REPORT OF THE MARIHUANA INVESTIGATION.*

(SUMMER OF 1937.)

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With the recent passage of the Marihuana Act, controlling the growth and traffic of the plant Cannabis sativa, there fell upon the Bureau of Narcotics, of the United States Treasury Department, the responsibility of assembling all significant data based upon which competent administration of the law might obtain.

In order that this Bureau might be in full possession of all pertinent information it was decided to plant a plot of ground and observe the growing of the plant and related phenomena. It was considered that, inasmuch as the Marihuana Act provided not only for the administration and control of legitimate agriculture but also for the stamping out of illicit agriculture and traffic in the plant, it would be appropriate to gather information based upon which the most effective pursuit of criminal activities might obtain. To assist in accomplishing these ends an extensive comparative study was also made of the findings of experts, including those who, in the past, had been consultants to the Health Committee of the League of Nations.

The various tests which had hitherto been found at least partially effective in the detection of the plant Cannabis sativa were applied to the crop obtained by this planting throughout its various stages of growth. This was done in an effort partly to determine the time and nature of development of the active principles in the plant and, in part, to gage the effectiveness of such control measures as proper administration of this law would require.

Early in the investigation it was discovered that the chemical methods hitherto developed and proposed for the identification of this plant were not universally effective. This factor, arising early in the investigation, compelled those engaged upon the research to partially modify their approach to the problem.

In submitting this report, therefore, it is desirable to stress the point that whereas the original purpose of the investigation, and the basis upon which it was planned, was a study of the growth of the plant and the time of appearance therein of those components which collectively are referred to as active principles, nevertheless, other factors having arisen during the progress of the research, the ultimate results obtained appear to challenge the previously proposed methods of analysis when used for identification purposes.

ORIGINAL PURPOSE OF THE INVESTIGATION.

The purpose of the investigation here reported originally was as follows:

1. To observe the growth of the plant Cannabis sativa.
2. To determine the time of appearance and disappearance in the plant of those substances responsible for the alkaline and acid Beam tests.
3. To determine whether or not the substances referred to exist in all parts of the plant and at all stages of growth.
4. To determine whether all plants, including male and female, grown in the same plot and dried under the same conditions, respond at each stage of growth to the alkaline and acid Beam tests.
5. To determine the effect of rapid drying on the capacity to respond to the alkaline and acid Beam tests.

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* United States Treasury Department, Bureau of Narcotics, in coöperation with the Department of Agriculture.

UNEXPECTED DEVELOPMENT.

Soon after the above program was initiated it was discovered that, contrary to expectation, Cannabis sativa plants were found which did not react to known chemical methods of identification. It was therefore concluded, as will be later developed in greater detail, that known chemical methods of testing could not be employed to observe certain of the Cannabis plants and their generation of active principle in their various stages of growth.

REVISED PURPOSE OF THE INVESTIGATION.

Under these circumstances the emphasis of the investigation was shifted and an effort was made to determine, if possible, what data could be gathered from an observation of those plants which gave tests as compared with those that did not. The investigation from this point onward was, therefore, broadly grouped under the following headings:

1. To observe the growth of the plant Cannabis sativa.
2. To standardize the method of testing the plant Cannabis sativa.
3. To observe statistical relationships, if any, obtaining between those plants which gave "positive" tests by chemical methods, and those plants which gave "negative" tests.
4. To determine the statistical relationship, if observable, between male and female plants which reacted to chemical tests.
5. To observe the time of occurrence in male and female plants of those compounds which caused "positive" reaction to chemical tests.
6. To determine the effect on the development of "positive" tests of variations in the drying of the plants.

The data developed in carrying through the above program required four months of activity by three chemists, with the occasional assistance of technologists of the Department of Agriculture. There were performed at least 3000 individual chemical tests. The farm was visited weekly and observations made. The climatic data was, of course, obtained from the Weather Bureau.

OBSERVATION OF THE GROWTH OF THE PLANT CANNABIS SATIVA.

On May 12, 1937, a plot of ground 130 feet long and 15 feet wide was selected in the lowland, near the Potomac River, of the Arlington (Virginia) Experimental Farm of the United States Department of Agriculture. Seeds of a Kentucky variety, usually cultivated for fiber, were planted in six rows, three feet apart. The young plants were not artificially thinned.

