	THC	CBG	Acids
2 hr pulse +				
0 hr chase	74	163	1,904	+
4 hr chase	140	673	-	+
6 hr chase	514	-	3,247	+
16 hr chase	776	2,836	2,258	+
22 hr chase	993	2,166	1,003	+

-, no data; +, present but not quantitated.

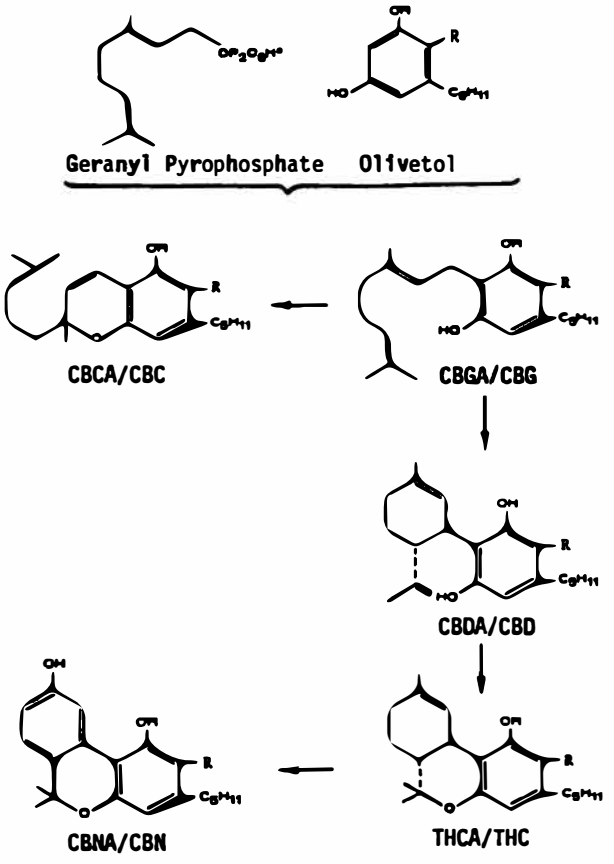
DISCUSSION

Previous studies using *Cannabis* seedlings as an assay system for cannabinoids have shown CBC to be the first cannabinoid detectable in the developing plant. Further, those studies indicated that the cannabinoid profile was developmentally dynamic with the ratio of cannabinoids gradually changing from a seedling profile with CBC as the predominant cannabinoid to a mature plant profile in which THC was the major cannabinoid characterizing this *Cannabis* strain (1, 2, 6).

The current study showed that seedlings were capable of incorporating both labeled sucrose and labeled olivetol into cannabinoids. However, the trend for sucrose toward a progressive increase in specific activity during the chase periods differed from the trend for olivetol. The trend for sucrose probably reflected incorporation into various compounds in other pathways in the plant with subsequent contribution to cannabinoid precursors during the chase periods. The trend noted for olivetol during the chase periods was indicative of cannabinoid turnover, although the ultimate products of such reactions are unknown at present.

The formation of both decarboxylated and carboxylated cannabinoids from olivetol is surprising but the mechanism of olivetol carboxylation is as yet unclear. It is possible that olivetol is directly carboxylated into the cannabinoids during incorporation or that it is degraded and subsequently recycled through earlier steps in the pathway which also include carboxylation of the precursors.

