STUDIES IN THE BIOSYNTHESIS OF DITERPENOID ALKALOIDS

By

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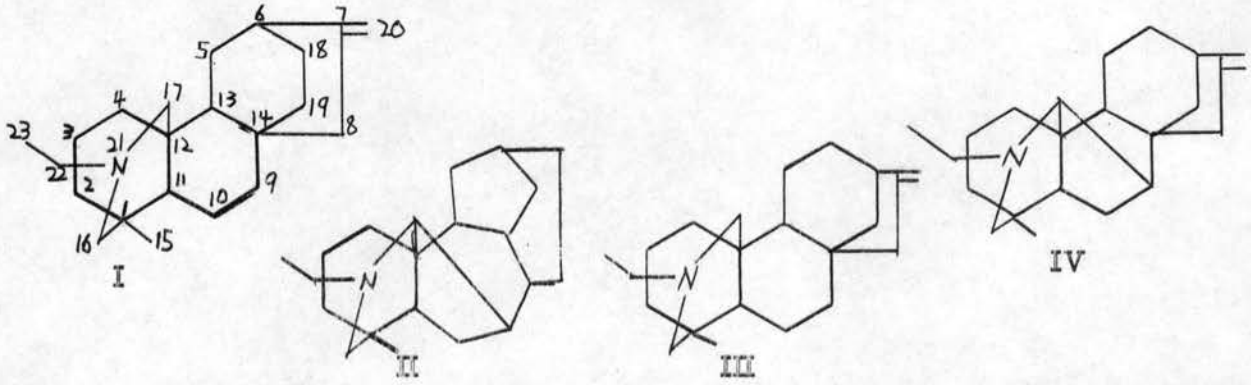
HISTORICAL AND INTRODUCTION

The many species of the genus Delphinium, a member of the plant family Ranunculaceae, have been the subject of numerous chemical investigations. This widespread interest was originally due to the plant's insecticidal properties. Later research was stimulated by the discovery that several species of Delphinium indigenous to the United States were responsible for the extensive poisoning of cattle in the western states.[1] These investigations led to the isolation of a number of complex alkaloids which are generally assumed to be derivatives of tetra- or pentacyclic diterpenes.

The occurrence of diterpenoid alkaloids is not restricted to the Delphinium genus. Similar bases have been found in the related genus Aconitum of the same family, the genus Garrya of the family Cornaceae, and in one species of the genus Inula in the family Compositae. In an extensive review, Boit[2] has given the source and physical properties of a large number of diterpenoid alkaloids isolated prior to 1960. The alkaloids may be classified as four distinct structrual types (I, II, III and IV), representatives of which are atisine, lycoctonine, veatchine, and napelline, respectively.



In recent years, during which much of the chemistry and structural relationships of these alkaloids have been elucidated, interest has increasingly been focused upon the mode of formation of these complex substances in the plant, that is, their biosynthesis. The structures of these alkaloids are strongly suggestive of a terpenoid origin. If such is the case the bases would be expected to be produced, in vivo, from acetate via the mevalonate pathway (Chart I) so well established for non-alkaloidal terpenes.[3] The mevalonic acid thus formed undergoes a series of phosphorylations and loses the carboxyl[4] carbon to form the biological isoprene unit (Chart II) which has been shown by Block's[5,6] group and by Lynen's[7] group to be Δ^3-isopentenyl pyrophosphate. As shown in Chart III, isomerization of Δ^3-isopentenyl pyrophosphate to dimethallyl pyrophosphate by an enzyme containing an -SH group leads to a carbonium ion-type polymerization of the biological isoprene unit. The combination of two units gives geranyl pyrophosphate, the precursor of the monoterpenes. Addition of a third unit gives farnesyl pyrophosphate which may give rise to the sesquiterpenes or may dimerize to give squalene which in turn may cyclize