Growth during the early season was exceedingly rapid. The chronology of growth was as follows:

May 12, 1937	Seed sown
By May 17, 1937	Plant appeared above ground
By June 15, 1937	Height was $1^1/_2$ feet
By July 2, 1937	Height was 6 feet
By July 9, 1937	Height was 7 feet
By July 16, 1937	Height was 10 feet
By July 23, 1937	Height was 12 feet
By August 15, 1937	Height was from 13 to 15 feet and all male plants could be distinguished.
By September 1, 1937	The fruit had appeared and males began to wither.
By October 1, 1937	The male plants had been reduced to bare stalks, retaining, however, portions of their flowering tops.

On October 25, 1937, when the leaves were mostly gone and the stalks were more readily visible, it was found that the plot contained approximately 3100 plants of which approximately 1900 were females, and approximately 1200 were males.

ESTIMATION OF YIELD OF FLOWERING TOPS.

Since the plants in the outside rows branched much more freely than those in the inside rows it is necessary to consider them separately for the purpose of estimating the yield of female flowering tops. It was found that the tops (including fruits) from 2 female plants in the outside rows weighed a total of 660 Gm. when wet and 350 Gm. when dried. The tops (including fruits) from 4 female plants in the inside rows weighed a total of 630 Gm. when wet and 310 Gm. when dry. Since the inside rows contained two-thirds of the plants, and the outside rows one-third, the yield from the plot is estimated as 400 Kg. of tops (including fruits) when wet, and 200 Kg. when dry.

STANDARDIZATION OF METHOD OF TESTING.

Choice of Method.—After examination and laboratory comparison of most of the chemical methods hitherto proposed for the identification of the plant (including those circularized through League documents) it was found that none yielded results of greater accuracy than the one finally adopted in this investigation.

In this respect it is worthy of note that none of the other methods examined ever gave a "positive" test in a plant which tested "negative" by the adopted procedure. In addition it is essential to note that no procedure so far employed was universally effective.

The Method of Testing.—The test used in the investigation, details of which appear below, was developed in this laboratory subsequent to the observation that hitherto proposed Beam tests, and modifications thereof, are apparently adversely affected by inert colored substances extracted from the plant by petroleum ether. Preliminary experiments revealed that such substances could be removed by adsorption on Norit (decolorizing carbon) but that in petroleum ether the carbon also removed from solution the substances which respond to the tests. In further experiments it was found that the desired compounds could be released from the carbon by washing with ethyl alcohol. This led to the conclusion that alcohol could be employed as the extracting solvent. Ethyl alcohol, however, in addition extracts a very large amount of colored material. A search was therefore instituted for a solvent which would extract comparatively little color and from which the color could be removed by adsorption on Norit without sensible loss of the compounds reacting to the Beam test. Ethyl acetate proved to be such a solvent and was consequently employed in the test finally developed.

The Method of Testing Ultimately Adopted.—1. A sample averaging 1 to 6 Gm. (depending on which part of the plant was tested) was crushed in a 250-cc. beaker and covered with 50 cc. of pure ethyl acetate.

2. After extracting a few minutes two 10-cc. portions were withdrawn through a filter.

3. One portion was divided equally between two white porcelain dishes and the solvent evaporated from each on a steam-bath before a fan.

4. The second 10-cc. portion was shaken for a moment with $^1/_2$ Gm. of Norit (decolorizing carbon) and filtered. The colorless filtrate was divided between two white porcelain dishes and the solvent evaporated from each on a steam-bath before a fan.

5. One of the resinous residues obtained in step 3 and one obtained in step 4 were treated with Beam's acid reagent (a saturated solution of hydrogen chloride in absolute alcohol) and the color produced observed.

6. The other residues obtained in steps 3 and 4 were treated with a few drops of Beam's alkaline reagent (2% alcoholic potassium hydroxide) and the color produced observed.

Comment on Method.—It was found in many cases that Norit treatment was not necessary for "positive" alkaline test response. However, when the acid test was employed no instance of a "positive" response was found in the absence of Norit treatment.

It was found that the above outlined method of testing was exceedingly sensitive when compared with other similar procedures. As little as 5 mg. of crushed marihuana gave a definite color with the alkaline reagent when extracted with 3 cc. of solvent and shaken with 10 mg. of Norit.

This method was compared with those involving extraction with petroleum ether. It was found that petroleum-ether extracts did not give "positive" reaction in plants which gave "negative" reaction on ethyl acetate extracts.

The presence of organic peroxides in the solvent did not appear to affect the reacting of the extracts.

Considered from all points of view the procedure involving extraction with ethyl acetate, followed by decolorization, appears preferable to that employing petroleum ether.

STATISTICAL RELATIONSHIP BETWEEN "POSITIVE" AND "NEGATIVE" PLANTS.