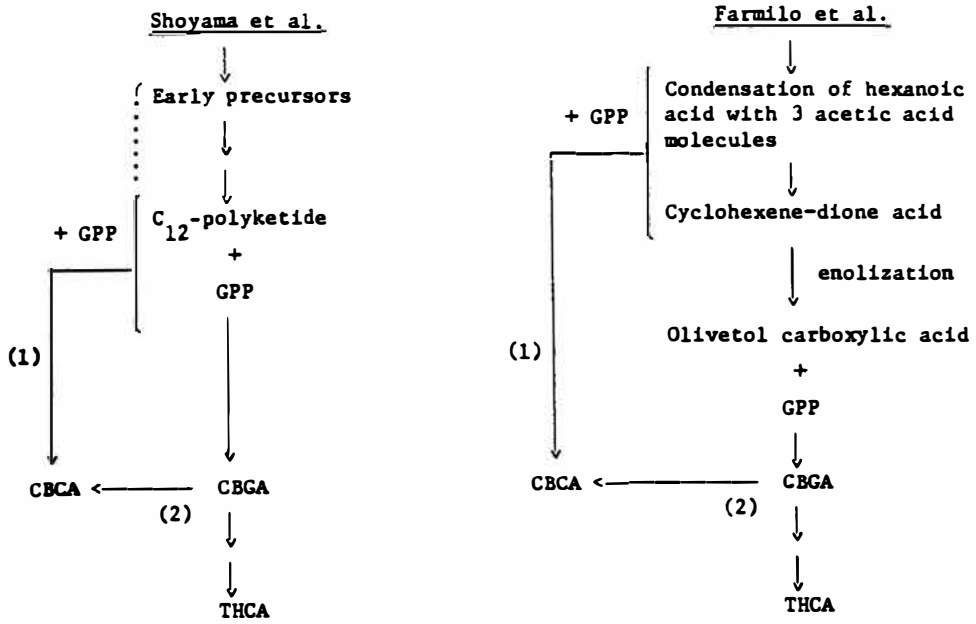
Fig. 1. PROPOSED CANNABINOID BIOSYNTHETIC PATHWAY



The low specific activities of CBC as compared to CBG and THC observed in this study, along with the results of previous studies on the seedling system, have lead us to reevaluate the cannabinoid biosynthetic pathway. The currently accepted pathway (fig. 1) (7) shows that CBGA/CBG is the first cannabinoid formed in the plant. However, no specific data exist on pathway events prior to CBGA/CBG. Farmilo et al. (8) and Mechoulam (7) proposed that olivetol carboxylic acid, from the fatty acid biosynthetic pathway, combines with geranyl pyrophosphate (GPP) to form CBGA (fig. 1). Shoyama et al. (9) suggested that a C_{12}-polyketide, a precursor to olivetol carboxylic acid, is the entity combining with GPP to form CBGA (fig. 2).

As another hypothesis, we propose that two pathways may exist in the developing plant. Initially, in early seedling development prior to the synthesis of other cannabinoids, CBCA/CBC may be formed from the condensation of GPP with a precursor of olivetol carboxylic acid. As plant development progresses, the pathway may change to that present in the older plant in which GPP combines with olivetol carboxylic acid to form CBGA/CBG and the other cannabinoids, with CBCA/CBC as a side branch (fig. 2).

Fig. 2. PROPOSED CANNABINOID PATHWAYS, AND OUR HYPOTHESIZED BIFURCATION:
(1) BRANCH IN SEEDLING DEVELOPMENT LEADING TO CBCA.
(2) BRANCH IN OLDER PLANT WHERE CBGA LEADS TO ALL CANNABINOIDS.



The basis for this possible early bifurcation in the pathway may be related to the availability in seedlings of substrates that differ from those in later stages of development. In seedlings, lipid reserves in cotyledons may contribute to CBCA/CBC synthesis, whereas during subsequent growth the precursors for cannabinoid synthesis are derived from products of photosynthesis. It may be the case that the low specific activities found for CBC reflect label incorporated only through the more mature pathway in which the larger quantities of CBC had been made earlier in the reserve pathway.

In summary, Cannabis seedlings provide a useful model system for studies of cannabinoid biosynthesis. Not only do they allow specific developmental steps to be analyzed, but they also incorporate labeled presursor into all detectable cannabinoids. The results presented here, showing incorporation of labeled olivetol into cannabinoid acids as well as a potential bifurcation in the cannabinoid pathway early in development, provide a basis for more in-depth investigations of these aspects.

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