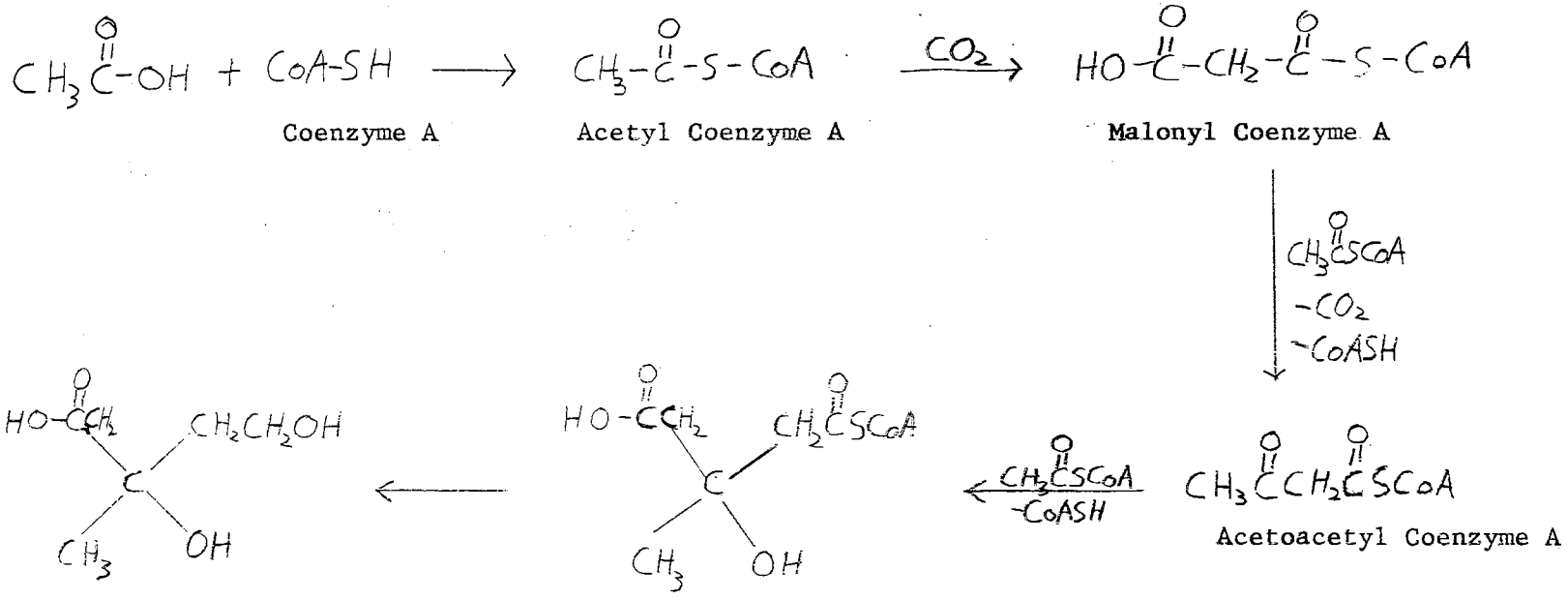


CHART I. CONVERSION OF ACETATE TO MEVALONATE

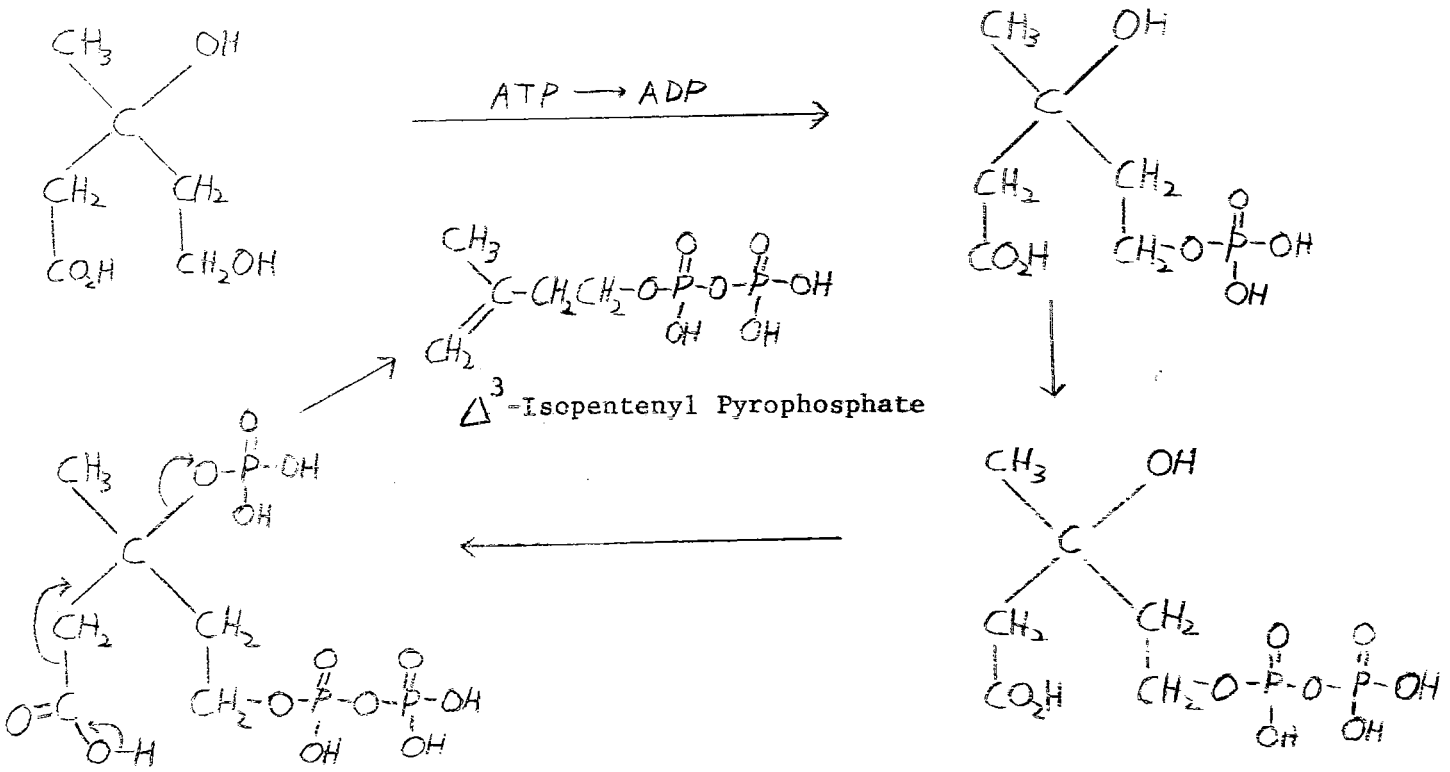


CHART II. CONVERSION OF MEVALONATE TO Δ^3-ISOPENTENYL PYROPHOSPHATE

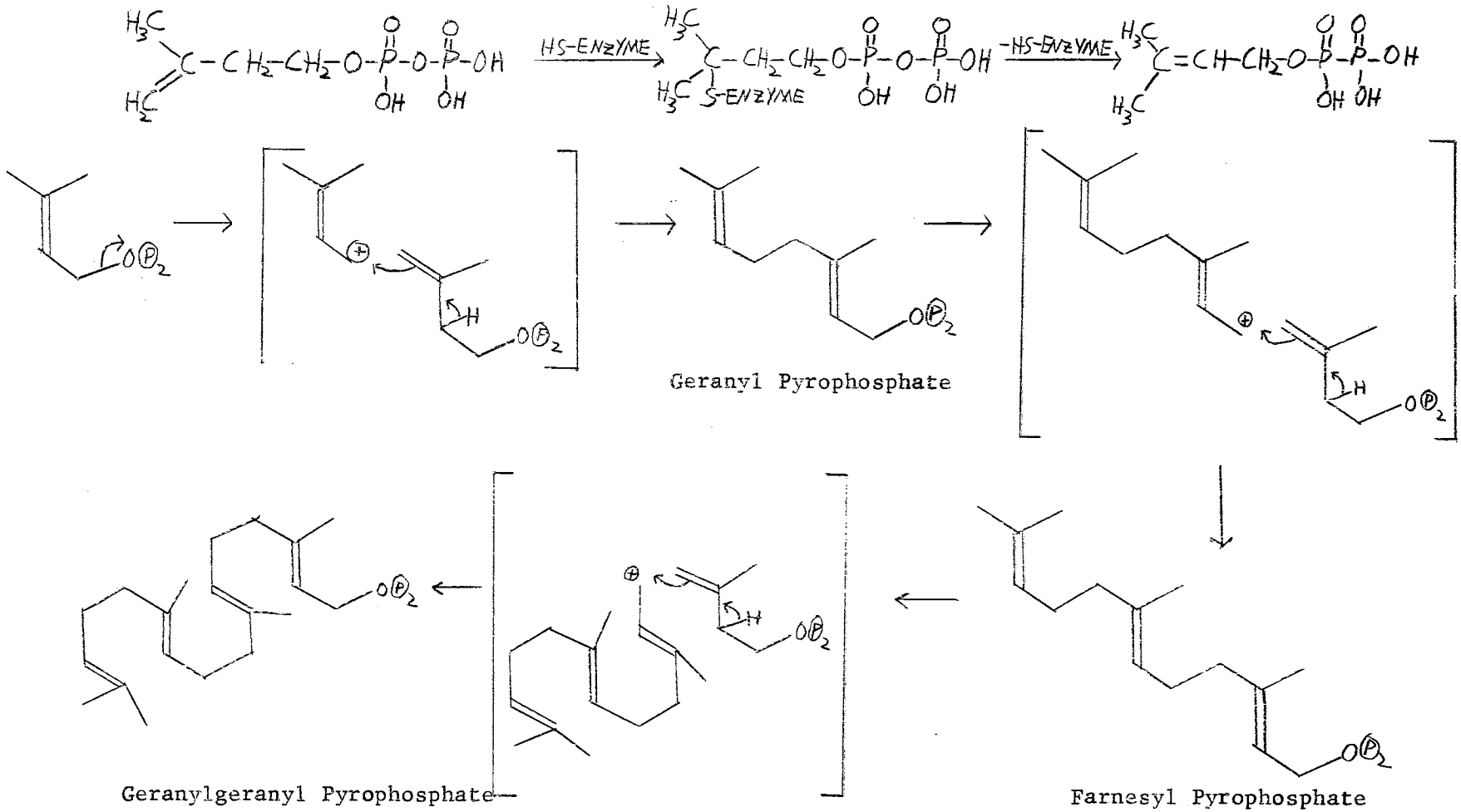
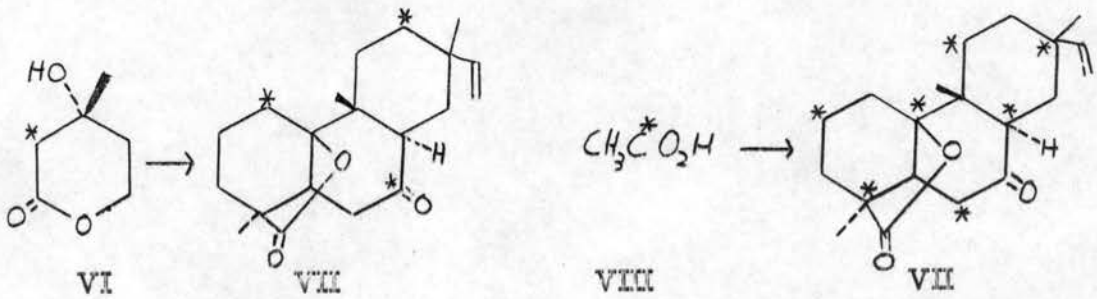


CHART III. POLYMERIZATION OF Δ^3-ISOPENTENYLPYROPHOSHATE

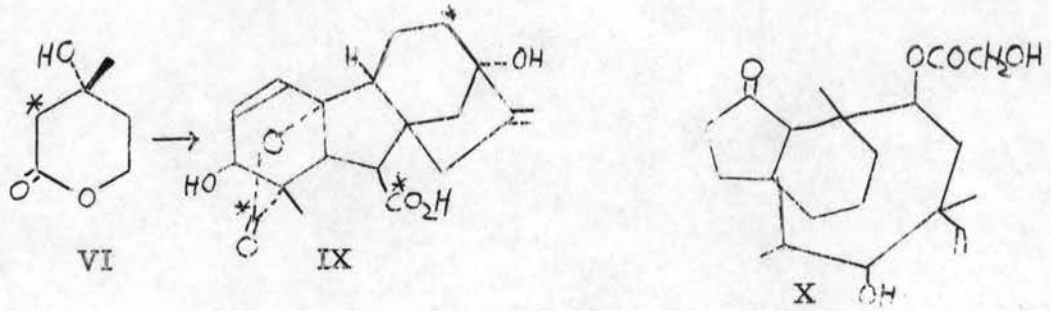
to give the triterpenes and steroids. Addition of a fourth unit affords geranylgeranyl pyrophosphate, which may dimerize, leading to formation of the carotenoids, or may cyclize to give diterpenes, which are thought to be the precursors of the diterpenoid alkaloids.

The postulated mechanisms of terpene biosynthesis are well established by numerous experiments in the case of the triterpenes. In the case of the diterpenes experimental evidence is limited to the studies by English[8] and Swiss[9] groups on the biosynthesis of rosenonolactone (VII) from mevalonic lactone-2-^{14}C (VI) and acetic acid-1-^{14}C (VIII), the incorporation of acetate-1-^{14}C and mevalonate-2-^{14}C into gibberellic acid[8,10] (IX), and the investigations on plueromutilin (X).[11,12,13] Rosenonolactone, as the result of a molecular rearrangement, possesses a structure which is not in accordance with the isoprene rule but nevertheless gave the pattern of labeling expected for a diterpene (VI→VII and VIII→VII), (* = ^{14}C). Gibberellic acid (IX) is also a modified diterpene which has lost an angular methyl group and undergone contraction of ring B. It also gave the pattern of labeling expected for a diterpene (VI→IX). The quite unusual diterpene pleuromutilin (X) in experiments with labeled precursors gave results in agreement with those expected for the postulated biosynthetic pathway.

Wenkert[14] has postulated that the biogenetic precursors of the tetracyclic diterpenes and of the diterpenoid alkaloids are pimaradienes of the type XI (only relative stereochemistry indicated). Production of this precursor by cyclization of geranylgeranyl pyrophosphate may

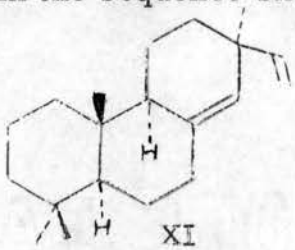


VI VII VIII VII



VI IX X

be envisioned as proceeding in the sequence shown in Chart IV.



XI

Three of the isoprene units are folded in two potential chair cyclohexane rings (XII) and the cyclization, initiated by H^+, proceeds in the manner of electrophilic addition to simple olefins, i. e. trans attack. The cation (XIII) formed loses a proton and may undergo attack by OH^- to form the alcohol (XIV). The mirror image of XIV occurs in nature and is known as manoöl. The alcohol (or its pyrophosphate ester) may then cyclize with loss of water to form the pimaradiene (XVI). The cyclization process may take other stereochemical courses leading to the mirror-image pimaradiene (XI) as well as C-7 epimers. Thus the homogeneous stereochemistry which occurs in the triterpenes and

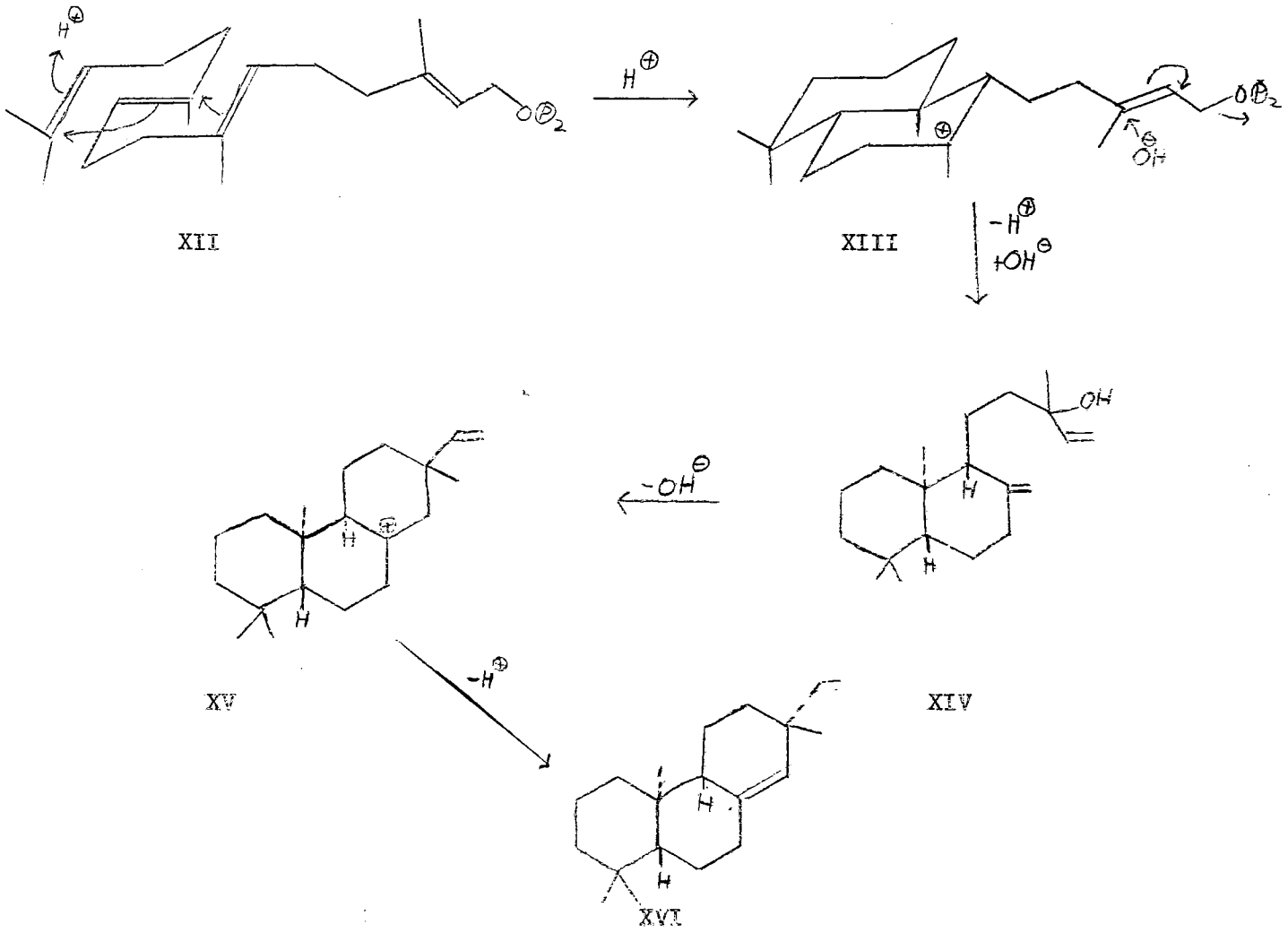
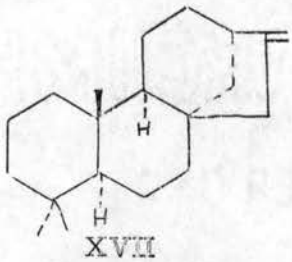
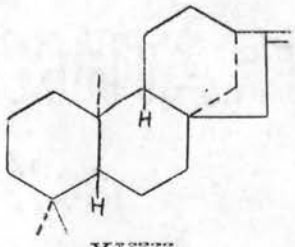


CHART IV. CYCLIZATION OF GERANYLGERANYL PYROPHOSPHATE

steroids is not found in the diterpenes as evidenced by the existence of such natural products as phyllocladene (XVII), with the normal A/B stereochemistry, and (-) kaurene (XVIII), with antipodal sterochemistry at the A/B ring fusion. The particular cyclization shown in Chart IV was chosen because the pimaradiene (XVI) possesses the configuration necessary for a precursor giving rise to the diterpenoid alkaloids, all of which possess an antipodal A/B stereochemistry. Whalley[15] has given a detailed discussion of the stereochemistry involved in the biosynthesis of the diterpenoid alkaloids. The postulated route by which the pimaradiene (XVI) leads to formation of these complex organic bases will be discussed later in connection with the specific examples investigated in the present work.