As indicated earlier in this report it developed that individual plants, though grown side by side, varied greatly in their capacity to respond to the above tests. Many responded in widely varying degree to both tests, a considerable number responded to only one, while still others responded to neither.

The problem was attacked as follows:

Each week 10 or more plants were brought to the laboratory and dried in the air for four days at room temperature (Circa 30° C.). A portion of the top or top leaves from each plant was subjected to both tests. Those which reacted strongly to the alkaline test were designated as "positive" and those which failed to react, or reacted exceedingly faintly, were designated as "negative."

One plant of each category was selected and broken into its various parts as will be explained below. Each part of each plant was then tested separately by both the acid and the alkaline tests.

Below are listed the various parts into which the plants were divided for the purpose of determining which respond to the test. The complete subdivision was, of course, dependent on maturity. Each subdivision was added as the part in question appeared during growth and, of course, deleted after it was no longer on the growing plant. For example, the lower leaves blew off early in the decadence of the plants.

1. Tops
2. Tops of branches
3. Upper leaves
4. Lower leaves
5. Upper stalk
6. Lower stalk
7. Stems of branches
8. Flowers (of male plants)
9. Bracts
10. Fruits
11. Roots

The attached diagram (page 33) indicates where these designations apply.

In every case where plants were found to be "positive" by the preliminary alkaline test subsequent examination of each of the subdivisions developed a "positive" alkaline test in each of such subdivisions excepting the pith, lower stalk and root.

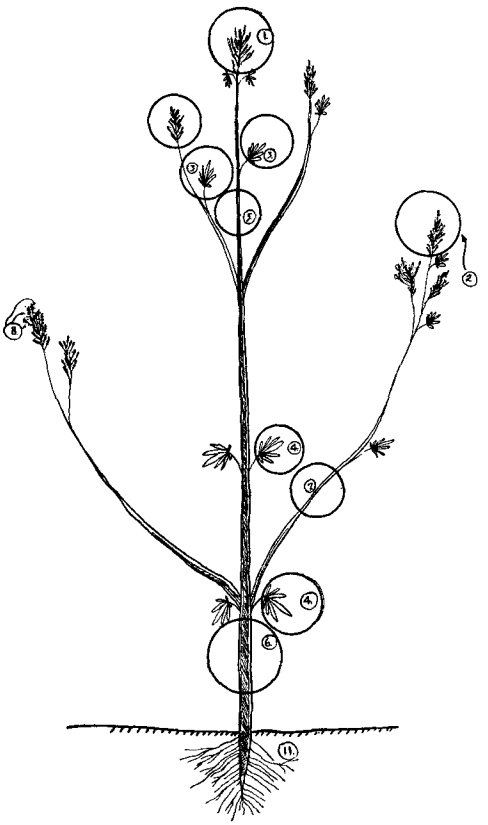
In every case where plants were found to be "negative" by the preliminary alkaline test subsequent examination of each of the subdivisions developed a

"negative" test in each of such subdivisions with, however, slight traces to be found in the upper leaves or tops.

It is to be understood, of course, that the separation into purely "positive" and purely "negative" plants is a highly empirical one and that quite a number were found between the absolutely "positive" and the absolutely "negative."

During the whole course of the research 241 plants of various ages were found to respond as follows:

120 gave both alkaline and acid tests
56 gave only the alkaline test
33 gave only the acid test
32 gave neither.



DIAGRAMMATIC SKETCH OF PLANT CANNABIS SATIVA.

1. Tops
2. Tops of branches
3. Upper leaves
4. Lower leaves
5. Upper stalk
6. Lower stalk
7. Stems of branches
8. Flowers of male plants*
9. Bracts (not shown)
10. Fruits (not shown)
11. Roots.

* These are only present on male plants but are included herewith for demonstration of position. Where flowers of male plant are referred to in report, and where tested, co-mingled leaves were removed. Where male flowering tops were referred to the entire top was tested.

Although the designation, by virtue of which plants were classified as "positive" and "negative," was made on the basis of the alkaline test, the acid test was also applied to each separate subdivision.

It appears that, where the acid test was "positive" in the tops, a corresponding "positive" acid test was generally obtained in the other subdivisions of the plant excepting, again, the pith, lower stalk and roots. *Not necessarily all of the several parts of any one plant would be "positive" in its reaction.* However, after all of these tests had been completed, it was found that each of the above enumerated parts, excepting the pith, lower stalk and roots, had yielded a "positive" test in one plant or another.

DISTRIBUTION OF "POSITIVE" AND "NEGATIVE" PLANTS WITH RESPECT TO SEX.

