A PHYTOCHEMICAL STUDY

OF

CANNABIS SATIVA LINNE

A thesis submitted to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

By

AMOS BLAINE COLBY

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INTRODUCTION

Purpose of the Investigation - The presence in Cannabis sativa Linne of one or more physiologically active constituents has been the cause of numerous phytochemical investigations. It has been known for some time that cannabis possessed principles which produced muscular incoordination in dogs. This property has been used as the basis for the bioassay of cannabis preparations in the United States Pharmacopoeia. Since the observation of the production of corneal anesthesia in rabbits by the administration of cannabis, reported by Gayer in 1928, considerable interest has been aroused to study the physiological activity of the drug.

This investigation was undertaken for the purpose of isolating, studying, and identifying the various constituents of cannabis. It was of particular interest to study the corneal anesthetic activity in comparison with the property of the drug to produce muscular incoordination. Use of the drug for its physiological activity has not been popular because of the unknown nature and properties of its constituents. An investigation of the plant from a chemical viewpoint is desirable, since the isolation and identification of a pure chemical compound with physiological activity must precede a pharmacological study. It was the purpose of this investigation to gather information and data which would be of value in reaching the point where a complete pharmacological investigation of the constituents could be undertaken.

1

Botanical Description - Dewey describes the plant as follows:

"The hemp plant, Cannabis sativa Linne, is an annual, growing each year from the seed. It has a rigid, herbaceous stalk, attaining a height of 3 to 16 feet, obtusely 4-cornered, more or less fluted or channeled, and with well-marked nodes at intervals of 4 to 20 inches. When not crowded it has numerous spreading branches, and the central stalk attains a thickness of 1 to 3 inches, with a rough bark near the base. If crowded, as when sown broadcast for fiber, the stalks are without branches or foliage except at the top, and the smooth fluted stems are 1-4 to 3-4 inch in diameter. The leaves, opposite except near the top or on the shortened branches, appearing fascicled, are palmately compound and composed of 5 to 11 - usually 7 - leaflets. The leaflets are dark green, lighter below, lanceolate, pointed at both ends, serrate, 2 to 6 inches long and 3-8 to 3-4 inches wide. Hemp is dioecious, the staminate or pollen-bearing flowers and the pistillate or seed-producing flowers being borne on separate plants. The staminate flowers are borne in small auxillary panicles, and consist of five greenish yellow or purplish sepals opening wide at maturity and disclosing five stamens which discharge abundent yellow pollen. The pistillate flowers are stemless and solitary in the exits of the small leaves near the ends of the branches, often crowded so as to appear like a thick spike. The pistillate flower is inconspicuous, consisting of a thin, entire green calyx, pointed, with a slit at one side, but remaining nearly closed over the ovary and merely permitting the two small stigmas to protrude at the apex. The ovary

is one seeded, developing into a smooth, compressed or nearly spherical achene 1-10 to 3-16 inch thick and 1-8 to 1-4 inch long, from dark grey to light brown in color and mottled. The seeds cleaned for market nearly always include some still covered with the green, gummy calyx. The seeds vary in weight from 8 to 27 mgm."

Scientific Names and Synonyms - The scientific names Cannabis sativa Linne and Cannabis indica Lamarck have often been used synonymously. Although the hemp plant of India has been considered by some as a distinct species and named Cannabis indica, it is now regarded merely as a geographical variety of Cannabis sativa Linne.[2]

The term "American Cannabis" or "Cannabis Americana" is used in reference to the Cannabis sativa grown in the United States. "African Cannabis" is a term applied to Cannabis sativa Linne growing in various sections of Africa.[3]

Common English synonyms are hemp, Indian hemp, hashish, and marihuana. The common German name is "Hanf", and the French equivalent is "Chanvre". Marihuana is also the common Mexican term. "Guaza" and "gunjah" are terms applied to a product from India, which is prepared by kneading the dried flowering tops in cylindrical or rounded resinous masses.[4] The drug known as "bhang" consists of the dried leaves and young twigs of Cannabis sativa Linne. The resin of the plant is separately collected and marketed in India under the name of "churrus" or "Charas". Many variations of these synonyms can be found in the literature, most of which have been collected and summarized by Walton.[5]

Etymology[6] - According to Tschirch, the word "cannabis" is derived from the two words from Sanskrit, cana, meaning a reed, and pis or pus, meaning a nettle. Literally a "reed-nettle".

The word "sativa" is from the Latin, meaning sown or planted.

Habitat[7] - Cannabis is a native of the Caucasus, Persia, and the hilly regions of Northern India. It is cultivated in all parts of the temperate zone.

Medicinal Uses[8] - The therapeutic application is more a matter of history than of present-day practice. There are no rational or indispensable uses of cannabis in modern medicine. Although preparations of cannabis appear to have been used since ancient times for the narcotic activity, the introduction of the drug for medicinal use did not occur until about 1840. Since that time it has been reported of value in numerous therapeutic applications, but because of the unknown nature and properties of its constituents it has never enjoyed much popularity as an essential drug. Recent legislation designed to curb the illegal use of the drug has now eliminated almost entirely the use of cannabis or its preparations for medicinal purposes in the United States.

Walton[9] has summarized the literature on the many therapeutic applications for which cannabis has been suggested. These uses are briefly reviewed as follows:

For spastic conditions, cannabis was recommended in the treatment of tetanus, hydrophobia, puerperal convulsions, chorea and strychnine poisoning. Its use as an analgesic was not uncommon, particularly in the treatment of headache and migraine.

The preparation, Compound Salicylic Collodion, in fifth and sixth editions of the National Formulary required 10 per cent of the Fluidextract of Cannabis. The sedative and hypnotic actions of cannabis have been compared with those of opium. Other therapeutic uses for which cannabis has been recommended are the treatment of mental conditions of a depressive nature, uterine dysfunction, as an oxytocic, and diagnostic purposes in psychiatric analysis. In current practice the sedative effects are used in veterinary work.[10] Wilks and Eichhorn state that one half ounce of the solid extract is sufficient to anesthetize a horse.

The use of cannabis for its narcotic activity has been common practice in many parts of the world for centuries. This particular use apparently was a folk custom of the earlier civilizations, and no exact data of its origin is known. Present-day misuse of the drug in this country for its intoxicating properties has been highly publicised, usually with a lack of data to substantiate the publicity. It is not within the scope of this investigation to discuss the illegal use and the sociological effects of the illegal use of cannabis. Further information and a bibliography on this subject may be found by referring to Walton.[8]

SUMMARY OF DATA IN U.S.P. OF CANNABIS

Official in:

U.S.P., 1870, 1880, 1890, 1900, 1910, 1920, 1930.

Official Latinized Title:

Cannabis Americana, 1870, 1880.

Cannabis Indica, 1870, 1880, 1890, 1900.

Cannabis, 1910, 1920, 1930.

Official English Title:

American Hemp, 1870

American Cannabis, 1880

Indian Cannabis, 1880, 1890, 1900

Cannabis, 1910, 1920, 1930.

Official Abbreviation:

Cannab., 1910, 1920, 1930.

Official Synonyms:

Indian Hemp, 1880, 1890

Cannabis Indica, 1910

Guaza, Gunzah, 1910

Cannabis indicae herba P.I. 1930.

Botanical Source:

Cannabis sativa Linne, 1870, 1880, 1890, 1900, 1920, 1930

Cannabis sativa Linne, var. Indica, 1870, 1910.

Natural Order:

Urticaceae, Cannabineae, 1880.

Urticaceae, 1890.

Family:

Moraceae, 1900, 1910, 1920, 1930.

Part or Product Used:

Flowering tops, 1870.

Flowering tops of the female plant, 1870, 1880, 1890.

Dried flowering tops of the pistillate plants, 1900, 1910, 1920, 1930.

Habitat:

North America, 1870.

Southern United States, 1880.

East Indies, 1880, 1890, 1900.

Time of Collection:

While flowering, 1880.

Gathered while the fruits are yet undeveloped, and carrying the whole of their natural form, 1900.

Purity Rubric:

Freed from the thicker stems and large foliage leaves and without the presence or admixture of more than 10 per cent of fruits and other foreign organic matter, 1910. Contains not more than 10 per cent of its fruits, large foliage leaves, stems over 3 mm. in diameter and not more than 2 per cent of other foreign organic matter, 1920, 1930.

Descriptions

1880, 1890, 1900, 1910, 1920, 1930.

Assay:

Biologically on the dog, 1910, 1920.

Preparations:

Extract of Cannabis, 1890, 1910, 1920, 1930.

Extract of Cannabis Indica, 1890.

Fluid Tincture of Cannabis Indica, 1890.

Fluid extract of Cannabis, 1910, 1920, 1930.

Tincture of Cannabis, 1910.

Dose:

0.065 Gm. - 1 grain, 1900, 1910, 1920.

SUMMARY OF DATA IN THE NATIONAL FORMULARY

Preparations:

Compound Salicylic Collodion, 1920, 1930.

Tincture of Cannabis, 1930.

Survey of the Constituents Isolated from Cannabis sativa Linne. -

The organic constituents which have been isolated from cannabis are briefly summarized below. Carotene was found in the leaves of cannabis by Arnaud[11] in 1889. Wood, Spivey, and Easterfield[12] investigated the exuded resin of cannabis in 1896 and reported the isolation of a terpene boiling between 170° - 180°C., a sesquiterpene boiling at 258° - 259°C., a paraffin melting at

62.5°C., and an impure red oil which they referred to as "cannabinol". The boiling point of the red oil was 265°C. Frankel[13] in 1903 isolated a paraffin melting at 70°C. with the formula $C_{28}H_{58}$ from a petroleum ether extract of flowering tops of the plants. He also obtained a red oil by distillation of the petroleum ether extract after a procedure similar to that of Wood, Spivey, and Easterfield. His analysis gave the formula $C_{21}H_{30}O_2$ for this red oil. In 1931 Cahn[14] isolated a pure compound from a similar red oil and determined the formula of this compound to be $C_{21}H_{26}O_2$. The name "Cannabinol" was retained. In 1940 Adams[15] prepared a red oil from the distillation of an extract of the leaves of cannabis. This oil contained besides cannabinol another phenolic compound of the formula $C_{20}H_{30}O_2$, which was named "cannabidiol". Neither cannabinol nor cannabidiol were physiologically active. In 1942, a physiologically active compound was isolated from the red oil by the United States Bureau of Narcotics Laboratory.[16] This product is an isomer of cannabidiol and is known as "tetrahydrocannabinol". The chemistry and relationships of these compounds is more completely discussed in the introduction to the experimental procedures.

The protein content of cannabis seeds was found by Osborne and Campbell[17] in 1896 to consist chiefly of edestin. A recent analysis of the fats found in the seeds of cannabis by Griffiths and Hilditch[18] indicated the presence of the following fatty acids:

Palmitic acid	5.8 per cent
Stearic acid	1.7 per cent
Arachidic acid	1.1 per cent
Oleic acid	14.1 per cent
Linoleic acid	65.3 per cent
Linolenic acid	16.1 per cent

19

In 1940 Adams, Pease, and Clark reported the isolation of Quebrachitol, l-inositol monomethyl ether, from the aqueous layer remaining after steam distillation of hemp extract.