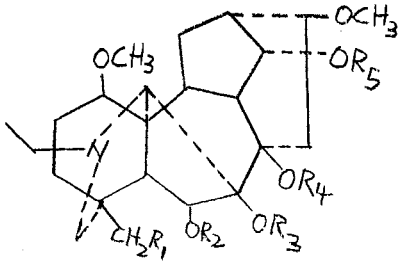
XVII



XVIII

Although there has been much speculation concerning the biosynthesis of the diterpenoid alkaloids, the quantity of experimental verification is quite limited. Only since the inception of the present investigation have the first two reports of biosynthetic studies appeared in the literature. In the first of these, Herbert and Kirby[16] failed to obtain incorporation of mevalonic acid-2-^{14}C into delpheline (XIX) biosynthesized in detached leaves of Delphinium elatum. These workers concluded that if delpheline was a true terpenoid then the

mevalonic acid was converted into non-basic terpenoids before reaching the site of alkaloid synthesis. More recently Benn and May[17] have succeeded in obtaining low incorporation of sodium acetate-1-^{14}C and -2-^{14}C as well as mevalonic acid-2-^{14}C into brownine (XX) and into lycoctonine (XXI) biosynthesized in intact plants of Delphinium brownii. These workers suggested that the primary site of alkaloid biosynthesis is the roots and ascribed Herbert and Kirby's failure to get incorporation to their use of detached leaves.



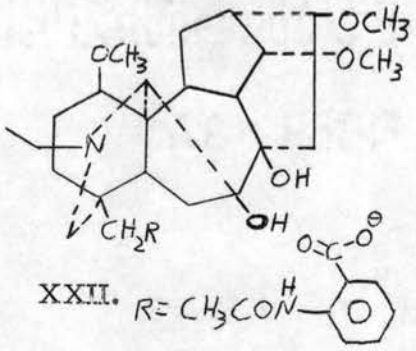
XIX. R_1=H, R_2=H, R_3+R_4=CH_2, R_5=CH_3

XX. R_1=OCH_3, R_2=CH_3, R_3=H, R_4=H, R_5=H

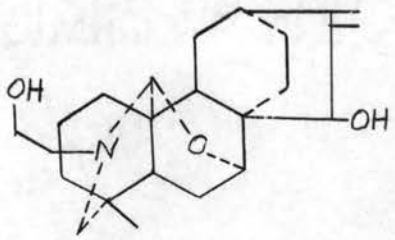
XXI. R_1=OH, R_2=CH_3, R_3=H, R_4=H, R_5=CH_3

DISCUSSION AND RESULTS

The species Delphinium ajacis was the plant chosen for the present investigation of diterpenoid alkaloid biosynthesis. Previous investigations of this species were restricted to extraction of the seeds rather than of the plant as a whole. In 1913, Keller and Volker[13] isolated two crystalline bases which they named ajacine and ajaconine. Ajacine was shown by Goodson[19] in 1944 to be N-acetylanthranoyl lycoctonine (XXII) and the structure of ajaconine (XXIII) was elucidated by Dvornik and Edwards[20] as recently as 1961.



XXII. R= CH_3CON



XXIII.

An investigation of the seeds of Delphinium ajacis in 1944 by Hunter[21] confirmed the presence of ajacine and ajaconine and led to the isolation of two additional alkaloids previously unreported in the literature. The first of these was named ajacinine and was obtained as a crystalline solid. The second was a yellow amorphous substance which was named ajacinoidine and which was almost certainly not a pure compound. A

fifth base isolated was unnamed but had properties resembling those of lycoctonine (XXI).

The following year the seeds of Delphinium ajacis were investigated by Goodson[22] who isolated ajacine, ajaconine, and three bases which he designated as "base B", "base C", and "base D". Alkaloids "B" and "C" were shown by the method of Herzig and Meyer[23] to contain an ethylimino group and three methoxy groups. Furthermore, alkaline hydrolysis of "base B" gave acetic acid and "base C".

The physical properties of the alkaloids mentioned above are given in Table I. "Base C" and Hunter's[21] ajacinine are seen to have similar properties but direct comparison[22] has shown them not to be identical.

The seeds used in the present investigation were found to contain approximately 0.5 percent total alkaloids. The crude mixture obtained from the seeds was separated into highly basic (19%) and less basic (81%) alkaloids as described in the experimental section. The highly basic portion yielded one crystalline base and three others were obtained from the less basic portion. The observed physical properties of the three identified alkaloids are given in Table II.

Ajaconine (XXIII) was crystallized from a concentrated ether solution of the highly basic portion of the seed extract. A comparison of Tables I and II shows that the melting point observed for this base agreed closely with the literature[2] value. Moreover, the infrared spectrum (Plate I) had characteristic hydroxyl and exo-methylene absorptions

TABLE I

RECORDED PHYSICAL PROPERTIES OF ALKALOIDS

Alkaloid	Formula	Melting Point	$[\alpha]_D$	Reference
Ajacine	$C_{34}H_{48}O_9N_2$	154°	+50°(ethanol)	2
Ajaconine	$C_{22}H_{33}O_3N$	167°	-122°(ethanol)	20
Ajacinine	$C_{22}H_{35}O_6N$	210-211°	+52°(chloroform)	2
Ajacinoidine	$C_{38}H_{56}O_{12}N_2$	120-126°	+46°(chloroform)	21
Base B	$C_{26}H_{41}O_8N$	193-195°	+34°(ethanol)	2
Base C	$C_{24}H_{39}O_7N$	203-204°	+57°(chloroform)	2
Base D	$C_{48}H_{64}O_{11}N_2$	97°	+59°(ethanol)	2

TABLE II

OBSERVED PHYSICAL PROPERTIES OF ALKALOIDS

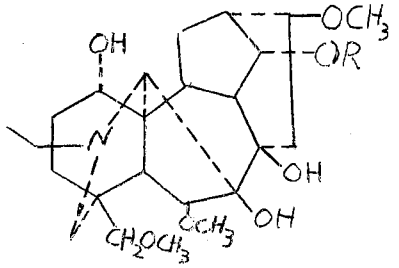
Alkaloids	Formula	Melting Point	$[\alpha]_D$
Ajaconine	$C_{22}H_{33}O_3N$	164-166°	-----
Delcosine	$C_{24}H_{39}O_7N$	206-208°	+49°(ethanol)
O-Acetyldelcosine	$C_{28}H_{41}O_8N$	191 -193°	+27°(ethanol)

which corresponded to those reported by Dvornik and Edwards.[20] The nuclear magnetic resonance spectrum of ajaconine (Plate V) was quite complex but that portion which could be interpreted (see Experimental) was consistent with the assigned structure. The mass spectrum of ajaconine (Plate IX) showed a molecular ion (M^+) at a mass to charge ratio (m/e) of 359 in agreement with the molecular formula $C_{22}H_{33}O_3N$. The base peak occurred at m/e 327. The interpretation of this and other major peaks is discussed in the experimental section.

Delcosine (XXIV) was isolated from the less basic portion of the seed extract by means of chromatography on alumina. It is a widely occurring alkaloid found in several species[2] of Delphinium and Aconitum plants and has been given several names by the various investigators. It is identical[24] with Goodson's "base C", the delphamine of Rabinowitsch[25], and the Takaobase I of Ochiai[26] and is probably identical to the lucaconine of Suginome.[27] The chemistry of delcosine closely parallels that of lycoctonine and the former was therefore assumed[24] to possess the lycoctonine skeleton. The structure (XXI) for lycoctonine has been established by x-ray analysis[28] and unpublished results[24, 29] on the x-ray investigation of delcosine hydrobromide show that it does possess the lycoctonine skeleton with the same orientation of substituents with the exception of the hydroxyl at C-4. This group was found to be on the same side of the ring as the nitrogen. Also ring A was found to possess a boat conformation in contrast to the chair form found in lycoctonine. The stability of this

conformation was attributed to the formation of a hydrogen bond between the C-4 hydroxyl and the nitrogen.[24]

The properties of the delcosine isolated in the present investigation were similar to those reported in the literature[2] as may be seen by a comparison of Tables I and II. The infrared spectrum of delcosine (Plate II) showed characteristic hydroxyl and ether absorptions. The nuclear magnetic resonance spectrum (Plate VI) showed absorptions characteristic of methyl, methoxyl, and hydroxyl groups (see Experimental for details). The mass spectrum of delcosine (Plate X) was much simpler than that of ajaconine and showed the molecular weight to be 453 in agreement with the formula $C_{24}H_{39}O_7N$. It gave a base peak with m/e 438 (M^+ -15). The discussion of the spectrum is given in the experimental section.



XXIV. R = H XXV. R = $COCH_3$

A second alkaloid isolated by chromatography on alumina of the less basic portion of the seed extract was found to have properties resembling those of Goodson's "base B" (see Tables I and II). The infrared spectrum (Plate III) showed hydroxyl and ether absorptions and also a carbonyl absorption suggestive of an acetic ester. The nuclear magnetic resonance spectrum (Plate VII) confirmed the presence of an acetic ester

and was otherwise very similar to the delcosine spectrum. Hydrolysis of the alkaloid with aqueous base gave a product which was identical with delcosine, thereby establishing the identity of the alkaloid as O-acetyldelcosine (XXV). Oppenauer oxidation of delcosine[30] gives a ketone in which the carbonyl is located in a five-membered ring. This secondary hydroxyl is therefore more available for reaction than the other. Acetylation[31] of delcosine with acetic and trifluoroacetic acids gives a monoacetyl derivative identical to the natural product while more vigorous conditions are required for formation of the diacetyl derivative. These facts establish the location of the acetic ester in the five-membered ring as shown in XXV.

The mass spectrum of O-acetyldelcosine (Plate IX) gave a molecular ion with m/e 495 in agreement with the formula $C_{26}H_{41}O_8N$. The base peak appeared at m/e 55. The spectrum is discussed in the experimental section.

A third crystalline solid which will be referred to hereafter as "less basic alkaloid three" (LBA-3) was isolated from the less basic portion of the seed extract by chromatography on alumina but has not been identified. Repeated recrystallizations gave no definite melting point (see Experimental) and the material probably is not a pure compound although it surely must be principally one substance. Thin layer chromatography showed one elongated spot. The infrared spectrum (Plate IV) showed hydroxyl and ether absorptions and the nuclear magnetic resonance spectrum (Plate VIII) was very similar to that of delcosine, the principal

differences being found in the methoxyl absorptions. The possibility must be considered that LBA-3 possesses the delcosine structure, differing only in the degree of methylation. Analysis of LBA-3 indicated that the substance is highly oxygenated, a characteristic of diterpenoid alkaloids possessing the lycoctonine skeleton (II), and suggested the molecular formula $C_{26}H_{43}O_7N$. This could possibly correspond to a dimethyl derivative of delcosine in which both secondary hydroxyls have become methyl ethers. Since the quantity of LBA-3 available was limited and of questionable purity it was not further investigated.

Preliminary to the biosynthesis studies, the investigations on occurrence of alkaloids in Delphinium ajacis were extended to include the whole plant. The aerial portions of plants grown in the field were found to contain approximately 0.06 percent less basic alkaloids and 0.01 percent highly basic alkaloids based on dry weight. Thin layer and gas chromatography of the extracts from various plots indicated as many as twelve components with, in most cases, four major ones, corresponding to the four alkaloids isolated from the seeds.

In order to determine the best time for injection of carbon-14 labeled precursors, a study was made of the rate of production of total alkaloids during the entire life cycle of the plant. This was accomplished by taking samples, including roots, at weekly intervals from a plot of plants grown in the greenhouse. The samples were then assayed by the usual method (see Experimental section) for total alkaloid content.

The seeds were planted on 9-29-64 and collection of samples was begun on 10-30-64, the thirty-first day. The results of this study are shown in Table III and Figures I and II. All assays were based on fresh plant weight and the data for alkaloid percentage as well as that for weight per plant are therefore subject to errors due to variations in water content of the plant. Also, because of a shortage of specimens, it was necessary to use only small, perhaps not accurately representative, samples near the end of the experiment. Nevertheless, certain obviously valid conclusions can be made and others may be cautiously expressed.

In general, the absolute quantity of alkaloid per plant increased throughout the life cycle, the greatest rate of increase coinciding with the period of rapid growth just prior to and during the early stages of flowering (about 160-180 days). Between days 188-202 the amount of alkaloid per plant remained essentially constant as did the weight of the plants after a noticeable drop due, perhaps, to a loss of water as the plants developed pods and began to dry up and deteriorate. During the last week of the study the amount of alkaloid per plant dropped considerably as the plants continued to lose their leaves and deteriorate.