Examination of 108 plants of known sex revealed no relationship between sex and capacity to respond to the test. Of 41 males 27 (or 66%) were designated as "positive" and 14 (or 34%) as "negative." Of 67 females 44 (or 66%) were found "positive" and 23 (or 34%) "negative."

These figures show that there are as many "positives" in proportion to "negatives" in the males as in the females. There can also be concluded from these figures that the concentration of reactive material in the males is as great as that in the females for, inasmuch as the test is empirical, if it had been otherwise a different ratio would have obtained.

TIME OF OCCURRENCE IN THE VARIOUS PARTS OF MALE AND FEMALE PLANTS OF THE COMPOUNDS WHICH CAUSE "POSITIVE" REACTION.

Subject to the limitations referred to in the foregoing discussion, the capacity of the Cannabis sativa under observation to react to the acid and alkaline Beam tests has been determined to be as follows:

1. Dry, old fruits do not give either alkaline or acid test.
2. Tiny plants, one inch above ground, do not respond to either test.
3. Some plants, 3 inches above ground, give both tests. The plants at this stage are too small to be tested separately.
4. From this time on both tests may be obtained from all parts of both male and female plants except the pith, lower stalk and roots. The strongest tests are given by the upper parts of the plant.
5. Capacity on the part of the male plants to give both tests disappears gradually as the mature plant disintegrates, disappearing last from the dried flowers which cling tenaciously after most of the leaves have blown away.
6. Capacity on the part of the female plants to give both tests increases in the flowering tops as maturity approaches. After the fruits are formed the flowering tops of all female plants respond in some degree to the alkaline test even though no other part of the plant may react "positive." This stage of development is reached at the time the male plants are disintegrating.
7. After the fruits are mature, capacity to respond to the tests disappears in the upper stalk.
8. Flowering tops give both alkaline and acid "positive" tests even after heavy frost.

EFFECT OF RAPID DRYING PROCEDURE UPON THE RESPONSE OF CANNABIS SATIVA PLANTS TO THE BEAM TEST.

For the purpose of this test 41 plants, the sex of some of which could be recognized, were selected. A portion of each (top of branches or top leaves) was heated in an oven at 100° C. in a current of air at the same temperature. These portions were removed from the oven after intervals of from 1 to 5 hours, as indicated in the accompanying table, and then tested.

The remaining parts of these same plants were dried in the air for four days after which a portion of their tops was tested.

Of the air-dried parts of plants 34 responded "positively" in varying degree to the alkaline test. Of the oven-dried parts of plants 39 responded "positively" in varying degree to the alkaline test. All of the male plants responded in varying degree both when air-dried and when oven-dried. In no case was capacity to respond to the test impaired by the heat treatment. The oven-dried parts of plants, on the contrary, gave stronger tests than the air-dried parts. Those which are shown in the table as "positive trace" have been referred to in other sections of this report as "negative" plants. The reason for our designating those very slight traces as "positive" in this phase of the investigation is based upon the fact that we were here looking for tendencies which a process of drying might have upon increasing or decreasing the reactivity of the plant. In the estimation of this laboratory such slight traces would not be sufficient justification for embarking upon criminological procedure and court action. However, for the purpose of scientific investiga-

tion they must needs be taken into account. These small traces fall within the category of bare indications of a "positive" Beam test.

RESPONSE OF 13- AND 14-WEEK OLD PLANTS TO ALKALINE BEAM TEST BEFORE AND AFTER HEATING IN A CURRENT OF AIR AT 100° C.