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EXPERIMENTAL INTRODUCTION

The first report of the isolation of an active substance from "charas", the exuded resin of Indian hemp, was that of Wood, Spivey, and Easterfield[1] in 1896. Although previous investigators had suggested that the physiological effect was probably caused by a volatile oil or an alkaloid, the product of Wood, et al, was a non-volatile, red oil obtained by fractionating alcoholic charas extract under reduced pressure. This oil was referred to as "cannabinol" or "crude cannabinol". From the constancy of composition of a number of preparations of this substance obtained from different samples of charas, it was believed to be a definite chemical compound of the formula $C_{18}H_{24}O_2$.

The character of the physiological activity of the red oil was described by these workers as follows: "The red oil is extremely active and taken in doses of 0.05 Gm. induces decided intoxication followed by sleep".

In 1898, these authors, after further examination of "crude cannabinol" found that it was a mixture or at least two compounds having similar physical properties, one of which, having the formula $C_{21}H_{26}O_2$, was isolated through acetylation. It was proposed to retain the name, cannabinol, for this compound although it was unknown whether or not it was a physiologically active material.

Later investigators[2,3] consistently failed to repeat the work of Wood, Spivey and Easterfield. Constant-boiling "resins" were obtained by several workers, and, although these "resins" yielded

only oily derivatives, homogeneity was claimed for each product. Cannabinol was the name appropriated in all cases to the resinous products.

A color test for the identification of hashish or its preparations was published in 1911 by W. Beam[4] of the Wellcome Tropical Research Laboratories, Khartoum, Egyptian Sudan. Beam observed that a rich purple color developed when one treated the residue from a petroleum ether extract of the drug with alcoholic potassium hydroxide. The chemistry of the color test was not investigated at that time, however. The test was accepted and used by government investigators in many countries as a method of identifying samples of cannabis in the attempt to stop the illicit traffic and use of the drug. The production of a purple color when an extract was treated with alcoholic potassium hydroxide was referred to as a positive Beam test. Apparently no attempt was made to determine whether or not the principle which produced the Beam test was physiologically active.

Biological Assays

In the absence of conclusive chemical data with which to develop a chemical method for the evaluation of cannabis, biological assay methods have necessarily been given preference in quantitative studies of the drug. Some of the more common methods employed by various workers are briefly reviewed in the following paragraphs.

Bioassay with Frogs[5] - Goodall tested cannabis preparations upon frogs. He reported that 1/4 grain doses of a standard extract was sufficiently active to deeply narcotize a 20 Gm. frog for 48 hours. The toxicity of the material to frogs was low.

Bioassay using Mice and Rats - The minimum lethal dose in white mice after intraperitoneal injection was measured by Wiechowski.[6] The fatal dose of petroleum ether extracts of the crude drug was found to be about 1.257 gm./kg. The hypnotic dose was not reported.

Attempts by Munch[7] to standardize fluid extract of cannabis by subcutaneous or by intraperitoneal injections in white rats gave inconsistent results. Munch concluded that rats and mice were not suitable for this assay.

Bioassay using Cats - The quantitative response of cats to the oral administration of cannabis preparations in comparison of the effect on dogs was studied and reported in 1898 by Marshall.[8] The effects in cats resembled those in dogs but were more prolonged. Indications of activity were depression and muscular weakness with swaying from side to side. Salivation was a usual symptom.

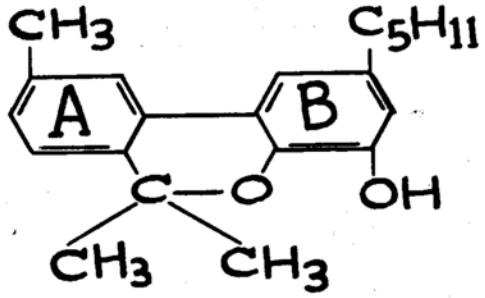
Bioassay with Dogs - The first quantitative tests using dogs were those of Houghton in 1897.[9] It was observed that when dogs were administered cannabis preparations they exhibited a type of incoordination apparently characteristic of cannabis. Typical effects have been described at length by a number of observers; the most recent and complete discussions are those of Munch[7] and Walton.[10] A characteristic symptom reported by all workers was the tendency of the animal to develop a swaying of the body which became progressively more intense; the direction of the sway was backward and forward as well as from side to side. The effect upon the general demeanor of the dog as well as other reactions has received considerable attention. However, no specific reaction

or series of reactions has been conclusively shown to be quantitatively characteristic of cannabis. Walton, Martin and Keller have defined six arbitrary stages of intensity of effects: first, a slight depression; second, a barely recognizable ataxia; third, an obvious ataxia; fourth, a marked ataxia in which the animal frequently pitches forward and barely catches itself; fifth, inability to stand alone; sixth, inability to rise and plunge about. The ataxia was chiefly manifested by swaying movements.

Bioassay with Rabbits - Incoordination was not exhibited by rabbits when administered cannabis preparations as was the case with dogs and cats, subsequently early workers concluded that rabbits were insusceptible to the action of cannabis. Ordinarily, they were quieted by the drug and showed a barely perceptible weaving motion. Gayer[11] made the observation that corneal anesthesia could be demonstrated after intravenous doses. The anesthesia was determined by the use of von Frey hairs, which would elicit the wink reflex when pressed against the cornea of normal rabbits but would have no effect in narcotized animals. Acetone extracts were used for the injection and it was claimed the usual amounts of acetone were without effect. If the preparation was given by mouth, it was necessary to increase the strength of the dose approximately ten times. When the extracts were dissolved in oil and administered orally, the drug was practically inactive. The behavior of cats toward cannabis by intravenous injection was apparently both qualitatively and quantitatively about the same as rabbits. With white mice, subcutaneous or oral administration of the acetone

extract produced cataleptic symptoms and corneal anesthesia. Gayer was unable to obtain corneal anesthesia in dogs and because of this fact the question has often arisen as to whether or not the substance which produces the corneal anesthesia in rabbits is the same as that which produces the incoordination observed in dogs.

In 1931, Cahn[12] confirmed the work of Wood, Spivey, and Easterfield. Using a high-boiling (distillation) fraction of the ether-soluble portion of the "resin" of Indian hemp, Cahn was successful in preparing crystalline cannabinol acetate by acetylation in pyridine solution with acetyl chloride. This compound was found to have the formula $C_{21}H_{25}O_2Ac$. Hydrolysis of the crystalline acetate yielded cannabinol as a colorless oil. Cannabinol was proven to be a dibenzo-pyran derivative having the following structure:



(?)3"hydroxy-2:2:5',trimethyl -(?)5" n amyl dibenzopyran

The location of the substituents of ring B was not conclusively proven.

Although Cahn did not report the isolation of a physiologically active principle, his work and findings were the basis for the immediate renewal of interest in the problem. Two independent groups of workers should be cited for their outstanding investigations into the chemistry of cannabis and cannabis resins. These were:

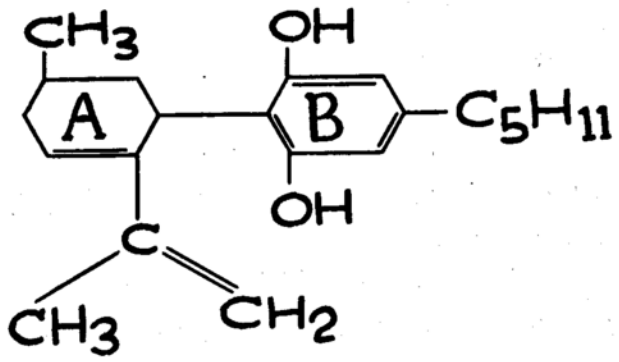
Roger Adams and coworkers[13] at the University of Illinois in collaboration with the Treasury Department, Narcotics Laboratory, Washington, D.C.; and several English workers under the direction of A.R. Todd[14] at the University of Manchester.

For the purpose of clarity the investigations and findings of these two groups of workers are summarized separately.

From wild hemp collected in Minnesota, Adams prepared an alcoholic extract by continuous extraction. The solid residue remaining after recovery of the alcohol was subjected to high-vacuum fractionation in a specially constructed still. They considered the highest-boiling fraction, which was physiologically active, to be "purified red oil" and worked this up in the following manner.

Using the method of Cohn, an attempt was made to isolate cannabinol by the preparation of an acetate or p-nitrobenzoate. No crystalline derivative could be obtained. Since the red oil contained substances with phenolic groups as shown by qualitative tests, other reagents for phenols were studied. This resulted in observing that a crystalline 3,5 dinitrobenzoyl derivative could be isolated in yields which corresponded to about 33 per cent of the purified red oil used. Analysis indicated this derivative to be a bis-3,5-dinitrobenzoate of a dihydric phenol of the formula $C_{21}H_{30}O_2$. Upon ammonalysis the isolation of a pure compound was accomplished. This product was optically active $[\alpha]_D^{22}$ - 119° and gave a strong alkaline Beam test. It was given the name cannabidiol. Cannabidiol possessed none of the physiological activity typical of marihuana.

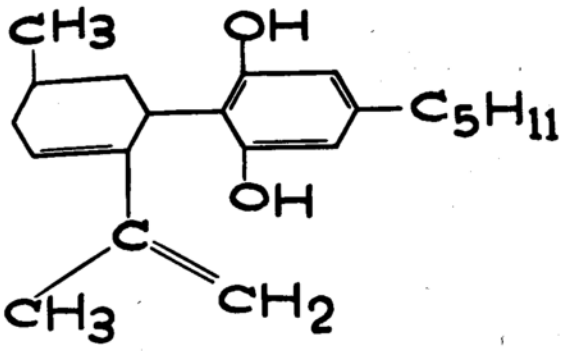
Comparison of the formula of cannabidiol with that of cannabinol as proven by Cahn showed that cannabidiol contained merely four more hydrogen atoms than cannabinol. From the various tests made and degradation products obtained, it appeared that the structure of cannabidiol resembled closely that of cannabinol. Working upon this supposition Adams synthesized cannabidiol and proved that it had the following structure with question as to the position of the double bond in ring "B".



Because cannabidiol was not the physiologically active principle, the investigation was continued upon the residue remaining after its removal from the "red oil". It was found that this residue no longer gave a positive Beam test. From this observation and the fact that cannabidiol gave a strong Beam test, it was evident that the Beam test could not be used for measuring the degree of biological activity. Although Adams had previously failed to isolate cannabinol from the Minnesota hemp extract, its isolation as the 3,5-dinitrophenyl urethane from the cannabidiol-free oil was accomplished. In agreement with previous findings, cannabinol failed to exhibit any physiological activity when administered to

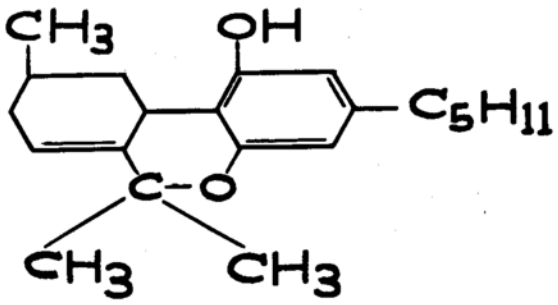
dogs. It was evident that the active constituent was present in the residue left after the removal of both the cannabidiol and cannabinol.