The percent alkaloid, as shown in Figure I, is quite erratic in the early stages of the life cycle. This may be a result of variations in the water content of the plant since, as previously stated, the data is based on fresh plant weight. It may be safely concluded however that the percentage of alkaloid was relatively high during the early life of the

TABLE III

GROWTH AND ALKALOID PRODUCTION

Number of Plant	Weight (gm.)	Weight per Plant (gm.)	Date Collected	Alkaloid Weight (mg.)	Alkaloid per Plant (mg.)	Percent Alkaloids
42	3.03	.07	10-30-64	2.8	0.07	.092
48	3.78	.08	11- 6-64	12.9	0.27	.341
40	7.00	.18	11-13-64	21.7	0.54	.310
8	10.83	1.35	11-20-64	5.6	0.70	.052
8	19.81	2.48	12- 1-64	7.9	0.99	.040
8	25.28	3.16	12- 8-64	27.2	3.40	.108
8	47.25	5.91	12-22-64	16.9	2.11	.036
8	90.10	I1.26	12-29-64	14.3	1.79	.016
8	114.65	14.33	1- 5-65	13.9	1.74	.012
8	132.62	16.58	1-12-65	18.1	2.26	.014
8	166.70	20.84	1-19-65	20.5	2.56	.012
8	181.60	22.70	1-27-65	17.2	2.15	.009
8	250.34	31.29	2- 2-65	36.7	4.58	.015
8	331.90	41.49	2- 9-65	27.2	3.40	.008
8	326.00	40.75	2-16-65	21.6	2.70	.007
8	388.00	48.50	2-23-65	54.0	6.75	.014
8	426.00	53.25	3- 3-65	52.1	6.52	.012
2	119.28	59.64	3- 9-65	12.6	6.30	.011
2	128.20	64.10	3-16-65	16.1	8.05	.013
2	186.20	93.10	3-23-65	34.3	17.15	.018
2	89.95	44.98	3-30-65	27.4	13.70	.030
2	108.12	54.06	4- 6-65	51.8	25.90	.048
2	98.55	49.28	4-13-65	51.3	25.65	.052
2	116.88	58.43	4-20-65	50.7	25.35	.043
2	86.30	43.15	4-27-65	32.8	16.40	.038

plant and decreased until about the ninetieth day. Then as the plant began to grow at a greater rate the formation of alkaloid kept pace and the percentage remained essentially constant. As the plant entered the flowering stage and the rate of formation of alkaloid exceeded the rate of plant growth, the percentage of alkaloid increased reaching a maximum at the one-hundred-ninety-fifth day which occurred during the period of pod formation. After this the percentage decreased as the plants began to deteriorate.

The biosynthesis of the alkaloids was investigated during each of three principal stages of the plant's life (pre-flowering, flowering and post-flowering) using two precursors, mevalonic acid-2-^{14}C and glycine-2-^{14}C. The precursors were injected as aqueous solutions into the stem cavities of the plants. A total of eighteen plants were treated, nine (three from each stage) with glycine-2-^{14}C and nine with mevalonic acid-2-^{14}C. Individual plants were harvested after one, four, and seven days incorporation time and extracted in the usual manner. A small portion of each extract was counted by the liquid scintillation method to determine incorporation of labeled precursors. The results are given in Table IV. Another portion of each extract was fractionated by thin layer chromatography. Sections of various widths were scraped from the chromatograms and the scrapings were added directly to the scintillation liquid for determination of activity. The results are shown in Figures III-XX. Probable locations of the various alkaloids are indicated by placing letters at the R_f values.

TABLE IV

INCORPORATION OF LABELED PRECURSORS

Plant Number	Green Weight (gm)	Alkaloid Weight (mg)	Stage	Precursor	Incorporation Time (days)	Percent Incorporation
01	25.5	8.2	PF	G	1	0.06
02	38.0	22.4	PF	G	4	0.09
03	44.0	17.4	PF	G	7	0.14
04	24.6	18.1	PF	MVA	1	0.22
05	25.5	14.0	PF	MVA	4	0.16
06	39.0	25.2	PF	MVA	7	0.14
07	22.0	16.3	F	G	1	0.04
08	26.0	24.7	F	G	4	0.22
09	31.0	24.3	F	G	7	0.04
10	47.0	32.0	F	MVA	1	0.14
11	30.0	31.7	F	MVA	4	0.13
12	32.5	23.5	F	MVA	7	0.06
13	31.0	24.9	PoF	G	1	0.04
14	23.5	17.0	PoF	G	4	0.14
15	22.5	16.6	PoF	G	7	0.03
16	39.5	33.9	PoF	MVA	1	0.15
17	39.0	41.4	PoF	MVA	4	0.14
18	26.5	14.7	PoF	MVA	7	0.13

PF = pre-flowering
F = flowering
PoF = post-flowering
MVA = mevalonic acid-2-^{14}C

determined for the pure alkaloids under identical conditions. Letter designations are: A = Delcosine; B = Ajaconine; C = Acetyldelcosine; D = LBA-3. Pertinent experimental information to be found on the graphs includes extract number, milligrams of extract chromatographed, and distance traveled by solvent front. The graph abcissa represents the length of the TLC plate in centimeters and the ordinate is disintergrations per minute per milligrams of extract divided by the widths of the scraped sections.

In the case of alkaloids derived from the mevalonic acid -2-^{14}C precursor incorporation is relatively high after one day in all three stages of plant development. For the pre-flowering plants incorporation on the fourth day dropped to 73% of its first-day value and to 64% on the seventh day. The flowering plants dropped only to 93% on the fourth day and then dropped drastically to 43% on the seventh day. The post-flowering plants also dropped to 93% on the fourth day and then only to 87% on the seventh day. These trends may be interpreted as suggestive of a competition such as Herbert and Kirby[16] suggested between alkaloidal and non-alkaloidal products for the mevalonic acid if not for the diterpene skeleton itself. It is possible that the diterpenoid alkaloids are deaminated to yield other diterpenoids. If these diterpenoids are considered to be in a period of rapid synthesis in the pre-flowering stages, incorporation in the alkaloids would rapidly drop from its initial value as observed. As the flowering stage was entered and the rate of alkaloid synthesis increased the

alkaloids would become more able competitors and finally in the post-flowering stage as synthesis slowed the incorporation would tend toward a more stable equilibrium value.

An additional conclusion which may be drawn from the data in Table IV is that there do appear to be operative modes of alkaloid degradation as well as synthesis.

Interpretation of Figures III-XX is made rather difficult by the fact that R_f values of the alkaloids vary considerably with the composition of the various extracts and with small variations in the TLC plates and solvents. The R_f values located by the letters at the tops of each graph should therefore be interpreted as designating the center of a considerable area in which the alkaloid concerned may be expected to be found. After perusual of the various graphs one may conclude that both glycine-2-^{14}C and mevalonic acid-2-^{14}C have been incorporated into each of the isolable alkaloids. Certain trends not readily apparent in the graphs are easily seen by means of autoradiography. For example, in the pre-flowering stage of the glycine-injected plants most of the activity on the first day was in one spot corresponding in R_f value to that of LBA-3 and the next most active spot corresponded to ajaconine. After four days the other alkaloids had become radioactive and the activity of LBA-3 relative to them had decreased, suggesting that LBA-3 might possibly be a precursor of the other alkaloids. After seven days the ajaconine and acetyldelcosine were the most highly labeled and the relative activity of LBA-3 had further

decreased. Delcosine and its acetate were not visible on the first day but appeared on the fourth with the acetate being of about the same intensity as ajaconine and LBA-3 while delcosine, though more highly labeled than it was on the fourth day, was still much less active than the other alkaloids. Essentially the same pattern was observed in the flowering and post-flowering stages except that ajaconine was observed to be by far the most active spot after the fourth day.

Autoradiography of the mevalonic acid-2-^{14}C derived alkaloids showed extensive trailing of radioactivity as is also apparent on the graphs. The largest portion of the activity in pre-flowering plants after the first day was in the area of LBA-3 with a considerable portion also in delcosine and a spot at R_f 0.16. After the fourth day the relative activity of ajaconine had increased while that of delcosine and R_f 0.16 had decreased and a new spot at R_f 0.75 had appeared. Not much change in the relative activities was observed after seven days. A similar pattern was observed for the flowering and post-flowering stages.

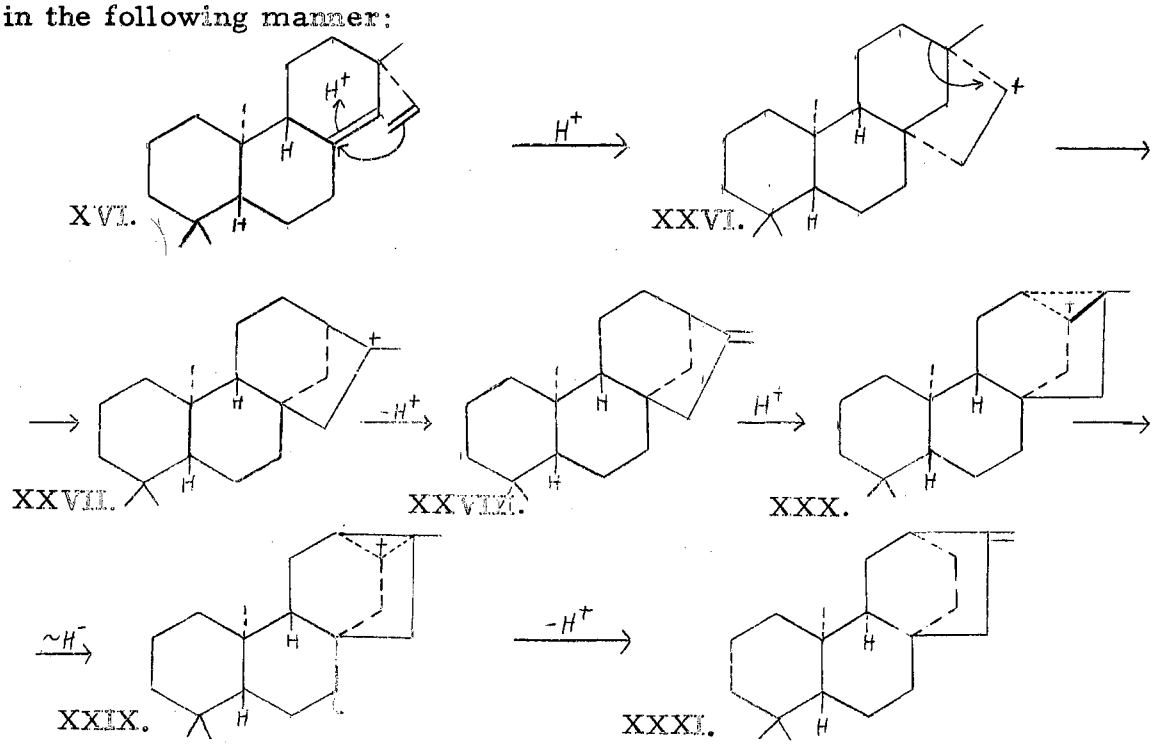
Specific activities for the three alkaloids of know composition were determined by fractionating the extracts by preparative thin layer chromatography followed by scraping off and eluting of the alkaloids from the silica gel (see Experimental for details). Positive identification of the eluate was made by spotting it and authentic samples on analytical TLC plates and developing in two different solvent systems (see Experimental). A known weight of the eluate was then counted by the scintillation method. Results are given in Table V.

TABLE V

SPECIFIC ACTIVITIES OF ALKALOIDS

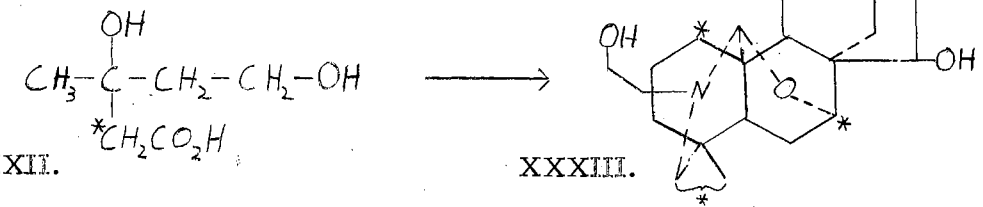
Percent Incorporation Total Alkaloids	Percursor	Alkaloid	Specific Activity
0.190	glycine-2-^{14}C	Delcosine	0.46μC/mM
		Ajaconine	0.49μC/mM
		Acetyldelcosine	0.73μC/mM
0.126	mevalonic acid-2-^{14}C	Delcosine	0.15μC/mM
		Ajaconine	0.28μC/mM
		Acetyldelcosine	0.63μC/mM

As mentioned previously (p. 6), Wenkert[14] has postulated pimaradienes of the type XI as being precursors of the diterpenoid alkaloids. Also a mode of cyclization by which the necessary isomer (XVI) might be formed from geranylgeranyl pyrophosphate was given in Chart IV. The subsequent steps leading to the diterpenoid alkaloids may be visualized as proceeding in the following manner:



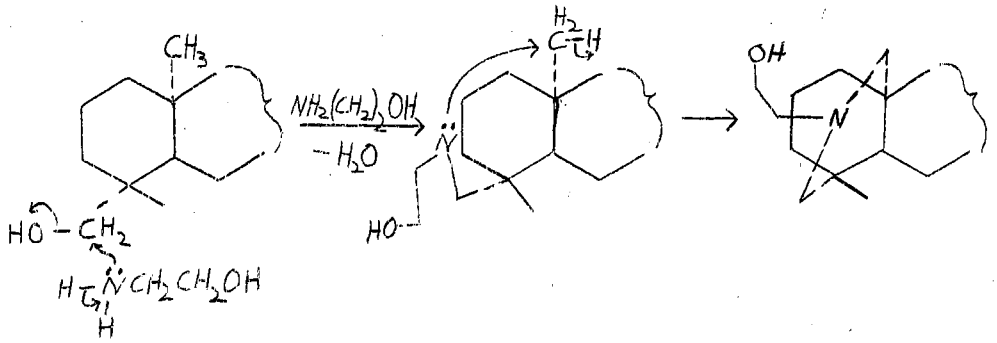
Protonation of the 8:14 double bond leads to formation of the bicyclo- [3. 2. 1] carbonium ion XXVI which rearranges to the ion XXVII. Loss of a proton leads to the formation of (-) kaurene (XXVIII) which possesses the veatchine skeleton (III). Alternatively carbonium ion XXVII may rearrange and undergo a hydride shift to give the carbonium ion XXX. Loss of a proton from XXX gives the atisine or ajaconine skeleton (XXXI). The steps necessary for completion of the biosynthesis of ajaconine (hydroxylation at C-8, insertion of the nitrogen and oxygen bridges, etc.) may occur at any convenient stage.