Plant No.	Sex	Time Heated.	Response Before.	Response After.
2	Male	1 hour	"Positive"	"Positive"
4	Male	1 hour	"Positive"	"Positive"
11*a*	Male	1 hour	"Positive"	"Positive"
12*a*	Female	1 hour	"Positive"	"Positive"
13*a*	Female	1 hour	"Positive"	"Positive"
14*a*	Female	1 hour	"Positive"	"Positive"
15*a*	Female	1 hour	"Positive"	"Positive"
1	Unknown	1 hour	"Positive"	"Positive"
3	Unknown	1 hour	"Positive"	"Positive"
5	Unknown	1 hour	"Positive"	"Positive"
12	Male	2 hours	"Positive"	"Positive"
14	Male	2 hours	"Positive" trace	"Positive"
1*a*	Male	2 hours	"Positive"	"Positive"
2*a*	Female	2 hours	"Positive" trace	"Positive"
3*a*	Female	2 hours	"Positive" trace	"Positive"
4*a*	Female	2 hours	"Positive" trace	"Positive"
5*a*	Female	2 hours	"Positive"	"Positive"
11	Unknown	2 hours	"Positive" trace	"Positive"
13	Unknown	2 hours	"Positive"	"Positive"
15	Unknown	2 hours	"Positive" trace	"Positive"
16	Male	3 hours	"Positive"	"Positive"
17	Male	3 hours	"Positive"	"Positive"
20	Male	3 hours	"Positive"	"Positive"
19*a*	Male	3 hours	"Positive" trace	"Positive"
16*a*	Female	3 hours	"Positive" trace	"Positive"
17*a*	Female	3 hours	"Negative"	"Positive"
18*a*	Female	3 hours	"Negative"	"Positive"
18	Unknown	3 hours	"Negative"	"Negative"
19	Unknown	3 hours	"Positive"	"Positive"
21	Unknown	3 hours	"Negative"	"Negative"
20*a*	Unknown	3 hours	"Negative"	"Positive"
7*a*	Male	4 hours	"Positive" trace	"Positive"
9*a*	Male	4 hours	"Positive"	"Positive"
6*a*	Female	4 hours	"Positive" trace	"Positive"
8*a*	Female	4 hours	"Negative"	"Positive"
10*a*	Female	4 hours	"Negative"	"Positive"
10	Male	5 hours	"Positive"	"Positive"
6	Unknown	5 hours	"Positive"	"Positive"
7	Unknown	5 hours	"Positive"	"Positive"
8	Unknown	5 hours	"Positive"	"Positive"
9	Unknown	5 hours	"Positive"	"Positive"

Two samples of decolorized resin, produced in the course of analysis, were divided and a portion of each heated four hours at 100° C. in a current of air at the same temperature. Response to the Beam tests was not affected. Both samples reacted "positive" to the alkaline test both before and after the heat treatment. One reacted slightly to the acid test before and after heating, and the other reacted "negative" before and after heating.

	Alkaline Test.		Acid Test.	
Sample.	Before Heat.	After Heat.	Before Heat.	After Heat.
A	"Positive"	"Positive"	Slightly "Positive"	Slightly "Positive"
B	"Positive"	"Positive"	"Negative"	"Negative"

SUMMARY.

A survey based upon observation of the growth of three thousand plants of Cannabis sativa in one area has been completed. From this survey the following major conclusions may be drawn:

1. That the alkaline Beam test, as employed and elsewhere described in this report, only gave a "positive" reaction on two-thirds of the plants.
2. That the proportion of male plants reacting "positive" to the alkaline Beam test is the same as the proportion of female plants.
3. That at no time during the growth of the plant was "positive" alkaline or acid Beam test to be obtained from the pith, lower stalk or roots.
4. That plants as small as three inches above ground have the capacity of giving the alkaline Beam test.
5. That the alkaline Beam test and the acid Beam test may result from more than one compound, or may be affected by the presence of other inhibiting compounds which result in a non-uniformity in the degree to which both tests are obtained.
6. That the dried, old fruits give neither test.
7. That neither the alkaline or acid Beam test, either as hitherto proposed or as developed to date, offer any assurance as means of identification from a criminological viewpoint.

PARA-AMINOBENZENESULFONAMIDE.*

NOTES ON THE COLORIMETRIC ASSAY.

BY ASA N. STEVENS AND EDWARD J. HUGHES.[1]

Marshall, Emerson and Cutting (1) have recently published an excellent method for the determination of para-aminobenzenesulfonamide in urine. This is accomplished by using dimethyl-α-naphthylamine to produce a red color which may be estimated quantitatively in a colorimeter after diazotization with sodium nitrite.

Because of the presence of a reddish color in our available supply of dimethyl-α-naphthylamine, and also in a newly obtained sample of this reagent, we found it impossible to make a satisfactory colorimetric determination until this color had been removed by means of fractional distillation.

It is therefore our custom, in all cases where the dimethyl-α-naphthylamine is not of a clear straw color, to provide freshly distilled reagent as an essential part of the colorimetric procedure.

We have found also that it is exceedingly important to use no more than the prescribed 1 cc. of 0.1 per cent sodium nitrite solution for the diazotization, in order to avoid the development of a brown color which gives low results. Furthermore it has been our experience that although the color produced by para-aminobenzenesulfonamide in pure water is stable for several hours, the color produced in urine reaches its maximum intensity in about five minutes and should be compared with a suitable standard within ten minutes. Longer standing tends to produce an orange color which yields lower results.

* Scientific Section, A. PH. A., New York meeting, 1937.

[1] Control Laboratories, Eli Lilly and Company.