During their studies on the structure of cannabidiol Adams and coworkers heated cannabidiol with pyridine hydrochloride and obtained a tetrahydrocannabinol as shown by the following equation:



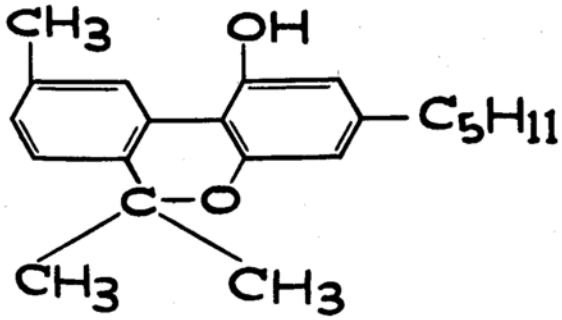
CANNABIDIOL
(inactive)

ISOMERIZE



TETRAHYDRO-
CANNABINOL
(active)

DEHYDROGENATE



CANNABINOL
(inactive)

This tetrahydrocannabinol was tested for biological activity upon dogs and it was observed that it possessed an activity many times that of the original purified red oil. Following this observation, Adams synthesized several tetrahydrocannabinols which all exhibited physiological activity in dogs although the degree of activity was less than that of the product obtained by isomerization of cannabidiol. These products were assumed to be isomers of the original product.

Studies to determine which portion of the molecule influences the activity of the tetrahydrocannabinol were conducted by synthesizing and bioassaying several similarly constituted substances. An increase in the degree of activity of the tetrahydrocannabinol resulted by substituting either a n-hexyl or a n-heptyl group for the n-amyl radical. Substitution of the n-amyl radical by octyl or nonoctyl groups tended to decrease the degree of activity. Data on this phase of the chemistry is as yet very incomplete.

The investigations of Todd were begun in 1939. A petroleum ether extract of Egyptian hashish was subjected to molecular distillation to obtain a red oil possessing physiological activity as determined by the Gayer test on rabbits. This red oil yielded cannabinol as the p-nitrobenzoate and cannabidiol was the bis-3,5-dinitrobenzoate according to the method employed by Adams. Likewise the material which remained after removal of these two compounds was physiologically active, and it was claimed by Todd that this contained all of the material giving a positive Gayer test. This residue was to be worked up separately and described elsewhere; as yet no reports have appeared concerning its investigation.

Todd isomerized cannabidiol to tetrahydrocannabinol in a manner similar to that of Adams and then was able to obtain corneal anesthesia when he administered it to rabbits. The synthesis of tetrahydrocannabinol by Todd was accomplished by an entirely different process than that of Adams; however, the products of both groups of workers were found to be the same when comparative tests were completed. The degree of corneal anesthesia in rabbits produced by the synthetic product of Todd was not discussed.

In comparing the work of Adams with that of Todd, the most important point of discussion lies in the fact that different methods of bioassay were employed by the two groups; the production of motor ataxia in dogs was used by Adams and the production of corneal anesthesia in rabbits by Todd.

In 1941 the United States Bureau of Narcotics Laboratory[15] reported that motor ataxia had been produced in dogs by the administration of an alcohol-soluble fraction of the non-saponifiable portion of an extract prepared from the fruits of Cannabis sativa l.c. Linne. A chemical investigation of this material was not reported.

The isolation of a naturally-occuring tetrahydrocannabinol from the drug was recently reported by the same laboratory. Adams and Todd had succeeded in preparing several isomeric tetrahydrocannabinols by isomerization of cannabidiol and by synthesis, but had been unable to obtain a naturally-occuring product. This product was isolated from a crude red oil by acetylation and passage of the acetylated product over a series of adsorption columns followed by high-vacuum fractionation of the non-adsorbed fraction. A specially constructed high-vacuum fractionating column

was employed in this procedure because the usual types of fractionating columns were found to be ineffective. The main fraction collected, amounting to about 85 per cent of the acetylated material, was an optically active, colorless oil. Attempts to obtain a crystalline product were unsuccessful. It was observed that on standing, especially in thin layers in contact with air, the material developed a yellow color and its potency dropped. Analysis indicated that these changes were due to oxidation. On comparison with the products of Adams, the natural product proved to be isomeric with the synthetic tetrahydrocannabinol, but were not identical as indicated by boiling points and optical activity. In the discussion of this work, the Narcotics Bureau Laboratory advanced the supposition that the red oil is composed essentially of cannabinol, cannabidiol, and various isomeric tetrahydrocannabinols, the proportions of which may vary widely. It would thus be possible to account for the extremely variable character of cannabis extracts and of "red oil", which has been observed pharmacologically and which is frequently referred to in the chemical literature.

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In 1940 Haagen-Smit by the use of high-vacuum fractionation prepared an oil which produced "typical activity" in dogs by oral administration. When a solution of this oil in a methanol-acetic acid mixture was cooled, some crystalline material was obtained. This crystalline material, termed "cannin" for convenience, caused incoordination of movement in a dog in a dosage less than that of the potent red oil necessary to cause a comparable effect. An insufficient amount of this active substance was obtained to establish its chemical identity.

A report of the continued investigations of Haagen-Smit and coworkers[19] was published in 1942. In attempts to isolate the most physiologically active fraction of the Cannabis preparations, the Gayer rabbit test was initially employed as the bioassay method. Literature on the subject had reported a close correspondence between the dog ataxia test and the Gayer test in working with distillation fractions of Cannabis. The use of rabbits was chosen because it was less cumbersome and less tedious. However, after a number of experiences in which relatively mild handling procedures appeared to result in a practical disappearance of the corneal anesthetic activity in rabbits, certain work was repeated using dog test methods. Contradictory results were obtained. (It was observed that a Cannabis extract which had been allowed to age for about six months had not lost any of its potency to produce motor ataxia in dogs, but the capacity of the extract to produce corneal anesthesia in rabbits had been lost.) Similar results were recorded when ethanol extracts were subjected to heating in the presence of a current of air. From these observations, it appeared probable that these two methods of testing were measures of different types of activity. The authors offered as a possible explanation of their findings the hypothesis that some active principle or principles which exhibit the rabbit corneal anesthesia activity in a marked degree are present in the drug but are deteriorated more rapidly than are the active principles which exhibit the dog incoordination activity.

An alternative hypothesis was advanced on the basis that no new active principle is involved, but that both the oxidized and unoxidized

forms act almost equally in the dog, while only the unoxidised form acts in the rabbit.

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EXPERIMENTAL

Source of Material - The material used in this investigation was obtained from the Department of Agronomy, College of Agriculture, University of Wisconsin. It was grown on the Hill Farm three miles west of Madison, Dane County, Wisconsin, in the 1940 season from seed which had been collected from the 1939 crop on the same area. The original seed had been obtained in 1938 from the United States Department of Agriculture, Washington, D.C., as the Roumanian variety of Cannabis sativa Linne. The container in which the original seed was received from the United States Department of Agriculture bore the label, "Spantov, Roumania, 1937".

The material, collected in September, 1940, consisted of the leaves, fruits, and branches up to about 3.5 mm. diameter of the female plant. Immediately after collection of the plant, it was dried in a corn drier for three days and then stored in a dry atmosphere for two months. The resulting dry material was ground to a coarse condition in a small hammermill. Investigation of the drug was started in March, 1942. Thus the drug was approximately eighteen months old from the time of collection.

Preliminary Examination

Ash Content[1] - The ash content was determined on two samples of the drug. The following results were obtained.

Total Ash		Acid-insoluble Ash	
Sample I	9.05 per cent	Sample I	4.39 per cent
Sample II	10.31 per cent		3.90 per cent
Average	9.68 per cent	II	4.14 per cent

Moisture Content - The moisture content of the ground drug was determined according to the toluene distillation method.[2] The results of two determinations were as follows:

Sample I	6.01 per cent
II	6.21 per cent
Average	6.11 per cent

Extraction Using Selective Solvents - Two 75 gm. samples of the drug were extracted to completion successively with the following selective solvents in the order listed: (1) Skelly-solve A, (2) Skelly-solve B, (3) chloroform, (4) ether, (5) alcohol, (6) water, (7) hydrochloric acid, 2 per cent, (8) sodium hydroxide solution, 1 per cent. Soxhlet extractors were employed except with the water, acidulated water, and sodium hydroxide solution. In these cases the drug was digested in a round bottom flask at a temperature of about 60°C. for 24 hours, the supernatant liquid poured off, and the process repeated with the same solvent until completely extracted. The marc after each extraction was allowed to drain and dry thoroughly before the succeeding extraction was started. The solvent was recovered from each extract and the per cent of solids was found to be as follows:

Solvent	Per cent Solids	
	I	II
1. Skellysolve A	11.33	12.00
2. Skelly-solve B	.80	.80
3. Chloroform	.28	.90
4. Ether	1.60	3.33
5. Alcohol	11.06	6.13
6. Water	5.60	4.26
7. 2 p.c. HCl	23.33	26.00
8. 1 p.c. NaOH	16.66	19.33
Total	70.64	72.75

Examination of the Skelly-solve A Extract - The solvent was recovered from the extract leaving an oily, dark-green residue containing some crystalline material. This residue was soluble in cold ether, warm alcohol, warm methanol, and warm acetone. Three solutions of the residue in the last three solvents listed above were prepared as follows:

Solution I	2 Gm. in 10 cc. of alcohol
Solution II	2 Gm. in 10 cc. of methanol
Solution III	2 Gm. in 10 cc. of acetone

These solutions were cooled in an ice bath for an hour. Solid material precipitated from each solution; however, the largest amount appeared to form in the methanol solution. Following this observation, 8.5 Gm. of the above residue was dissolved in 25 cc. of methanol, and the solution was chilled in a dry ice-acetone bath for 1 hour. The solid material, which formed, was removed by filtration and then warmed

on a steam bath for an hour to evaporate the traces of solvent. A solid, waxy residue amounting to 4.3 Gm. was collected.

The filtrate remaining from above was heated on a water bath to remove the methanol. A thick, oily, dark-green residue resulted. One Gm. of this oil was dissolved in 25 cc. of methanol and refluxed with .5 Gm. of norite for a half hour. A reddish-orange filtrate was collected. After recovery of the solvent from the filtrate, a red oil remained. The amount was too small for further investigation.

Skelly-solve B Extract - The recovery of the solvent from the Skelly-solve B extraction left a soft, sticky, dark-brown residue. It gave an acid reaction and possessed a pleasant hay-like odor.

Chloroform Extract - The residue remaining after recovery of the chloroform was a thick, dark-brown oil and possessed a strong pungent odor. Exposure of the residue to air for a few weeks increased the odor to such an extent that it had a slight sternutatory effect.

Ether Extract - The removal of the ether from the ether extract resulted in a soft brown residue with a granular consistance indicating the presence of crystalline material. Attempts to separate the crystalline material were unsuccessful. The residue possessed a pleasant hay-like odor.

Alcohol Extract - The alcohol was recovered from the extract leaving a black semi-solid residue. It was very sticky and hardened on exposure to air.

Aqueous Extract - A dry powdery residue remained after evaporation of the water on a steam bath. A faint odor of carmelized sugar was evident.

Extract prepared with 2 per cent Hydrochloric Acid Solution - A dark-brown extract was obtained which gave a soft black residue on evaporation of the water. The odor was pleasant, resembling that of molasses.

Extract Prepared with 1 per cent Sodium Hydroxide Solution - Evaporation of the water from this extract left a brown sticky residue which became dry and was easily powdered after drying on a sand bath at 50°C. for three days.