When mevalonic acid-2-^{14}C (XXXII) is the precursor one expects the pattern of labeling shown below for ajaconine (XXXIII).

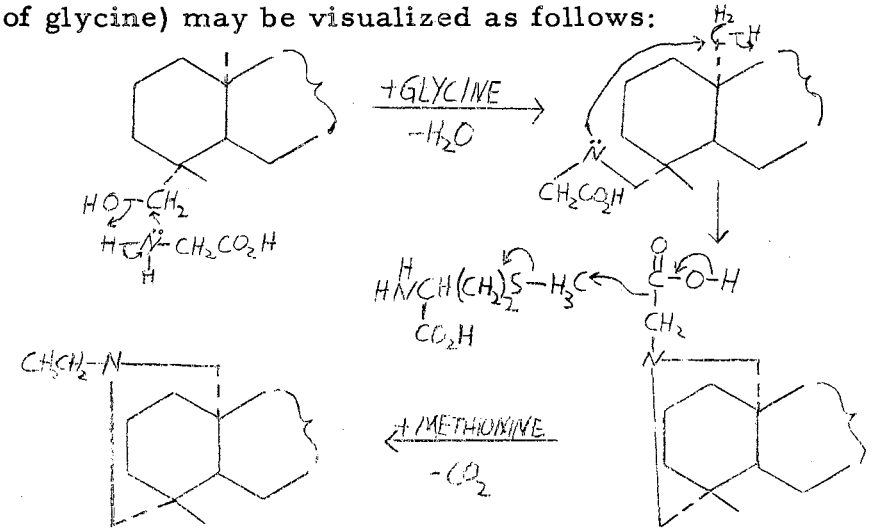


XXXII. XXXIII.

Use of glycine-2-^{14}C would be expected to give ^{14}C in the hydroxyethyl group at the position adjacent to nitrogen if the function of this precursor is amination. Whalley[15] has visualized the biosynthesis of ring E as proceeding by the sequence:

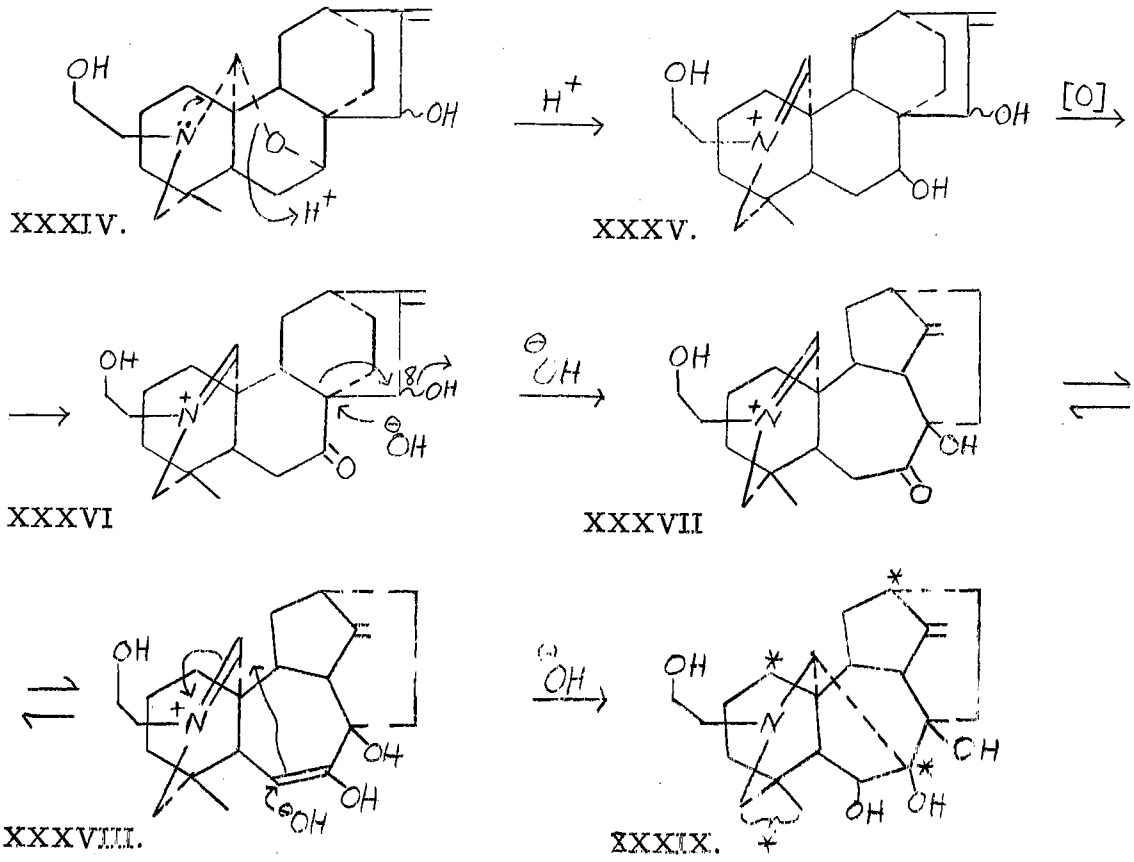


Herbert and Kirby[16] observed incorporation (0.025%) of methionine-CH_3-^{14}C into delpheline (XIX), 88% of the activity being located in the methoxyl groups and 11% in the N-ethyl group. Since the incorporation was very low and "some contamination by methoxyl was possible" it can not be definitely concluded that methionine is a precursor for the N-ethyl group. If it actually is a precursor the mode of incorporation of methionine (and of glycine) may be visualized as follows:



This process results in formation of an unsubstituted ethyl side-chain such as that found in delcosine and the other alkaloids having the lycoctonine skeleton. A simple oxidation process may then produce the hydroxyethyl side-chain of ajaconine and other atisine-type alkaloids.

It has been postulated[32, 33] that alkaloids possessing the atisine and ajaconine skeletons are precursors of those with the lycoctonine and delcosine skeletons. The process may be visualized as proceeding in the following manner (XXXIV → XXXIX). The pattern of labeling in XXXIX is that expected for incorporation of mevalonic acid-2-^{14}C.



XXXIV. XXXV. XXXVI XXXVII XXXVIII. XXXIX.

This sequence provides the necessary structural rearrangements to give the delcosine skeleton although the order of the steps is of course arbitrary. The step (XXXIV→ XXXV) is an acid cleavage of the carbinol-amine-ether system resulting in formation of the immonium salt (XXXV). Such a reaction has been observed[20], in vitro, when ajaconine is treated with a solution of hydrogen chloride in methanol. Oxidation of XXXV affords the ketone XXXVI which undergoes rearrangement to the ketone XXXVII. As may be seen by inspection of molecular models, the double bond in the seven-membered ring of the enol tautomer (XXXVIII) is ideally situated for formation of the 9-17 bond. This ring closure forms XXXIX which has the delcosine skeleton with several of the necessary substituents in place and possessing the necessary stereochemistry. The

molecule may be completed by removal of the OH on the N-ethyl group (perhaps by the sequence suggested for incorporation of glycine and methionine) and a series of oxidation and methylation reactions.

It should be noted that the orientation of the C-8 hydroxyl is important in the rearrangement step. The steric course of this reaction requires that this hydroxyl be α-oriented. Whalley[15] has suggested that the atisine structures possessing an 8-β-hydroxyl, as does ajaconine, survive in the plant only because they have the wrong configuration at C-8 and cannot undergo rearrangement.

EXPERIMENTAL

Melting points were taken on a Fisher-Johns apparatus and are uncorrected. The infrared spectra were recorded in KBr pellets using a Beckman IR-5A spectrophotometer. Nuclear magnetic resonance spectra were recorded with the Varian A-60 spectrometer, using tetramethylsilane as an internal reference ($\delta = 0$) and deuteriochloroform as the solvent. Mass spectra were provided by Professor Carl Djerassi of Stanford University using a Consolidated Electrodynamics Corporation mass spectrometer No. 21-103C with direct insertion of the sample into the ion source. All determinations of radioactivity were with the Tri-Carb Series 314A Liquid Scintillation Spectrometer.

For column chromatography Woelm neutral alumina was used as the adsorbent. For thin layer chromatography, the adsorbent was Mallinckrodt Silicar-7G and the developing solvent was benzene: ethyl acetate: diethylamine (7:2:1) unless otherwise stated. Detection was made with Dragendorff's reagent (Munier modification).[34]

Extraction of Plant Material

The alkaloids were extracted from the commercially acquired seeds and from the air-dried and ground plants grown in the field by soaking

the material in 5% acetic acid for about a week. The filtered acid solution was cooled in ice and cold aqueous sodium hydroxide was added until the pH of the solution was greater than 13. The mixture, which had become cloudy, was then extracted with chloroform. The chloroform extracts were washed with water, dried over anhydrous magnesium sulfate, filtered, and evaporated under reduced pressure. The yield of crude extracts was about 0.4% of the seeds and 0.07% of the dried plants.

The residue left after evaporation of the chloroform was redissolved in 5% acetic acid and the solution adjusted to pH 8 by addition of aqueous sodium hydroxide. Extraction of the solution with chloroform and removal of the chloroform as above gave the less basic alkaloids which accounted for approximately 80% and 95% of the seed and plant extracts, respectively. The solution was then adjusted to pH 13 and extracted with chloroform to give the highly basic alkaloids.

In obtaining the data given in Tables III and IV the entire extract was used and the separation of highly basic and less basic alkaloids was not made.

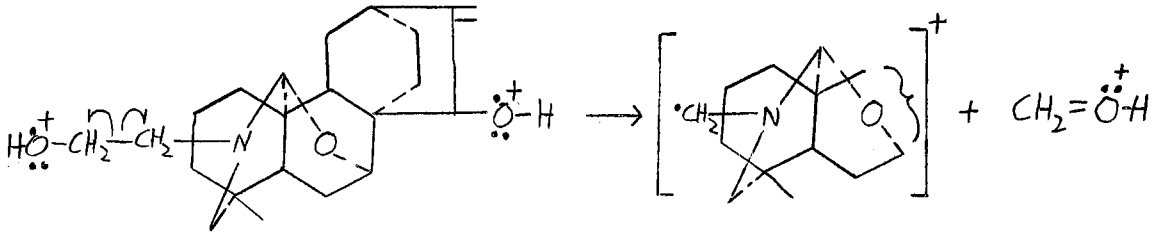
Isolation and Properties of the Alkaloids

Ajaconine

Crystallization of 3 grams of highly basic extract of the seeds from a concentrated ether solution gave 79 mg. (0.002% of seeds) of ajaconine,

which was recrystallized from aqueous methanol to give fine needles melting at 164-166°C. The infrared spectrum showed hydroxyl absorption at 3375 and 3290 cm^{-1}. Absorptions characteristic of an exocyclic methylene occurred at 3090, 1660 and 888 cm^{-1}. The N.M.R. spectrum showed a sharp singlet at δ 0.75 for the methyl group and a singlet at δ 4.58 for the exocyclic methylene.