Chemical Investigation

Extraction with Skelly-solve B - An extract from 6.5 Kg. of the dried drug was prepared by continuous percolation with Skelly-solve B for 100 hours in a Lloyd extraction apparatus. The solvent was recovered from this extract leaving a greenish-black oily residue amounting to 590 Gm., which corresponds to 9.07 per cent of the total drug.

Removal of Volatile Constituents - The 590 Gm. of residue were distilled with steam for 5 hours until the distillate was a clear, colorless, and odorless solution. Five liters of distillate were collected during the entire procedure. The aqueous solution remaining in the distillation flask was removed from the non-volatile residue. Both the distillate and the aqueous solution thus obtained were placed in tightly stoppered containers and set aside. The black, oily residue was dried in a vacuum oven at 60°C. for two days. It weighed 500 Gm.

Determination of the Saponification Value - The determination of the saponification value according to the method in the U.S.P. XV

gave the following results:

Sample	I	156.37
	II	148.13
Average		152.25

It is evident that these results do not closely agree. The large amount of pigmented material made it very difficult to observe the end point in the titration.

Saponification of the Non-volatile Residue - The remaining non-volatile residue amounting to 490 Gm. was saponified for 8 hours in 2000 cc. of 80 per cent alcohol with an excess of potassium hydroxide calculated on the basis of the saponification value obtained above.

Separation of the Non-saponifiable from the Saponified Material - After saponification, the alcohol was recovered from the reaction mixture by distillation under reduced pressure. Three liters of water were added to the residue, and the mixture was thoroughly agitated. The mixture was placed in a continuous extraction apparatus and then extracted with ether for about 30 hours. The ethereal extract collected at the end of the procedure was only slightly colored indicating complete extraction of the non-saponifiable material.

The soap solution remaining after the separation of the ether-soluble constituents was thoroughly mixed and a 50 cc. sample was shaken with 50 cc. of ether in a separatory funnel. The ether layer remained colorless and left a negligible residue on evaporation indicating that the non-saponifiable material had been entirely removed from the soaps. The aqueous soap solution was stored in a tightly stoppered bottle under an atmosphere of carbon dioxide. The

examination of the soap solution for fatty acid content is reported in the following chapter.

EXAMINATION OF THE SAPONIFIABLE MATERIAL

Separation of the Fatty Acids - The aqueous solutions of the saponifiable material obtained from the saponification of 905 Gm. of the Skelly-solve B extract were combined for a study of the fatty acids present.

To the warmed aqueous solution, hydrochloric acid (10 per cent) was added until the solution was distinctly acid to litmus. The free fatty acids which separated in a greenish-black supernatant layer were removed by shaking out the solutions with several portions of ether. Traces of hydrochloric acid were removed by shaking this ethereal solution with distilled water and finally drying over anhydrous sodium sulfate, which was separated by filtration. The ether was recovered by distillation in the presence of carbon dioxide. The free fatty acids were dried under vacuum at 60° for 24 hours. The residue amounted to 683 Gm., which represented 75.57 per cent of the Skelly-solve B extract.

The aqueous solution resulting after shaking out with ether was set aside.

Separation of the Unsaturated from the Saturated Acids - For the separation of the unsaturated acids from the saturated acids, the dried fatty acid material was treated according to the procedure suggested by Hilditch[4] based upon the Twitchell[5] modification of the Gusserow-Varentrapp[6,7] lead salt process.

Four hundred Gm. of the fatty acid material and 280 Gm. of lead acetate were dissolved in 3500 cc. of boiling alcohol containing 1.5 per cent of glacial acetic acid and the solution was boiled for

10 minutes. This solution was then cooled to 15°C. and kept at that temperature for 24 hours, resulting in the precipitation of a considerable quantity of solid material. This solid material was removed by filtration and purified by recrystallization from alcohol. The mother liquors from the above procedures were combined for the determination of the unsaturated fatty acids present.

Isolation of the Unsaturated Fatty Acids - The alcohol was recovered from the combined mother liquors and the resulting residue then dissolved in 2000 cc. of ether. The ethereal solution was shaken vigorously 3 times with 2000 cc. portions of water for the removal of the lead acetate formed in the reaction between the lead salts and the acetic acid. The aqueous solutions were set aside. To insure complete decomposition of the lead salts, the ether solution was shaken with 200 cc. of dilute hydrochloric acid followed by shaking twice with water to remove the excess hydrochloric acid or acetic acid. Water was removed from the ethereal solution by drying over anhydrous sodium sulfate. The ether was recovered by distillation in the presence of carbon dioxide leaving a dark-brown, oily residue consisting of the unsaturated acids. The yield was 230 Gm., which represents 57.5 per cent of the totall fatty acid material.

Bromination of the Unsaturated Fatty Acids - Bromination of the unsaturated acids obtained above was carried out according to the procedure of Hazura[5] as described by Rosenthaler[6]. A 100 Gm. sample of the unsaturated acids was dissolved in a solution of 1000 cc. of glacial acetic acid and 500 cc. of ether. This mixture was placed in an ice bath, and when cold, a solution of one part of bromine in two

parts of glacial acetic acid was added slowly from a dropping funnel until the bromine was present in excess. Addition of the bromine solution required about 2 hours, during which the reaction flask was shaken repeatedly. A flaky crystalline precipitate began to form during the procedure. The mixture was kept at approximately 4°C. for 12 hours in an ice chest allowing for the deposition of a considerable quantity of the crystalline material.

Isolation of Linolenic Acid Hexabromide - The precipitate which settled out upon cooling was removed by filtration and washed first with 500 cc. of a cold solution of equal parts of glacial acetic acid and ether and then with 200 cc. of water. The residue was collected and dried resulting in 21.64 Gm. of a white crystalline powder. The melting point was 177°-179°. Purification of this material was accomplished by repeated recrystallizations from absolute alcohol. The product obtained was a white finely-divided, crystalline substance with a melting point of 181°C. Recrystallization from ether failed to raise the melting point above 181°C. The recorded melting point of linolenic acid hexabromide is 180°-181°C.[6]

Isolation of Linoleic Acid Tetrabromide - The mother liquor from the above procedure was combined with ether-glacial acetic acid solution used for washing the hexabromide and the composite was then investigated for the presence of linoleic acid tetrabromide. This material was poured in 3000 cc. of water with stirring, and the mixture was allowed to stand in an ice chest for 2 hours. A dark-red precipitate containing crystals settled out from a yellow aqueous solution. The aqueous supernatant layer was decanted from the residue and set aside. The residue was washed several times with water, dissolved

in 500 cc. of ether and dried over anhydrous sodium sulfate for 12 hours. The sodium sulfate was removed by filtration and the ether was recovered from the filtrate by distillation, leaving an oily residue. This was dissolved in 1000 cc. of Skelly-solve B and placed in the ice chest for 10 hours. A light-brown deposit on the side of the flask resulted. The clear red mother liquor was poured off and chilled again for 5 hours to allow for further crystallization. No additional material precipitated. The above precipitate was washed with cold Skelly-solve B and dried on a porous plate. A yield of 10.5 Gm. of dry material was obtained. The melting point was determined and found to be 106°C. Recrystallization of the material from Skelly-solve B failed to yield a white product. The impure material was dissolved in 100 cc. of alcohol and refluxed with 5 Gm. of norite for 30 minutes and then filtered. The alcohol was recovered from the filtrate and the residue was crystallized from Skelly-solve B. A product was obtained consisting of fine white needles with a melting point of 114°C. This melting point coincides with the recorded melting point of linoleic acid tetrabromide.[6]

Debromination - The mother liquors remaining after the removal and purification of the tetrabromide were combined and distilled to recover the Skelly-solve B. The residual, dark-brown, oily material was dried in a vacuum oven at 60°C. for 3 hours. This material, when dry, weighed 93 Gm.

Debromination of this material was carried out according to the procedure employed by Hazura.[8] A 40 Gm. portion of the oil was dissolved in 600 cc. of 90 per cent alcohol and refluxed with 200 Gm. of zinc dust for 4 hours. A considerable quantity of zinc remained

unreacted in the bottom of the flask. The clear supernatent solution was decanted and filtered and the remaining zinc was washed with 200 cc. of alcohol. The alcohol used for washing the zinc residue was also filtered and then added to the original filtrate. Five hundred cc. of alcohol was removed by distillation from the combined filtrates thus concentrating the volume to about 150 cc. This concentrated solution was poured in 3000 cc. of water with agitation and the mixture was acidulated by adding 400 cc. of dilute sulfuric acid and warming on a water bath for 20 minutes. A dark-brown oily layer formed on the top of the solution. Separation of the oily layer from the aqueous portion was accomplished by shaking with ether. The aqueous solution which separated from the ether solution was set aside. The ether was recovered from the solution and the residue saponified by refluxing for 2 hours with 200 cc. of half-normal alcoholic potassium hydroxide. The alcohol was recovered from the reaction mixture by distillation under carbon dioxide. The soaps were dissolved in 2000 cc. of water and decomposed again by warming with 200 cc. of dilute sulfuric acid for 20 minutes. The freed fatty acid material, thus formed, was taken up with ether and dried over anhydrous sodium sulfate. The ether was recovered by distillation leaving a thick reddish-brown residue amounting to 14 Gm. Attempts to determine the congealing point of this material were unsuccessful.

A method employed by Brown and Shinowara[9] to prepare pure oleic acid was used in an attempt to purify the syrupy liquid obtained above. A 7 per cent acetone solution of the oily material (14 Gm. in 200 cc.) was chilled in a dry ice-acetone bath for one hour. The solid material which formed during the chilling was removed by filtration through a

chilled funnel. The light-brown solid residue was removed and liquified at about 8°C. to a reddish-brown oil. This was recrystallized from acetone and then from absolute alcohol by chilling in a dry ice bath. The solid material obtained by this procedure liquified to a light brown oil at room temperature (23°C.). Traces of solvent were removed by drying in a vacuum oven at 60°C. for two hours after which the residue solidified at room temperature to a tan wax-like mass. The weight of the dried material was .83 Gm. Melting point of the solid material was 35°-39°C. The mother liquor was again chilled in a dry-ice acetone bath for several hours without the formation of additional solid material. One hundred cc. of acetone were recovered from the combined mother liquors obtained above thus concentrating the volume to about 100 cc. This concentrated solution was again chilled in the dry ice bath for several hours. The solid material was removed by filtration through a cooled Buechner funnel. At room temperature the material liquified to a brown oil. Traces of solvent were removed from this oil by heating it in a vacuum oven at 60°C. for 2 hours. An attempt to determine the presence of oleic acid by the solidification point of the oil was unsuccessful. The oil remained in a liquid state when chilled in an ice-salt bath for 6 hours. The solidification point of oleic acid is 4°C. Final weight of the oily fraction was 9.6 Gm.

Because of the apparent absence of oleic acid in the debrominated material, it was desirable to investigate the remaining brominated material. A sample of 53 Gm. of the brominated oil was dissolved in 40 cc. of Skelly-solve B and chilled in an ice bath for 12 hours with the intention of precipitating traces of linoleic acid tetrabromide

which appeared to be present from the results obtained in the first attempt at debromination. A solid amorphous deposit was formed during the cooling of the solution. The material was removed by filtration and dried on a porous plate. The dry product weighed 6.4 Gm. and melted at 103°-105°C. indicating that it was probably impure linoleic acid tetrabromide.

The concentration and further chilling of the mother liquors remaining from above did not increase the yield of the solid material.

The solvent was recovered from the solution and the residue was dissolved in 600 cc. of alcohol (90 per cent). The debromination was conducted by refluxing with 200 Gm. of zinc dust for 4 hours and then following through the procedure as described in the first attempt at debromination.

The final debrominated fatty acid material which was obtained by this procedure was a dark-brown oil weighing 16.8 Gm. An attempt to solidify this oil in an ice bath was unsuccessful, indicating that the material was not oleic acid. This oil was dissolved in 200 cc. of acetone and chilled in the dry-ice bath for 6 hours. The solid material, which separated, was removed by filtration through a chilled funnel and dried in the vacuum oven at 60°C. After removal of traces of the solvent, a brown oil remained, which was liquid at the temperature of an ice-salt bath.

From these observations it is possible to conclude that the unsaturated acids consisted chiefly of linolenic and linoleic acids. It is not improbable that traces of oleic acid was present, although it was not possible to isolate it by the method employed.

Isolation of the Saturated Fatty Acids - The fraction of lead salts that were insoluble in alcohol should contain the saturated fatty acids. The liberation of the fatty acids was accomplished by adding 240 Gm. of the solid lead salts to 3000 cc. of boiling 10 per cent hydrochloric acid. The mixture was boiled and stirred for 10 minutes causing an oily layer to separated on top of the aqueous solution. This oily layer which solidified on cooling was taken up in ether, and the aqueous portion containing precipitated lead chlorids was discarded. Traces of hydrochloric acid present in the ether solution was eliminated by washing the solution several times with distilled water. Anhydrous sodium sulfate was added to dry the ether solution and was later removed by filtration. The ether was recovered by distillation leaving a greenish-black residue of the saturated acids amounting to 128 Gm. This represented 31.1 per cent of the original fatty acid material.

Fractionation of the Saturated Fatty Acids - Because of the colored material present, it was necessary to remove as much of the color as possible. A sample of 90 Gm. of the above residue was dissolved in 600 cc. of alcohol and refluxed with 25 Gm. of norite for 2 hours. Considerable colored material had been removed by the norite, as the filtrate possessed only a light brown color.

Separation of the solid fatty acids was attempted by a process of fractional crystallization. The filtrate was cooled to 15°C. for 2 hours and no solid material separated. It was then kept in an ice bath for 12 hours, during which time a small amount of material solidified. The material was removed by filtration and recrystallized from alcohol. After drying on a porous plate, the product weighed

0.47 Gm. and melted at 69°-70°C. It is probable this fraction was chiefly stearic acid, which melts at 69.3°C.[10] The chilling of the mother liquors from above was continued for another 12 hours but no more solid material was formed. The solution was concentrated from 580 cc. to 500 cc. by evaporation of a portion of the alcohol and was again placed in the ice bath for 12 hours. Another fraction of solid material separated and was removed and dried. Recrystallization from alcohol and then from acetone of this second fraction yielded 0.74 Gm. of solid material melting at 58°C.

Concentration of the mother liquor to 400 cc. and chilling the concentrate as in the above procedure gave a third fraction. Fraction 3 was recrystallized from alcohol and then from acetone. The melting point was 53°-55°C. and the yield was 0.45 Gm.

Attempts to obtain further fractions of the solid acids from this procedure were unsuccessful because further concentration of the mother liquor caused the formation of a jelly-like mass at 0°C. No solid material could be separated from this jell-like mass.

Determination of the Acid Value of the Saturated Fatty Acid Material - The alcohol was recovered from the mother liquor and the residue was dried in a vacuum oven at 60°C. for 2 hours. The acid value of the dried residue was determined,[11] and was found to be 88.80. This indicated that the material analyzed consisted of a considerable amount of substances other than free fatty acids.

Saponification of the Saturated Acids - The remaining fatty acid material, amounting to 37.5 Gm., was saponified with alcoholic potassium hydroxide using an excess of the alkali. After saponification, the alcohol was recovered, and the residual soaps were dissolved in water.

The non-saponifiable matter was separated from the soap solution by shaking with successive portions of ether. The ether solution was washed with water, and the water used for washing was added to aqueous soap solution. Removal of the ether left an oily residue of non-saponifiable material amounting to 6.1 Gm. This material was set aside. The soap solution remaining from the above was acidified by the addition of 50 cc. of 5 per cent sulfuric acid and warming for 20 minutes on a steam bath. The fatty acids separated as an oily layer on top of the aqueous solution and were taken up in ether. The ether solution was dried over anhydrous sodium sulfate and then distilled to remove the ether. The residue amounted to 19.6 Gm. of a dark-brown oil.

The oily residue was dissolved in 50 cc. of alcohol and cooled in an ice bath for four hours. The solid material which formed was removed by filtration and recrystallized several times from alcohol and then from anhydrous ether. A yield of 2.8 Gm. of solid acids was obtained with a melting point of 44°-45°C.

Further chilling of the filtrate in the ice bath did not increase the yield of solid acids. Twenty-five cc. of acetone were added to the filtrate and the solution was chilled in a vacuum bottle containing dry ice for 1 hour. The solid material which had formed was removed by filtration and recrystallized from acetone. This fraction of saturated fatty acids weighed 0.8 Gm. and melted at 44°-45°C., which was melting point of the previous fraction.

The alcohol-acetone solution remaining from above was evaporated to dryness on a steam bath. The residual oil was dissolved in 50 cc. of acetone and then chilled in the dry ice bath for 1 hour. A considerable quantity of solid material formed during this chilling

and was removed by filtration through a cooled funnel. At room temperature this material liquified to a brown oil, indicating that it consisted chiefly of unsaturated acids.

Investigation of the Non-saponifiable Material - The ethereal solution of the non-saponifiable material possessed a brownish-red color and was observed to be somewhat cloudy when held directly in a beam of light. This cloudiness was eliminated by washing the ether solution in a separatory funnel with 1000 cc. of a 3 per cent solution of potassium hydroxide and then with several successive portions of water. The aqueous solutions used for washing were collected and set aside in stoppered bottles under carbon dioxide. A clear red ethereal solution of the non-saponifiable material was dried over anhydrous sodium sulfate. The ether was recovered by distillation and the residue of non-saponifiable material amounted to 100 Gm. which represents 20 per cent of the original extract used in the saponification. This residue had a dark reddish brown color and a pleasant rather characteristic odor. It was very thick and sticky.

A determination of the saponification value was made on the sticky residue and was found to be 0, indicating that complete saponification had been accomplished and the residue possessed no free acids or acids combined as esters.[12]

Determination of Physiological Activity - The determination of the physiological activity of the non-saponifiable material from the fruits of Cannabis Sativa Linne was reported by Matchett and Loewe.[13] The Gayer test was employed by these workers, using acetone as the vehicle for the extract. It was claimed that the intravenous injection of acetone in a small quantity would cause no significant response.

Blank tests with acetone were made on two rabbits and a marked response was observed in each case. The amount of acetone injected approximates that used by other workers.

Test Number 1 - A rabbit weighing 1.9 Kg. was injected intravenously with 1.75 cc. of acetone. The respiration rate increased very rapidly and within 3 minutes the animal did not react to pinching of the tail or legs. A convulsion occurred after five minutes from which the rabbit soon recovered. The depression following the convulsion lasted for about 30 minutes. After two hours no effects were observed. Conjunctival and corneal reflex responses could be obtained at any time during the two hour period.

Test Number 2 - A rabbit weighing 2.3 Kg. was injected intravenously with 1 cc. of acetone. The respiration rate increased rapidly. The animal collapsed and died two minutes after the injection.

From the above tests it appeared that acetone was not necessarily a reliable medium for intravenous injection of the extract. On the suggestion of Dr. A. L. Tatum, propylene glycol was used as the vehicle for the injection of the various fractions which were tested.

Inasmuch as it was necessary to know that the material being used was physiologically active, tests were carried out on the crude non-saponifiable material before a more complete chemical investigation was started. A solution of the non-saponifiable material in propylene glycol was prepared by adding 0.5 Gm. of the material to 10 cc. of the glycol and heating the mixture on a steam bath. The entire 0.5 Gm. of non-saponifiable material did not dissolve in the small amount of glycol used and the excess of material was removed. With solutions prepared in this way it was possible to determine the fractions which were physiologically active.

A rabbit weighing 2.1 Kg. was injected intravenously with 3 cc. of the filtrate obtained above. A slight swaying of the body and the

production of corneal anesthesia occurred within 5 minutes. The corneal anesthesia* lasted for 5 hours after which the corneal reflex returned. This indicated that the physiologically active principle or principles were present in the non-saponifiable residue.

Treatment with Methanolic Potassium Hydroxide Solution - A portion of the active non-saponifiable material amounting to 43 Gm., was tested to determine its reaction to the Beam test. A drop of the material was dissolved in 10 cc. of Skelly-solve B, 3 cc. of 2 per cent methanolic potassium hydroxide solution was added, and the mixture was agitated. A clear brown solution resulted. The addition of a few cc. of water caused the separation of two distinct layers; a brown Skelly-solve B layer on top and a deep purple aqueous-methanol layer on the bottom. The production of the purple color is considered a positive Beam test.

An attempt was made to separate the material producing the Beam test from the other constituents in the non-saponifiable material. Fraction 1 was dissolved in 1500 cc. of Skelly-solve and thoroughly mixed with 500 cc. of 2 per cent methanolic potassium hydroxide solution. This mixture was transferred to a large separatory funnel and sufficient water was added to cause the separation of the two layers as observed in the Beam test. Approximately 400 cc. of water were needed to cause this separation. The purple aqueous methanol layer was separated from the brown Skelly-solve solution and transferred to a second separatory funnel. The remaining Skelly-solve solution was treated

* Corneal anesthesia in this investigation was considered positive when it was possible to press a bristle of horse hair or a blunt glass rod against the cornea without producing the characteristic response.

with successive 50 cc. portions of 2 per cent methanolic potassium hydroxide solution and then with water until a purple color was no longer produced by such treatment. This indicated that the material producing the Beam test had been entirely removed from the Skelly-solve solution. The several aqueous methanol fractions collected from the above procedure were combined and washed with successive portions of Skelly-solve until the solvent was only slightly colored. (All of the Skelly-solve fractions were combined to form a composite solution.) Thus in the above procedure two composite solutions were obtained: (1) a purple aqueous methanolic solution of the material which produced the Beam test and (2) a Skelly-solve solution of the remaining constituents of the non-saponifiable material. The Skelly-solve composite was transferred to a tightly stoppered bottle and set aside. The investigation of the aqueous methanol solution is described in the succeeding paragraphs.

Investigation of the Aqueous Methanol Phase - This solution was made slightly acid by the addition of 10 per cent hydrochloric acid. The color of the solution changed from purple to yellow and on standing a brown oily layer collected on top of the solution. The oily layer was taken up in ether and separated from the aqueous layer. The aqueous solution was set aside. Water was removed from the ether solution by drying over anhydrous sodium sulfate after which the ether was recovered by distillation leaving a dark brown resinous material amounting to 6.2 Gm. This represents 14.41 per cent of the original non-saponifiable material.

The above resinous material was assayed to determine if it was physiologically active. A solution of this material was prepared by

adding 500 mg. of the material to 10 cc. of propylene glycol and warming the mixture on a steam bath. Intravenous administration in the doses given did not produce corneal anesthesia.