The mass spectrum gave the molecular weight as 359 and showed the base peak at m/e 328 (M^+ - 31). The base peak probably arises as a result of the loss of $CH_2{=}\overset{+}{O}H$ from the doubly charged ion radical (XL) as shown below (single-headed arrows indicate one-electron shifts).

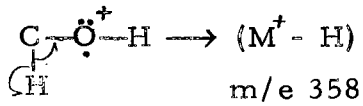


XL. m/e 328 m/e 31

Other major peaks and their possible modes of formation are given below.

m/e 358 (M^+ - 1) 74%

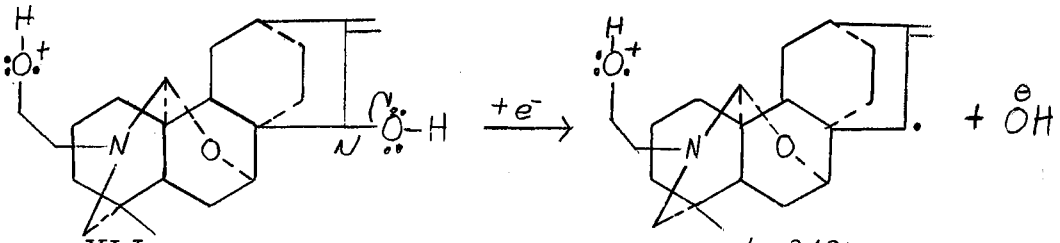
This peak probably arises by loss of a hydrogen from the primary or the secondary hydroxyl:



m/e 358

m/e 342 (M^+ - 17) 47%

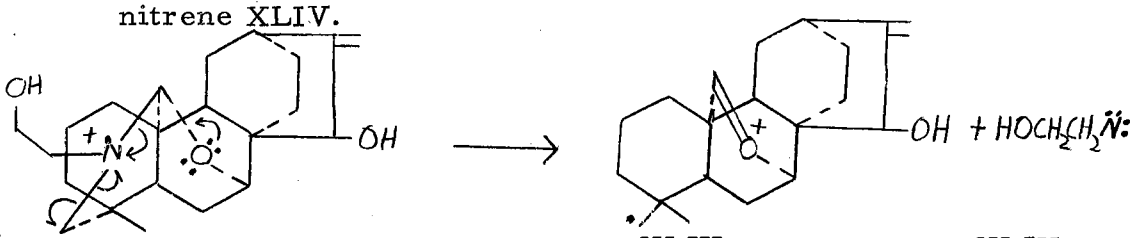
This peak corresponds to loss of OH which likely comes from the primary or the secondary hydroxyl. It is probably lost as OH^- from the ion radical (XLI).



XLI. m/e 342

m/e 300 (M^+ - 59) 99%

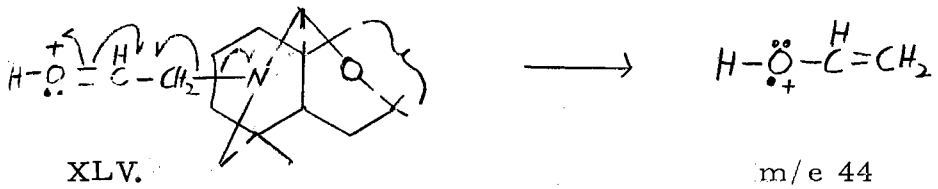
This peak may arise as a result of the loss of one of the non-bonding electrons on nitrogen giving the ion radical (XLII) which may fragment giving the ion radical XLIII and the neutral nitrene XLIV.



XLII. XLIII. XLIV.

m/e 44 75%

This peak may result from the M^+ - 1) peak XLV.



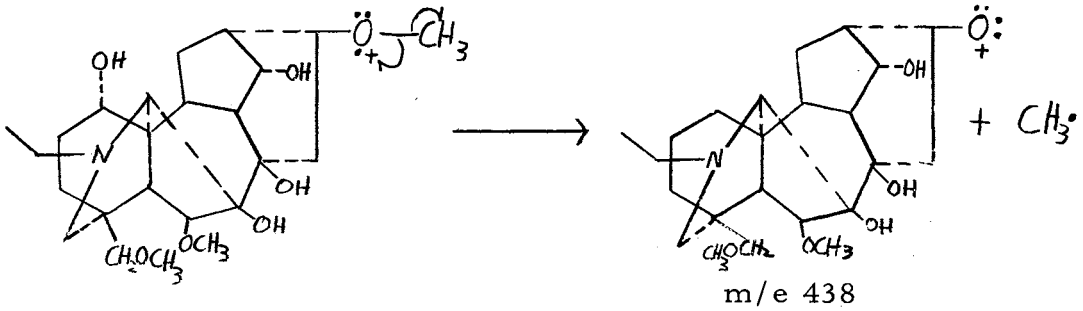
XLV. m/e 44

Delcosine

Column chromatography of the less basic extract of seeds gave delcosine (0.128% of seeds) in the chloroform fraction. Evaporation of the solvent and recrystallization from acetone gave large prisms, m.p. 206-208°, $[\alpha]_D$ +49° (c= 1g/100ml., ethanol). The infrared spectrum showed hydroxyl absorptions at 3356 and 3460 cm^{-1} and strong absorptions at 1087, 1097, and 1115 cm^{-1} due to the methyl ethers. The N.M.R. spectrum showed the methyl protons of the N-ethyl group as a triplet centered at δ 1.09 (J=7cps)

and singlets at δ 3.33 and δ 3.35 corresponding to 3 and 6 protons, respectively.

The mass spectrum gave the parent peak at m/e 453 and the base peak at m/e 438. The base peak is the result of α-cleavage of one of the three methoxyl groups. An example is shown below:

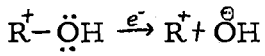


m/e 438

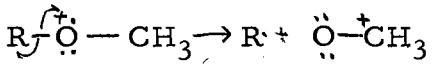
When one considers the number of methoxyl groups in the molecule it is not surprising that this should be the base peak.

Other major peaks and their possible modes of formation are given below.

m/e 436 (M^+ - 17)34%
This corresponds to loss of one of the hydroxyls from the molecular ion.

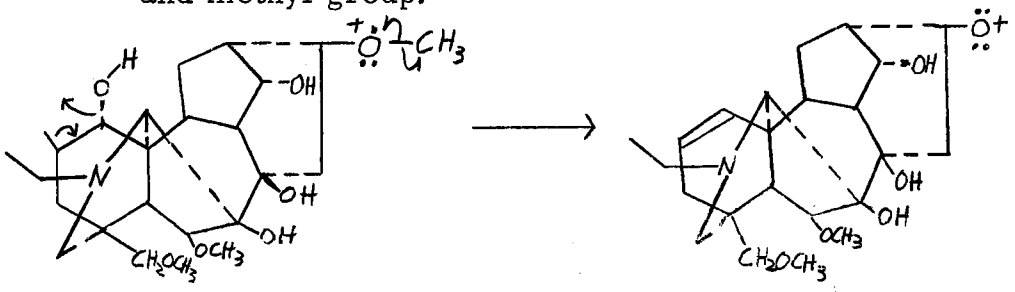


m/e 422(M^+ - 31)54%
This peak results from loss of a methoxyl group by α-cleavage in the direction opposite to that which produced the base peak.



m/e 420 (M^+-33) 60%

This peak corresponds to the loss of water and methyl group.

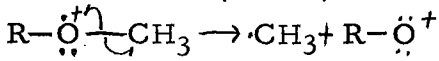


O-Acetyldelcosine

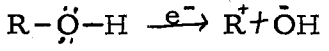
Column chromatography of the less basic extract of seeds gave O-acetyldelcosine (0.008% of seeds) in the benzene-ether fraction. Evaporation of solvent and recrystallization from acetone gave large prisms, m.p. 191-193°, $[\alpha]_D$+27° (c= 1g/100 ml., ethanol). The infrared spectrum showed absorption at 1742 and 1242 cm^{-1} which is indicative of an acetic ester, hydroxyl absorption at 3460 cm^{-1} and broad ether absorption at 1080 cm^{-1}. The N.M.R. spectrum showed the methyl protons of the N-ethyl group as a triplet centered at δ 1.09 (J=7 cps), a singlet at δ 2.05 for the methyl of the acetate group, and a singlet at δ 3.33 for the methoxyl protons.

The mass spectrum gave the molecular ion at m/e 495 and the base peak at m/e 55.

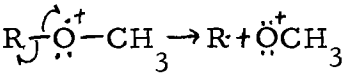
m/e 480 (M^+-15)



m/e 478 (M^+- 17)



m/e 464 (M^+ - 31)



m/e 462 (M^+-33)
Loss of H_2O and $CH_3^\cdot$

Hydrolysis of O-Acetyldelcosine

A solution of 1N NaOH (0.1 ml) was added to a solution of 42.3 mg. of O-acetyldelcosine in 0.3 ml. of ethanol. The mixture was refluxed two hours and then diluted with 2 ml. H_2O and cooled. The solid (29.8 mg.) which then crystallized from the solution, when filtered out and recrystallized from acetone, had melting point 202-205°. The infrared spectrum was identical with that of delcosine and the mixed melting point showed no depression.

LBA-3

Column chromatography of the less basic extract of the seeds gave LBA-3 (0.005% of seeds) in the ether-chloroform fraction. The substance has proven to be quite difficult to purify and melting points have ranged from 210-220° with slight darkening beginning at about 180°. The infrared spectrum showed hydroxyl absorption at 3420 cm^{-1} and ether absorption at 1083 and 1105 cm^{-1}. The N.M.R. spectrum showed a triplet centered at δ 1.10 (J= 7 cps) for the methyl protons of the N-ethyl group and singlets at δ 3.37, 3.41 and 3.46 for methoxyl protons.

Gas Chromatography

The instrument used was the Perkin-Elmer 801 gas chromatograph equipped with a hydrogen flame detector. A 10' x 1/8" glass column of 1% JXR on Gas Chrom Q (Applied Science Laboratories, Inc., State College, Pennslyvania) was used at a temperature of 240° and a helium flow rate of 43 cc/min. Under these conditions the following retention times (T_R) were observed:

Alkaloid	Ajacine	LBA-3	Delcosine	O-Acetyldelcosine
T_R	2.5 min.	5 min.	7 min.	7 min.

Biosynthesis Studies

Plants were grown in the greenhouse and the precursors were injected directly into the plant stems as aqueous solutions. Purity of the precursors was checked by paper chromatography. The glycine had the R_f values of 0.13 and 0.14 in 1-butanol-acetic acid-water(4:1:1) and 2-propanol: concentrated NH_4OH: water (80:1:19), respectively. The mevalonic acid had the R_f values 0.84 and 0.29 in 1-butanol-acetic acid-water (4:1:1) and 1-butanol-1.5 M NH_4OH (3:1), respectively. After suitable periods the plants were subjected to extraction by the previously described method with the exception that no separation of less basic and highly basic alkaloids was made. The values for percent incorporation and the specific activities of the three alkaloids of known constitution are given in Table V.

Specific Activities

Glycine-2-^{14}C

Fifty microcuries of glycine-2-^{14}C of specific activity 0.86 mC/mM were injected into each of six plants in the flowering stage. After four days the alkaloids (45.5 mg.) were extracted from the plants in the usual manner. The extract was chromatographed on three 8 x 14-inch preparative TLC plates and the solvent front was allowed to advance 25 cm. Radioactive spots were located by autoradiography. An active band at 7-8.7 cm was scraped from the plate and eluted from the silica gel with chloroform. The eluate was shown to be delcosine by TLC. In both benzene-ethyl acetate-diethylamine (7:2:1) and chloroform-acetone-diethylamine (5:4:1) solvent systems

the eluate showed one spot at the same R_f as an authentic sample of delcosine. In a like manner active bands at 9.4-11.4 and 13.4-15cm were shown to be due to ajaconine and O-acetyldelcosine, respectively. Small samples of the eluates were then counted to determine specific activities of the pure alkaloids.

Mevalonic Acid-2-^{14}C

The precursor was released from its N, N'-dibenzylethylene-diammonium salt by addition of aqueous sodium hydroxide to a water solution of the salt. The free amine thus released was extracted with ether and the remaining aqueous portion, containing the mevalonic acid-2-^{14}C, of specific activity 1.7 mC/mM, was adjusted to pH 6. Twenty-seven microcuries of this solution were injected into a plant in the flowering stage. After four days the plant was subjected to extraction, yielding 31.7 mg. of basic material. The extract was chromatographed on an 8 x 14-inch preparative TLC plate and the solvent front was allowed to advance 31 cm. Bands were located by spraying a strip about one-quarter of an inch wide along the length of the plate with Dragendorff's reagent. Four bands were located at 12.2-13.1, 15-15.8, 18-18.7 and 12.3-22cm. The center one-third of the first three bands were shown by the method previously described to be delcosine, ajaconine, and O-acetyldelcosine, respectively. Determination of radioactivity by the usual method led to the values listed in Table V for the specific activities of the alkaloids derived from mevalonic acid-2-^{14}C.

Sample Calculations

Compound: Delcosine from band one (12.2-13.1 cm.) of extract from the plant treated with mevalonic acid-2-^{14}C.

Formula: $C_{24}H_{39}O_7N$ (Molecular Weight 453)

Weight: 0.44 mg. = $9.7 \cdot 10^{-4}$ millimoles

Measured Counts:

Background (30 minutes)

First Measurement	553
Second Measurement	617
Average	585

Sample (30 minutes)

First Measurement	4882
Second Measurement	4914
Average	4898

Efficiency Correction:

Disintegrations (30 minutes)

(Sample-Background) ÷ (Efficiency, 44%)=

(4898-585) ÷ (0.44) = 9803

Disintegrations per minute (dpm)

(9803) ÷ (30) = 327 dpm

Specific Activity:

(327 dpm) ÷ ($9.7 \cdot 10^{-4}$ mmoles) = 337,000 dpm/mmole

(337,000 dpm/mmole) ÷ (220 dpm/mμc) = 152 mμc/mmole = 0.15 μc/mmole

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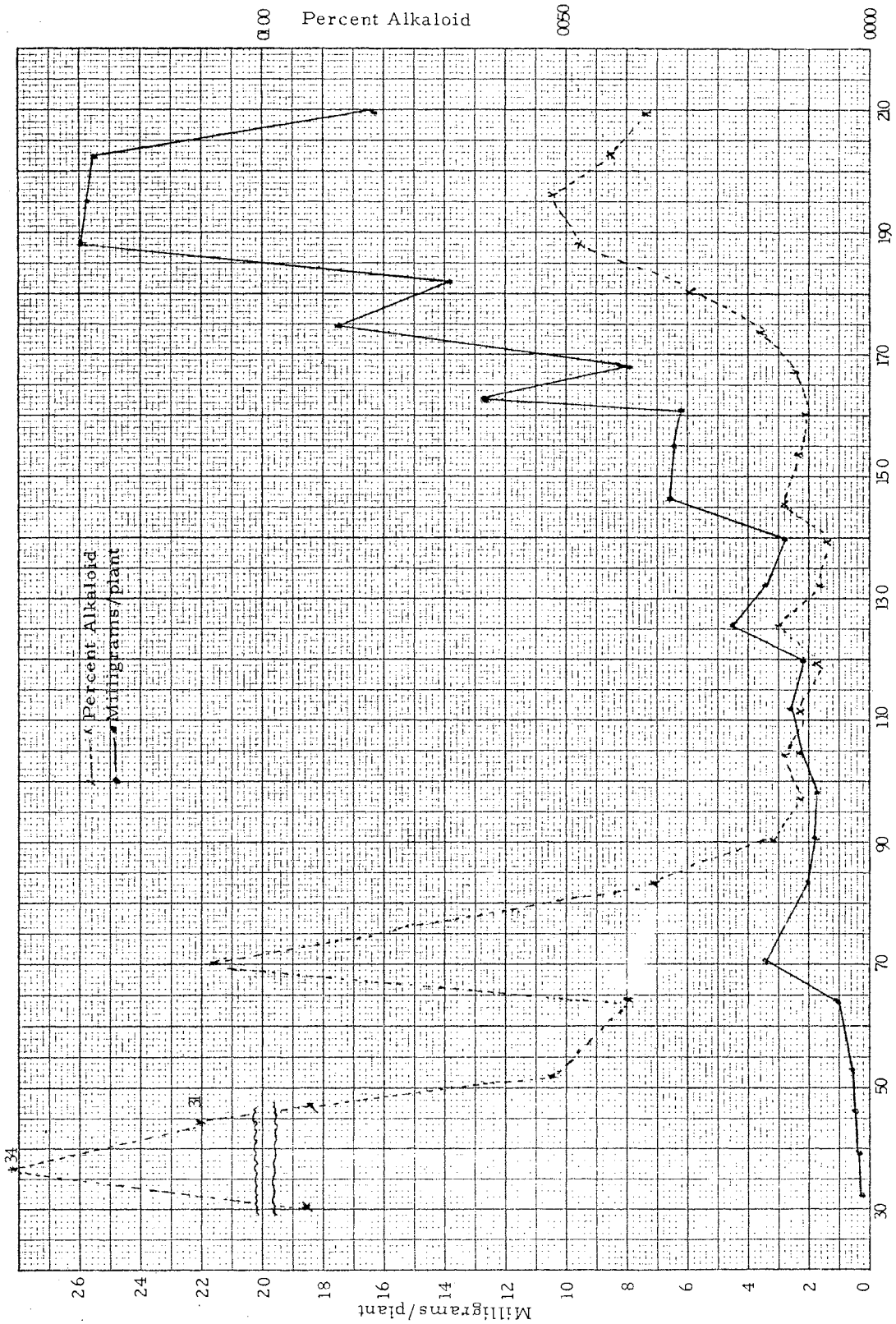

Percent Alkaloid
Milligrams/plant
Percent Alkaloid
Milligrams/plant
Age (Days)

Figure 1.

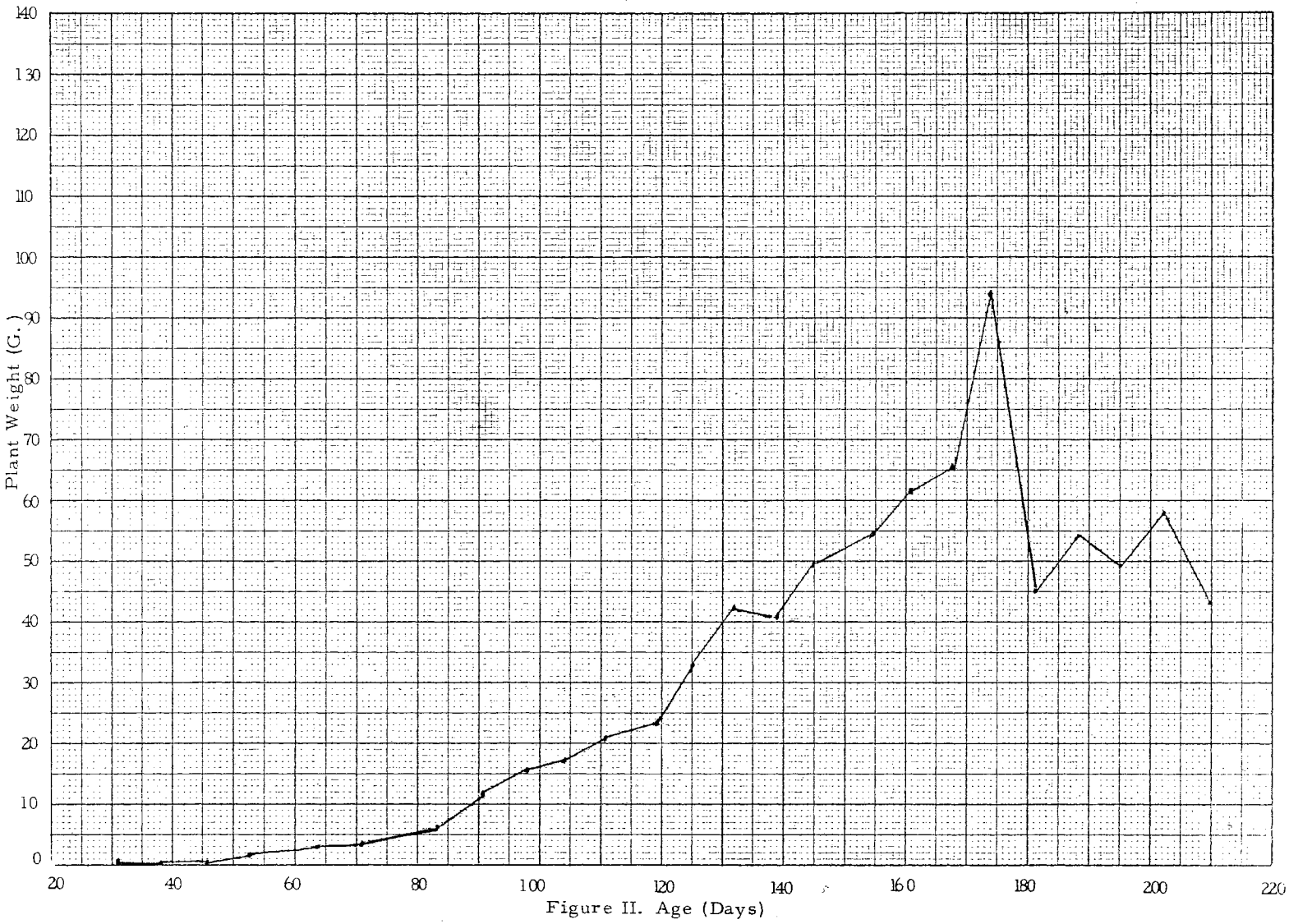


Figure II. Age (Days)