Test number 1

Weight of rabbit: 2.4 Kg. Dose: 2.4 cc.

Corneal Reflex present.

Test number 2

Weight of rabbit: 1.9 Kg. Dose: 2 cc.

Corneal Reflex present.

(These doses were comparable to those of the crude non-saponifiable material).

Attempt to Prepare a Derivative of the Non-active Material -

An attempt was made to prepare a dinitrobenzoate from the non-active material by the procedure described below. The 3,5-dinitrobenzoyl chloride was prepared by the addition of 4 Gm. of phosphorus pentachloride to 3 Gm. of 3,5-dinitrobenzoic acid and warming of the mixture slightly until the evolution of hydrochloric acid had ceased. The warm liquid was poured on a glass plate where it immediately solidified. The phosphorus oxychloride formed in the reaction was removed from the solid 3,5-dinitrobenzoyl chloride by absorption on a porous plate.

The non-active resinous material, amounting to 5.2 Gm. was dissolved in 25 cc. of anhydrous pyridine, and to this was added the freshly prepared 3,5-dinitrobenzoyl chloride. The mixture was heated on a steam bath with agitation for 30 minutes and then poured while

warm into 100 cc. of cold water. This was cooled for an hour in an ice-salt bath. The resulting flocculent precipitate was removed by filtration and recovered as a gummy mass. The traces of pyridine which were still present in the gummy material were eliminated by dissolving the material in 100 cc. of benzene and washing this solution with several successive portions of 10 per cent hydrochloric acid until the odor of pyridine was not evident. Hydrochloric acid was removed by washing the benzene solution with successive portions of a 10 per cent solution of sodium carbonate. The benzene solution was finally washed with distilled water. An attempt was made to obtain a crystalline product by chilling the benzene solution in an ice-salt bath for 12 hours. No solid material was formed. The solution was concentrated to half of its volume by distillation of about 40 cc. of benzene. The remaining solution was again chilled in the ice-salt bath for 12 hours but no solid material was obtained. Spontaneous evaporation of the remaining benzene on exposure to the air at room temperature resulted in a brown gummy residue. Solutions of the gummy material in several other volatile solvents were prepared and allowed to evaporate slowly in further attempts to obtain a crystalline product. Solvents used were ether, absolute alcohol, dioxane, methyl acetate, ethyl acetate, and methanol. No crystalline material was formed with any of these solvents. The resinous products resulting from the concentration and chilling of each of the above solutions were combined and set aside.

Since it had been indicated by the bioassays that the material producing the Beam test was not physiologically active, a more complete investigation of this material was not attempted.

Examination of the Skelly-solve B Solution of the Non-Saponifiable Material remaining after the Material producing the Beam Test had been removed. - Separation of a Red Oil - The combined Skelly-solve solutions were distilled at 50°C. under reduced pressure to recover the solvent. A red oil remained in the distilling flask. This was removed and dried in the vacuum oven. The weight of the residue was 28.8 Gm., representing 66.27 per cent of the non-saponifiable material.

Because of the fact that the residue from the aqueous-methanol phase was physiologically inactive, it appeared probable that the physiological activity could be traced to the red oil from the Skelly-solve solution. A solution of the oil, suitable for intravenous administration was prepared by adding 0.45 Gm. of the oil to 10 cc. of propylene glycol, warming the mixture on a steam bath, and then filtering. A clear yellow filtrate was obtained. This was administered intravenously to two rabbits, and the following data was recorded:

Test number 1

Weight of rabbit: 1.9 Kg. Dose: 2 cc.

Observations: Corneal anesthesia was evident within 5 minutes. A slight tendency for the animal to sway from one side to the other was observed.

The animal responded to a pinching of the tail or leg. The period of corneal anesthesia was approximately 6 hours.

Test number 2

Weight of rabbit: 1.8 kg. Dose: 2 cc.

Observations: Corneal anesthesia was evident within a

few minutes. The swaying of the body was not pronounced. Reflexes other than the corneal appeared normal. The duration of the corneal anesthesia was 5 to 6 hours.

Conclusions from the above tests - The above data indicated that the material remaining in the Skelly-solve solution possessed the principle or principles which caused corneal anesthesia in rabbits.

Solubility Tests on the Red Oil Residue from the Skelly-solve Solution - The relative solubility of the red oil in several organic solvents was determined with the purpose of finding a solvent in which it was only partially soluble.

Alcohol - 0.5 Gm. of the red oil was dissolved in 10 cc. of alcohol at room temperature. This was chilled in an ice-salt bath for 8 hours. A small amount of a flocculent precipitate formed during this period. The amount of precipitate was too small to recover and examine further.

Methanol - 0.5 Gm. of the oily material was dissolved in 10 cc. of methanol and cooled in the ice bath for 8 hours. A small amount of a dark green precipitate was obtained. This was removed and dried on a small piece of filter paper. It had a wax-like consistency and melted from 55° - 60°C.

Skelly-solve B - A solution of 0.5 Gm. of the oily material in 10 cc. of Skelly-solve B was chilled for 8 hours. No separation was effected.

Methyl Acetate - A solution of 0.5 Gm. of the red oil in 10 cc. of methyl acetate was chilled in the ice bath for 8 hours. The solution became cloudy but no separation occurred.

Acetone - 0.5 Gm. of the oil was dissolved in 10 cc. of acetone.

This solution was chilled for 8 hours resulting in the formation of a considerable quantity of slight green substance which was removed by filtration. The material was dried and found to possess a wax-like consistency very similar to that of the material obtained from the methanol solution. However, the amount was greater than that from the methanol solution.

The results of the above tests indicated that a solid material could be removed from the red oil by chilling an acetone solution of the oil. Of the solvents tested acetone appeared to be the most selective.

Separation of a Substance Insoluble in Cold Acetone - A sample of the red oil amounting to 25 Gm. was dissolved in 200 cc. of acetone. It was necessary to warm this slightly in order to obtain a clear solution. This was cooled in the ice bath for 8 hours to cause the precipitation of the insoluble material. This material was removed by filtration and washed with cold acetone. Concentration and further chilling of the mother liquor did not increase the yield. The melting point of the material after drying was 66°-69°C. A yield of 2.6 Gm. was obtained, which represented 10.4 per cent of the sample of red oil. The identification of the solid material was not attempted at this point because of the small amount of material obtained. The results of the investigation of a solid material obtained by a similar procedure is reported later in this thesis under the heading, Investigation of the Acetone-insoluble Portion of the Non-saponifiable Material (see page 57).

Bioassay on the Acetone-soluble Material - The acetone was recovered from the filtrate collected above and a residue of 20.5 Gm.

of reddish brown oil was obtained. A bioassay by the Gayer method upon this residue from the mother liquor indicated that the acetone soluble material was still physiologically active. A propylene glycol solution of the oil was prepared as has been described. A rabbit was injected intravenously with this solution and the following data was recorded:

Weight of rabbit: 2.2 Kg. Dose: 1.5 cc.

Observations: Corneal anesthesia was produced at once. The duration of action was about 5 hours. At times during this period the rabbit appeared to be hypersensitive to touch and sound.

Isolation of β -Sitosterol - As was indicated by the solubility tests which were made on the red oil previous to the removal of the acetone-insoluble material, it appeared probable that a substance insoluble in cold alcohol could be removed by chilling an alcoholic solution of the oil. The red oil amounting to 20 Gm. was dissolved in 100 cc. of alcohol by warming on a water bath. This solution was then chilled in an ice-salt bath for 16 hours resulting in the precipitation of a dark brown resinous mass which contained a quantity of light yellow crystals. The precipitate was removed by filtration, and a few of the crystals were separated mechanically. Microscopic examination showed them to possess a fine needle-like structure. The melting point was 137°C. By concentration and further chilling of the mother liquor an increase in the yield of the crystalline material was obtained. The precipitate was removed and the process repeated 3 times until no more solid material could be separated. The precipitates so obtained were combined and dissolved in 50 cc. of absolute alcohol. Several recrystallizations were necessary in order to obtain a pure product. The yield of the final product was

450 mg. melting at 137°C.

A positive Liebermann-Burchard test[14] and a positive Salkowski test[15] indicated that the crystalline material was a sterol. The melting point agreed with that recorded for β-sitosterol. A melting point of 136°-137°C. was recorded for β-sitosterol isolated from cottonseed oil by Wallis and Chakravorty,[16] while Gloyer[17] reported the melting point of β-sitosterol from rye germ oil at 137.0°- 137.5°C.

Optical Rotation of Crystals - 50.6 mg. dissolved in 2 cc. of chloroform and viewed in a 1 d. tube gave $\alpha_D^{23} - 0.94$; $[\alpha]_D^{23} - 37.1$. The recorded rotation of β-sitosterol is $[\alpha]_D^{25} - 36.6$.[4]

Preparation of β-Sitosterol Acetate - The acetate of β-sitosterol was prepared by refluxing 250 mg. of the sterol in 5 cc. of acetic anhydride for 1 hour. A jell-like mass of crystalline material formed when the mixture was cooled to room temperature. This was added to 25 cc. of water, thoroughly agitated, and allowed to stand for 2 hours to decompose the excess acetic anhydride. The crystalline material was removed by filtration, washed with water, and dried on a porous plate. The yield was 230 mg., melting point, 125°C. Recrystallization from absolute alcohol and then from methanol raised the melting point to 127°-128°C. This corresponds with the melting point of β-sitosterol acetate as recorded by Gloyer.[17]

Preparation of β-Sitosterol Benzoate - To prepare the benzoate, 0.1 Gm. of the sterol was dissolved in 3 cc. of anhydrous pyridine, and 1 cc. of benzoyl chloride was added. After the initial reaction the mixture was warmed for a few minutes and poured, with vigorous stirring, into 10 cc. of water. The precipitate was allowed to settle and the supernatant liquid was decanted. Five cc. of 5 per cent sodium

carbonate solution were added to the precipitate and the mixture thoroughly stirred and then filtered. The residue was washed with water and recrystallized from acetone. The melting point was 131°C. which was raised to 146° - 147°C. after several recrystallizations from acetone. This coincides with recorded melting point for β-sitosterol benzoate.[17]

After the isolation and identification of β-sitosterol from the non-saponifiable material, the investigation of the remaining mother liquors was continued. Traces of the sterol which could be precipitated by chilling of the mother liquor were removed by filtration. The solvent was recovered from the filtrate, and a red oily residue was again obtained. The oil was dried in a vacuum oven at 60°C. for 5 hours and was then tested for physiological activity. A solution of red oil was prepared by adding 80 mg. to 4 cc. of propylene glycol and heating on a steam bath. Intravenous injection of 1 cc. of this solution in a rabbit weighing 2.2 Kg. produced corneal anesthesia within 5 minutes. The duration of action was about 4 - 5 hours, after which the animal appeared normal.

In order to obtain a larger amount of the active red oil, the remaining crude non-saponifiable material was processed in a manner which was essentially the same as that employed in the initial investigation. Fifty Gm. of the solid non-saponifiable material were dissolved in 200 cc. of acetone with aid of gentle heat. The solution was chilled in an ice-salt bath for several hours. The resulting solid material was removed by filtration and dried. The yield was 4.1 Gm. representing 8.2 per cent of the non-saponifiable material. The melting point was 64° - 68°C. This material was

combined with similar material from the initial investigation. The examination is described in the following paragraphs.

Examination of the Acetone-insoluble Portion of the Non-Saponifiable Material - A sample of 14.4 Gm. of the solid material which had formed in the cooling of an acetone solution of the non-saponifiable material was added to 300 cc. of methanol and refluxed for 30 minutes. The solution was filtered, and the residue was treated in a similar way with an equal quantity of methanol. The final residue which remained after treating with methanol for a second time consisted of a hard, black, wax-like material. This weighed 7.3 Gm. It was set aside.

Isolation of a Hydrocarbon - The methanol solutions collected above were combined and cooled in an ice bath. A yellow flocculent precipitate was formed. This was removed and, when recrystallized from methanol, gave a melting point of 68^{0}-70^{0}C. Concentration and cooling of the mother liquors gave a further yield of solid material, which amount to a total yield was 5.84 Gm. This was dissolved in 50 cc. of purified petroleum ether and refluxed with 1 Gm. of activated alumina for 15 minutes. The alumina was removed by filtration, and a colorless filtrate was obtained. The solvent was recoverd, and the residue was purified by crystallization from a mixture of equal parts of glacial acetic acid and purified petroleum ether. Recrystallization of the material from chloroform and then from acetone gave a white flaky product melting at 69^{0}-70^{0}C.

Results of the following tests carried out on the crystalline material indicated that it was a paraffin:

1. Negative Liebermann-Burchard test.[14]
2. Negative Salkowski test.[15]

3. Not affected by either fuming sulfuric acid or fuming nitric acid.
4. A 5 per cent bromine solution in carbon tetrachloride was added from a dropping funnel to a carbon tetrachloride solution of the hydrocarbon. No decolorization of the bromine solution occurred.
5. A sample of the material was refluxed for 2 hours with acetic anhydride. The compound was recovered and its melting determined. No change in melting point had occurred.

Treatment with Methanolic Potassium Hydroxide Solution - The acetone was recovered from the mother liquor remaining after removal of the hydrocarbon, and a brown oily residue amounting to 45.3 Gm. was obtained. This oil gave a positive Beam test. The entire quantity was dissolved in 500 cc. of Skelly-solve B and treated with successive 50 cc. portions of 2 per cent methanolic potassium hydroxide solution and then with water until the Skelly-solve solution gave a negative Beam test. The aqueous-methanol solutions thus obtained were washed with Skelly-solve B. Addition of a few cc. of 10 per cent hydrochloric acid to the aqueous methanol solution rendered the solution acid to litmus and caused the formation of an oily layer on the top of the solution. This was taken up in ether and separated from the aqueous portion. Removal of the ether left 14.4 Gm. of a residue which appeared the same as that obtained from fraction 1 of the non-saponifiable material. Corneal anesthesia was not observed when two cc. of a saturated solution of the residue in propylene glycol were injected intravenously in a 2 Kg. rabbit. The remainder of the residue was

set aside.

The solvent was recovered from the Skelly-solve solution obtained above leaving an oily residue. After drying in a vacuum oven at 60°C. for an hour, 39.5 Gm. of the red oil remained. This material was assayed for physiological activity according to the Gayer test. In agreement with the observations made in the initial investigation this oil was found to possess the physiologically active principle or principles producing corneal anesthesia in rabbits. The solution used for the bioassay was prepared according to the method previously described using propylene glycol as the vehicle. Administration of 2 cc. of this solution to a 2 Kg. rabbit produced corneal anesthesia within 5 minutes.

Removal of β -Sitosterol - The sterol was removed from the oil by dissolving it in 100 cc. of absolute alcohol and chilling at -20°C. for 24 hours. The crystalline material which had formed during this time was removed by filtration and dried on a porous plate. The filtrate was concentrated to half its volume and chilled for another 24 period. The solid material formed was removed and added to the first fraction. A total yield of 490 mg. was obtained. Repeated recrystallizations from absolute alcohol gave a white product melting at 137°C. agreeing with the previous determination on β -sitosterol.

When no more solid material could be removed by further chilling, the solvent was recovered from the mother liquor and 28.7 Gm. of the oily residue remained. This was dried in a vacuum oven and tested for physiological activity. A rabbit weighing 1.9 Kg. was administered 2 cc. of a propylene glycol solution of the oil, and pronounced corneal anesthesia was observed within a few minutes.

The results of this investigation of the non-saponifiable material verified the results of the initial investigation. Based upon the data from this investigation, the calculated percentage yield of the active red oil from the crude drug amounted to 0.8 per cent.

Attempt to Prepare an Acetate from the Active Red Oil - One Gm. of the oil was mixed with 1 Gm. of powdered fused sodium acetate and 10 cc. of acetic anhydride. The mixture was heated on a steam bath for 2 hours, and poured with stirring into 100 cc. of ice water. After the mixture had been allowed to stand for 3 hours to allow for the hydrolysis of the excess acetic anhydride, an oily layer remained on top of the aqueous solution. The oily layer was dissolved in ether and allowed to evaporate slowly, resulting in a non-crystalline residue. Attempts to obtain the acetate were unsuccessful.

As has been mentioned in the introduction to this thesis, Cahn was successful in preparing an acetate which he identified as cannabinol acetate. Adams, et al, and Todd, et al, also attempted the preparation by similar procedures but were unable to obtain an acetate.

Attempt to Prepare a Dinitrobenzoate from the Active Red Oil - Todd, et al, succeeded in separating cannabinol as the p-nitrobenzoate, but the attempt by Adams to prepare a p-nitrobenzoate was unsuccessful. The separation of cannabinol by Adams was accomplished by preparing a 3,5-dinitrophenyl urethane from the cannabidiol-free oil. Since it was apparent that we had removed cannabidiol by the treatment with methanolic potassium hydroxide it seemed possible that cannabinol might be separated as the 3,5 dinitrobenzoate. - *

One Gm. of the oil was dried in the vacuum oven at 60°C. for 24 hours and mixed with 0.5 Gm. of dinitrobenzoyl chloride and 1 cc.

of pyridine. The mixture was heated on a steam bath for 1 hour. After cooling in an ice bath a small amount of crystalline material settled out. This was removed and identified as dinitrobenzoic acid (M.P. 202°C.)[18]. Excess dinitrobenzoyl chloride and pyridine was eliminated by treatment of the filtrates from above with 10 cc. of 5 per cent sodium bicarbonate solution. An oily layer was formed on top of the aqueous solution. This was dissolved in an ether solution which was allowed to evaporate slowly. A brown oily product was obtained. The product was dissolved in 10 cc. of alcohol and chilled. No solid material was formed. Concentration and further chilling of the solution failed to give a crystalline product. A similar procedure was employed using acetone as the solvent, but resulted only in a thick brown oil.

Chromatographic Adsorption of the Active Red Oil - An attempt was made to further purify and analyze the oil by the process of chromatographic adsorption. Two columns, each 1.5 cm. by 22 cm., were packed with activated alumina and moistened with purified petroleum ether. A solution of 2.5 Gm. of the active oil in 50 cc. of purified petroleum ether was run through each column very slowly. All of the colored material was adsorbed in the upper half of the columns. Three color bands were observed at this point; however, there was no distinct line of separation between the bands of the chromatogram. Development of the chromatogram into more distinct bands was accomplished by percolation of the columns with 100 cc. portions of purified petroleum ether. The developed chromatograms were removed from the columns and separated mechanically into the three differently colored fractions. Corresponding fractions from

the 2 columns were combined to give 3 composite fractions from the chromatograms. The first fraction or top layer was light brown; the second or middle layer was yellow, and the third or bottom layer was a bright orange. Each fraction was extracted and examined separately.

The petroleum ether solution which had passed through the columns were combined and evaporated to dryness to recover a possible fourth fraction of the non-adsorbed material. No residue was left after removal of the solvent indicating that all of the constituents of the red oil had been adsorbed on the column of activated alumina.

Examination of Fraction 1 - An eluant consisting of petroleum ether with 1 per cent of alcohol was used for extracting each fraction of the chromatogram. Fraction 1 was extracted with 50 cc. of the eluant. An orange colored solution was obtained, and the residual aluminum oxide was set aside. The solvent was recovered from the solution leaving .285 Gm. of a brown, wax-like residue. Bioassays of this residue gave a negative Gayer test in rabbits and failed to produce muscular incoordination in a dog at a dose of 10 mg. per Kg.

A 1 Kg. rabbit was injected intravenously with 2 cc. of a saturated solution of the above residue in propylene glycol. Corneal anesthesia was not produced.

A male dog weighing 11.5 Kg. was injected intravenously with 115 mg. of the above residue dissolved in 2 cc. of alcohol. None of the characteristic symptoms or reactions of cannabis was exhibited during the observation period of 8 hours. These tests indicated that fraction 1 of the chromatogram did not contain physiologically active material. Fraction 1 was set aside.

Examination of Fraction 2 - Fraction 2 was extracted and recovered by the same procedure as fraction 1. The yield of fraction 2 was 4.22 Gm. of a red oil. This material was found to be physiologically active.

A determination of the minimal active dose in rabbits was made on this material in order to compare its potency with that of the original red oil which had not been chromatographed. From the data recorded in table number 1 it had been found that the original red oil was active in a dose of 3 mg. per Kg. Table number 5 records the results of the bioassays upon the material obtained from fraction of 2 of the chromatogram.

Table Number 5

Determination of Minimal Active Dose of Fraction 2 of the Chromatogram.

Administered intravenously in propylene glycol.

Rabbit No.	Weight of Rabbit	Dose mg. per Kg.	Dose Administered	Results
1	1.0 Kg.	0.5 mg.	0.5 mg.	negative
2	1.1 Kg.	1.0 mg.	1.1 mg.	negative
3	1.1 Kg.	2.0 mg.	2.2 mg.	negative
4	1.0 Kg.	3.0 mg.	3.0 mg.	positive
5	1.2 Kg.	3.0 mg.	3.6 mg.	positive
6	1.1 Kg.	3.0 mg.	3.3 mg.	negative

It is apparent that this material was no more potent than the original red oil.

The oil from fraction 2 was tested upon a dog and found to produce

muscular incoordination in a dose of 10 mg. per Kg. administered intravenously. A dose of 115 mg. of the oil in 1 cc. of alcohol was injected into a dog weighing 11.5 Kg. The typical swaying motion and staggering walk were evident within 15 minutes. Normal posture and walk were not regained until about 6 hours after administration of the drug. Corneal anesthesia was not observed at any time during this period.

Examination of Fraction 3 - The extraction and recovery of a light red oil from fraction 3 was accomplished by the same procedure used on fractions 1 and 2. The yield of the oily material from fraction 3 amounted to .320 Gm.

A saturated solution of this material in propylene glycol was prepared in order to test it for physiological activity. Two bioassays upon rabbits were made and both gave negative results.

Rabbit No. 1 weighing 1.2 Kg. was injected with 2 cc. of the above solution. Corneal anesthesia was not produced. A second rabbit weighing 1 Kg. was administered 2 cc. of the solution without producing corneal anesthesia. It was concluded that the oily material from fraction 3 was inactive in the dose given.

Purification of Fraction 2 - Because of the fact that the bioassays had indicated that fraction 2 was the only active fraction in the chromatogram an attempt was made to further purify fraction 2 by chromatographing a second time. 3.5 Gm. of the oil were dissolved in 100 cc. of purified petroleum ether and passed through a column of activated alumina. After development of the column with 100 cc. of petroleum ether, the column was removed from the tube and separated into 3 sections. A band of brown material 0.5 cm. wide was separated

from the upper portion of the column, and a bright orange band 0.2 cm. wide was separated from the lower portion of the column. These portions were too small to be extracted and examined for physiological activity. This material was set aside. It seemed probable that the active material would be found in the central portion of the column. This portion of the column, corresponding to fraction 2 of the first chromatogram, was extracted with 100 cc. of petroleum ether containing 1 per cent of alcohol. Recovery of the solvent left a reddish orange colored oil amounting to 3.2 Gm.

A saturated solution of this oil in propylene glycol was prepared and found to produce corneal anesthesia with a dose of 2 cc. per Kg. Since this indicated that the active material had been adsorbed by the middle portion of the column, it was of interest to determine the minimal active dose of this material and compare it with the dose of oil which had not been chromatographed. The results of the determination of the minimal active dose are recorded in Table number 6. X Cap

Table Number 6

Determination of minimal active dose from Fraction Number 2 of the second chromatogram. Administered intravenously in propylene glycol.

Rabbit No.	Weight of Rabbit	Dose mg. per Kg.	Dose Administered	Results
1	1.0 Kg.	1	1 mg.	negative
2	1.0 Kg.	1	1 mg.	negative
3	1.2 Kg.	1	1.2 mg.	negative
4	1.0 Kg.	3	3 mg.	negative
5	1.1 Kg.	3	3.3 mg.	positive

Results of the bioassays from fraction 2 of second chromatagram indicated that the potency of the oil had not been increased to a measurable degree. A small loss of active material resulted, and a small amount of non-active pigmented material was eliminated.

It is apparent from a comparison of the results of the bioassays non-chromatographed oil with the bioassays of the chromatographed oil that chromatographic adsorption of the red oil did not increase the potency of the oil to a measurable degree. Chromatographic adsorption was of value in the removing of small amounts of non-active, pigmented material.

Table Number 1

Determination of Minimal Active Dose of Active Red Oil

Administered intravenously in a propylene glycol solution.

Rabbit No.	Weight of Rabbit	Dose per Kg.	Dose Administered	Results
1	1.2 Kg.	1	1.0 mg.	negative
2	1.0 Kg.	1	1.0 mg.	negative
3	1.7 Kg.	1	1.7 mg.	negative
4	2.2 Kg.	2	4.4 mg.	negative
5	2.8 Kg.	2	5.6 mg.	positive
6	2.2 Kg.	2	4.4 mg.	negative
7	1.7 Kg.	2	3.4 mg.	negative
8	1.7 Kg.	3	5.1 mg.	positive
9	1.8 Kg.	3	5.4 mg.	positive
10	1.7 Kg.	3	5.1 mg.	positive
11	2.6 Kg.	4	10.4 mg.	positive
12	2.0 Kg.	4	8.0 mg.	negative
13	2.8 Kg.	4	11.2 mg.	positive
14	3.0 Kg.	5	15.0 mg.	positive

In the above tests a response was considered to be positive when it was possible to press a blunt tipped glass rod against the cornea without causing the characteristic response. Whenever the corneal reflex was normal or appeared to be only slightly depressed, the result was considered negative.

From the above data, it was concluded that the minimal active dose

by intravenous administration in propylene glycol was approximately 3 mg. per Kg.

Effect of Oxidation upon the Active Red Oil - From the report of Haagen-Smit[20] it appeared probable that the principle which produced corneal anesthesia in rabbits was not the same as that which caused the typical muscular coordination observed in dogs. The method of preparation of the material used by Haagen-Smit and coworkers was entirely different from that employed in this investigation. Because of this fact it was of interest to compare the effect of oxidation of the active red oil obtained in this investigation with the effect of oxidation of the material used by Haagen-Smit. The method of oxidation employed in this investigation was similar to that described by Haagen-Smit, which consisted essentially of bubbling a current of air through an alcoholic extract of the drug for 5 hours at a temperature of about 50°C.

Determination of the Minimal Active Dose Orally - Before treatment of the active oil with a current of air, it was necessary to determine the minimal active dose by oral administration. This was determined by administration through a stomach tube of alcoholic solutions of increasing strengths of the active red oil. The amount of alcohol given to each rabbit varied from 2 to 3 cc. From the data recorded in Table number 2, it was concluded that 35 mg. per Kg. of body weight was the minimal active dose.

Table Number 2

Determination of Minimal Active Dose
of the Active Red Oil
Administered orally in an Alcoholic Solution.

Rabbit No.	Weight of Rabbit	Dose Mg. per Kg.	Dose Administered	Results
1	1.8 Kg.	10	18 mg.	negative
2	1.0 Kg.	10	10 mg.	negative
3	1.2 Kg.	10	12 mg.	negative
4	1.0 Kg.	20	20 mg.	negative
5	1.7 Kg.	20	34 mg.	negative
6	2.2 Kg.	20	44 mg.	negative
7	1.1 Kg.	25	25 mg.	negative
8	1.3 Kg.	25	32.5 mg.	negative
9	1.2 Kg.	25	30 mg.	negative
10	1.0 Kg.	30	30 mg.	negative
11	1.2 Kg.	30	36 mg.	negative
12	1.2 Kg.	30	36 mg.	negative
13	1.7 Kg.	30	51 mg.	positive
14	1.1 Kg.	30	33 mg.	negative
15	1.1 Kg.	30	33 mg.	positive
16	1.2 Kg.	30	36 mg.	negative
17	1.0 Kg.	35	35 mg.	positive
18	1.0 Kg.	35	35 mg.	positive
19	1.0 Kg.	35	36 mg.	positive

Treatment of the Active Material with a Current of Air - A sample of 6.5 Gm. of the oil were dissolved in 75 cc. of alcohol in a flask fitted with a reflux condenser and a capillary tube extending to the bottom of the flask. A current of air was allowed to bubble through the solution while refluxing for 8 hours. The alcohol was then recovered leaving the oily residue. The oral dose of this residue required to produce corneal anesthesia was again determined. The data is recorded in Table Number 3.

Table Number 3

Determination of Minimal Active Dose after Treatment with a Current of Air Administered in alcoholic solution.

Rabbit No.	Weight of Rabbit	Dose mg. per Kg.	Dose Administered	Results
1	1.3 Kg.	35	45 mg.	negative
2	1.0 Kg.	35	35 mg.	positive
3	1.0 Kg.	35	35 mg.	positive
4	1.1 Kg.	35	39 mg.	negative
5	1.2 Kg.	35	42 mg.	negative
6	1.2 Kg.	35	42 mg.	negative
7	1.2 Kg.	40	48 mg.	positive
8	1.1 Kg.	40	44 mg.	positive
9	1.2 Kg.	40	48 mg.	positive

The degree of activity of the oil was not significantly lowered by treatment with a current of air. The slight difference in activity noted above is entirely within the range of animal variation.

Treatment of the Oil with Oxygen - The oil which remained from the above procedure was dissolved in 75 cc. of alcohol and refluxed as before for 5 hours with a current of oxygen. The alcohol was recovered and the minimal active dose of the residual oil was determined.

Table Number 4

Determination of Minimal Active Dose after Treatment with Oxygen Administered orally in alcohol.

Rabbit No.	Weight of Rabbit	Dose mg. per Kg.	Dose Administered	Results
1	1.2 Kg.	40	48 mg.	negative
2	1.2 Kg.	40	48 mg.	negative
3	1.0 Kg.	40	40 mg.	negative
4	1.0 Kg.	45	45 mg.	positive
5	1.2 Kg.	45	54 mg.	positive
6	1.4 Kg.	45	63 mg.	negative

A slight decrease in activity is evident from the above data. However, it appears that the material was fairly stable to treatment with air or a current of oxygen using the procedure employed in this investigation. The results recorded here are in contrast to those of Haagen-Smit and coworkers, who observed that treatment with air caused their material to entirely lose its corneal anesthetic activity.

A rabbit weighing 1 Kg. was injected intravenously with 8 mg. of the oil dissolved in 2 cc. of propylene glycol. Corneal anesthesia was observed within 5 minutes. This agreed with the observations made above when the material was administered orally.

Determination of the Effect of the Red Oil upon a Dog - A male dog, weight 11.5 Kg., was injected intravenously with 115 mg. of the oil which had been found to be active when administered to rabbits. The oil was dissolved in 1 cc. of alcohol for injection. The following observations were recorded:

Time	Observations
10:15 A.M.	- Time of administration of the drug.
10:20	- The animal developed a swaying motion with a tendency to fall forward.
10:40	- The swaying movements became more pronounced. A staggering walk was observed. Slight noise or touching of the dog caused the dog to exhibit signs of hypersensitivity. Corneal anesthesia was not produced.
11:00	- No apparent change was observed.

The dog was observed several times during an 8 hour period. At all times he was very inactive and appeared to be in a stupor. By 8:00 P.M. the dog had recovered his normal walk and posture and appeared normal.

Determination of the Effect of the "Oxidized Red Oil" upon a dog - Cap 115 mg. of the red oil which had been treated with air and oxygen was administered intravenously. The oil was dissolved in 2 cc. of alcohol for injection. Ten minutes after the injection the dog developed a swaying motion which lasted for about 2 hours. During this time the animal experiencedconsiderable difficulty in walking. Its gait was

very uneven and jerky. After about 3 hours the dog appeared to be in a stupor. This stuporous condition lasted for about 10 hours before the dog again acted normal. These observations indicated that the red oil was still physiologically active in dogs as well as rabbits. Treatment of the red oil by a current of air and a current of oxygen had little if any effect upon its physiological activity.

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Conclusions - An analysis of the fatty acids resulted in the isolation and identification of linolenic and linoleic acids. Small amounts of saturated fatty acids were present. From the physical properties of the material isolated it appeared probable that they consisted chiefly of palmitic and stearic acids.

The non-saponifiable material was found to consist of a paraffin hydrocarbon melting at 69°-70°C., a sterol, isolated and identified as β-sitosterol, and a physiologically active "red oil".

It was possible to remove cannabidiol from the "oil" by treatment with methanolic potassium hydroxide solution. Other non-active and pigmented constituents were eliminated by chromatographic adsorption on activated alumina. By these processes a partial purification of the physiologically active material was accomplished.

Propylene glycol was found to be a satisfactory vehicle for the intravenous administration of the material. This vehicle does not exhibit the depressant or narcotic activity characteristic of acetone, the vehicle, which has been used by other workers.

In contrast to the findings of Haagen-Smit and coworkers, it was found that the active "red oil" was relatively stable to the action of air or oxygen, and that treatment of an alcoholic solution of the "red oil" with air or oxygen did not result in a loss of the corneal anesthetic activity.

The minimal intravenous dose of the purified "red oil" for the production of corneal anesthesia in rabbits was found to be 5 mg. per Kg. Oral administration of the active material required 35 mg. per Kg. to

produce corneal anesthesia in rabbits.

Intravenous administration of 10 mg. per Kg. of the purified oil was sufficient to produce muscular incoordination in dogs but did not produce corneal anesthesia in dogs.

APPROVED BY: R. A. Kuhl

Professor of Pharmaceutical Chemistry

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