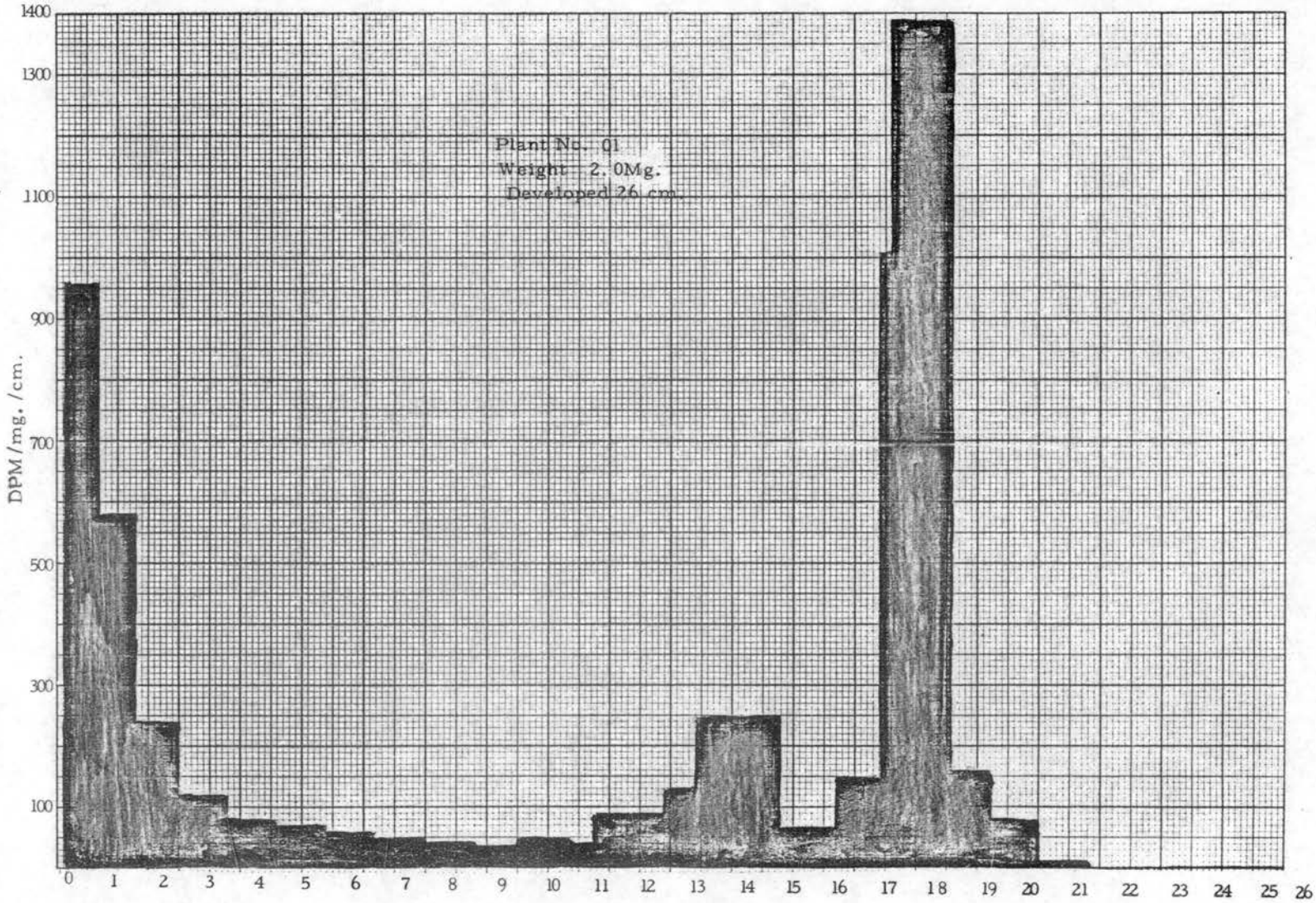


Figure III. Centimeters

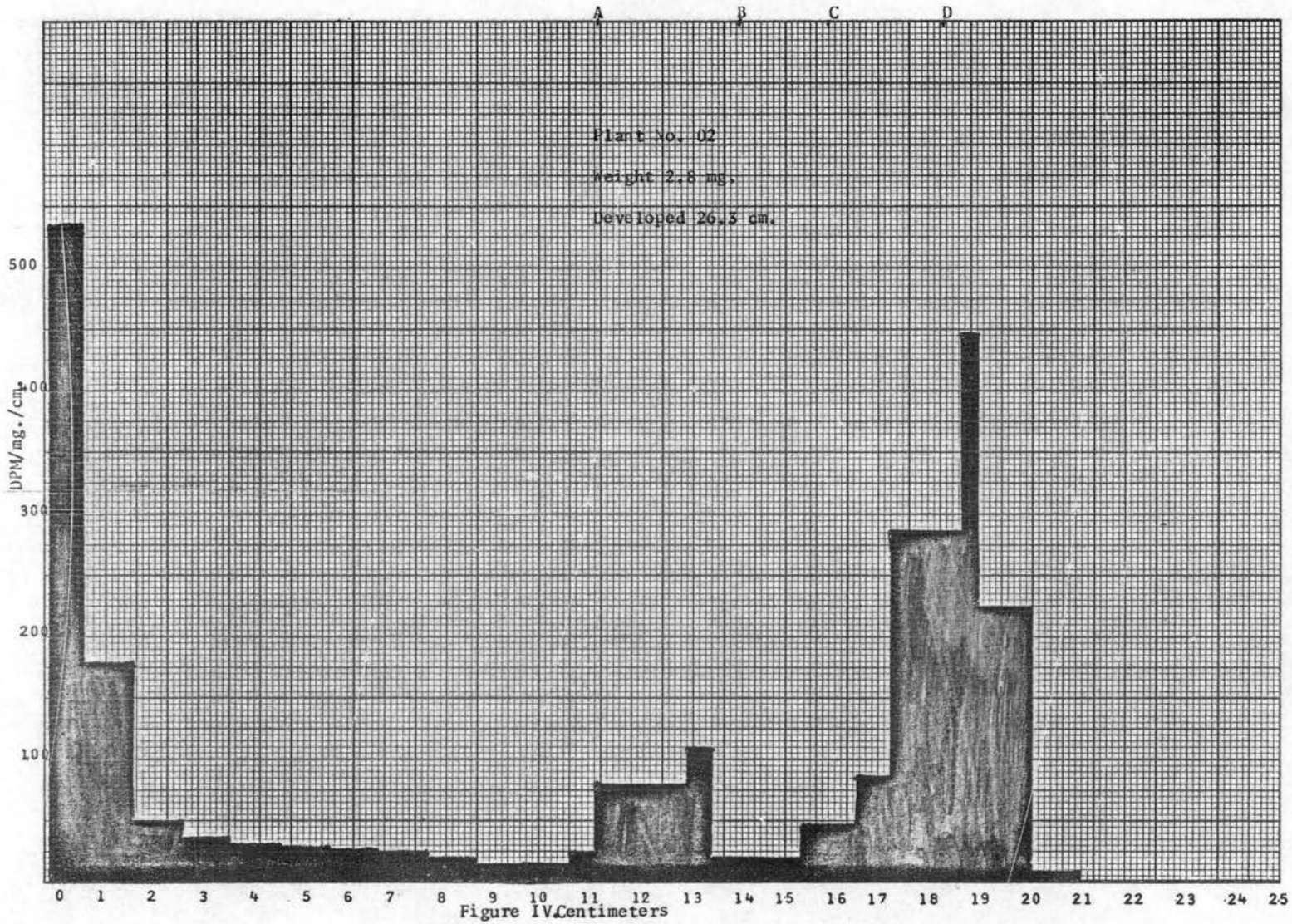

A
B
C
D
Plant No. 02
Weight 2.8 mg.
Developed 26.3 cm.
500
400
300
200
100
DPM/mg./cm.
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Centimeters

Figure IV

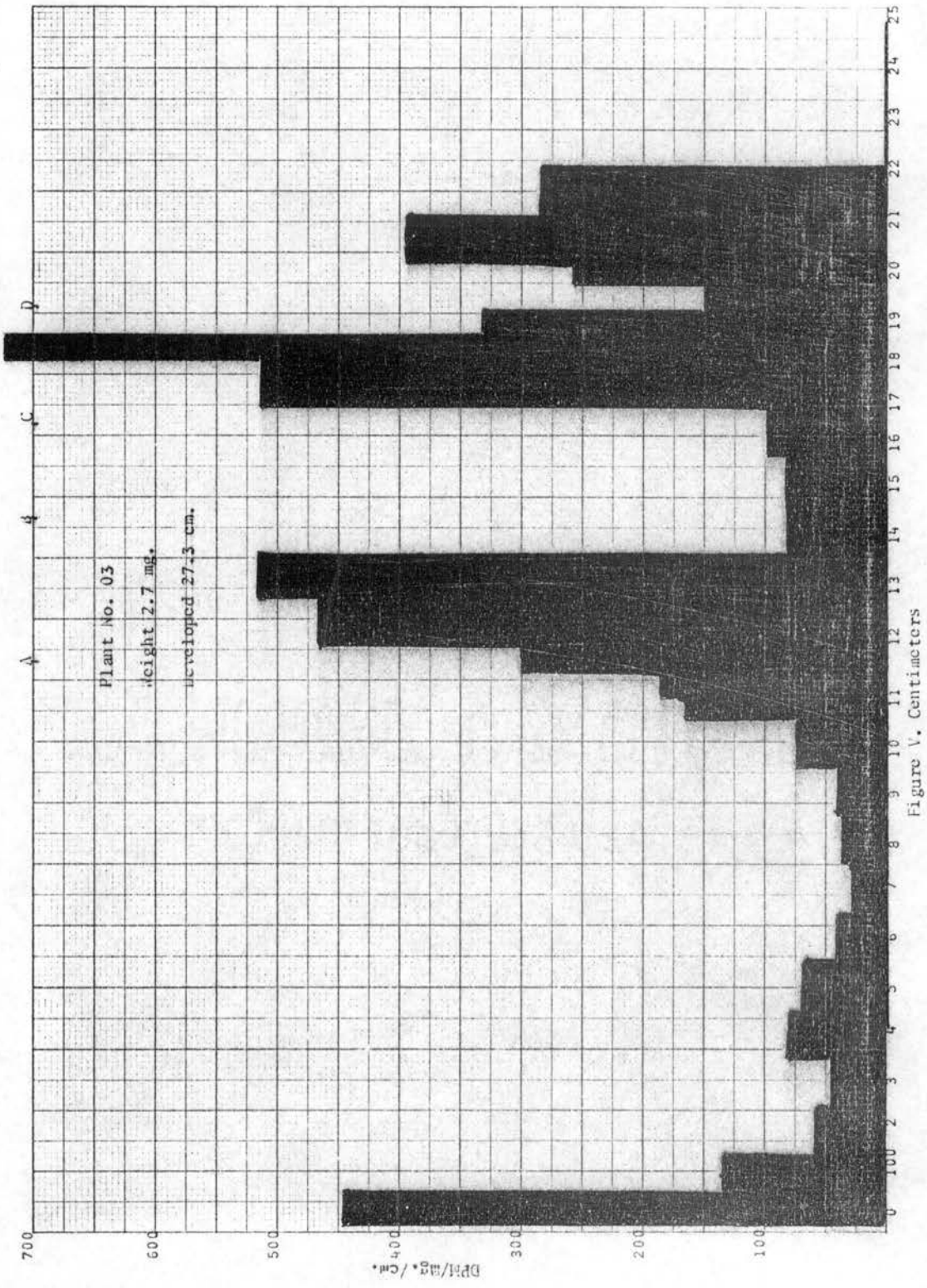


Figure V. Centimeters

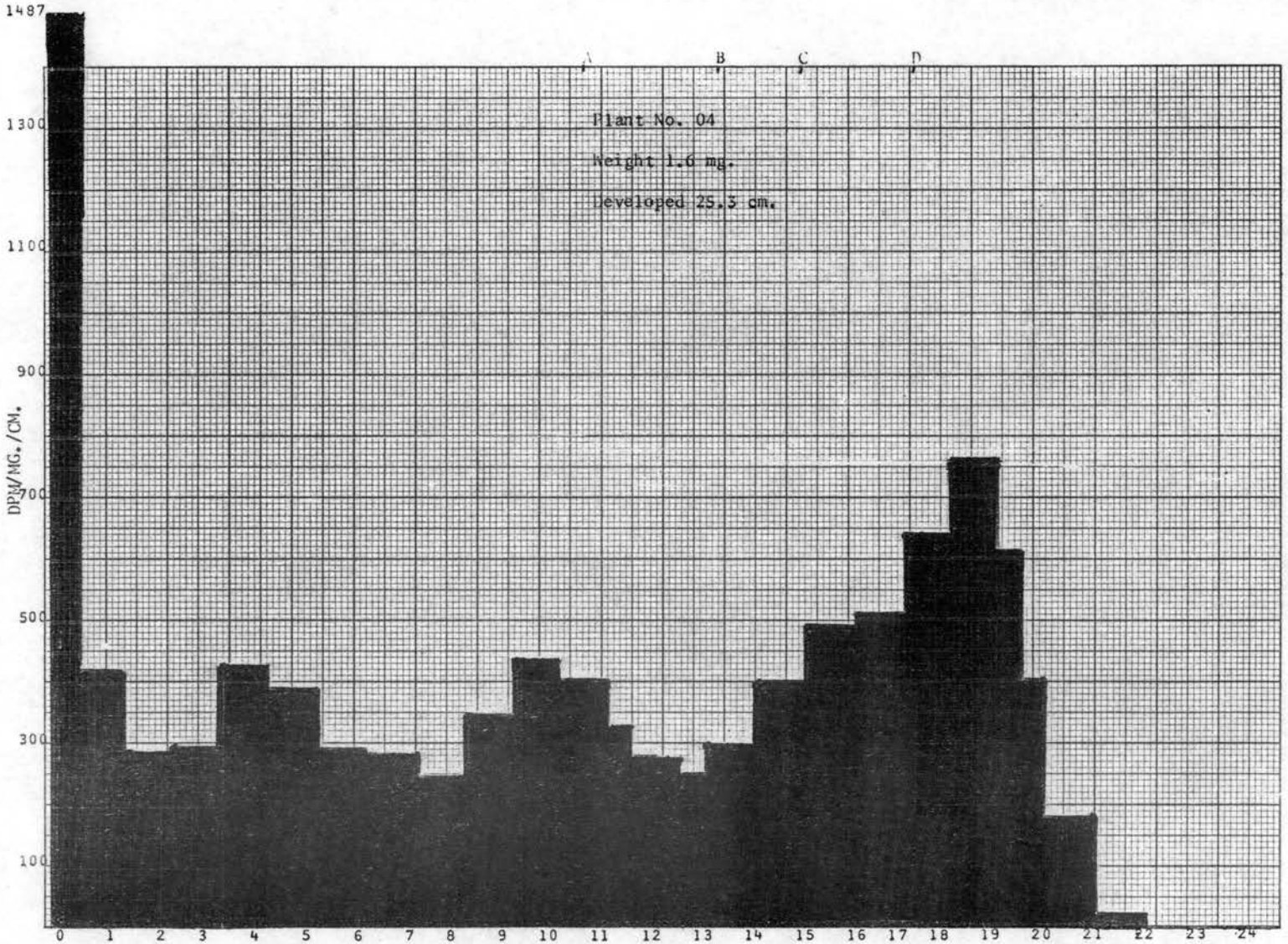


Figure VI. Centimeters

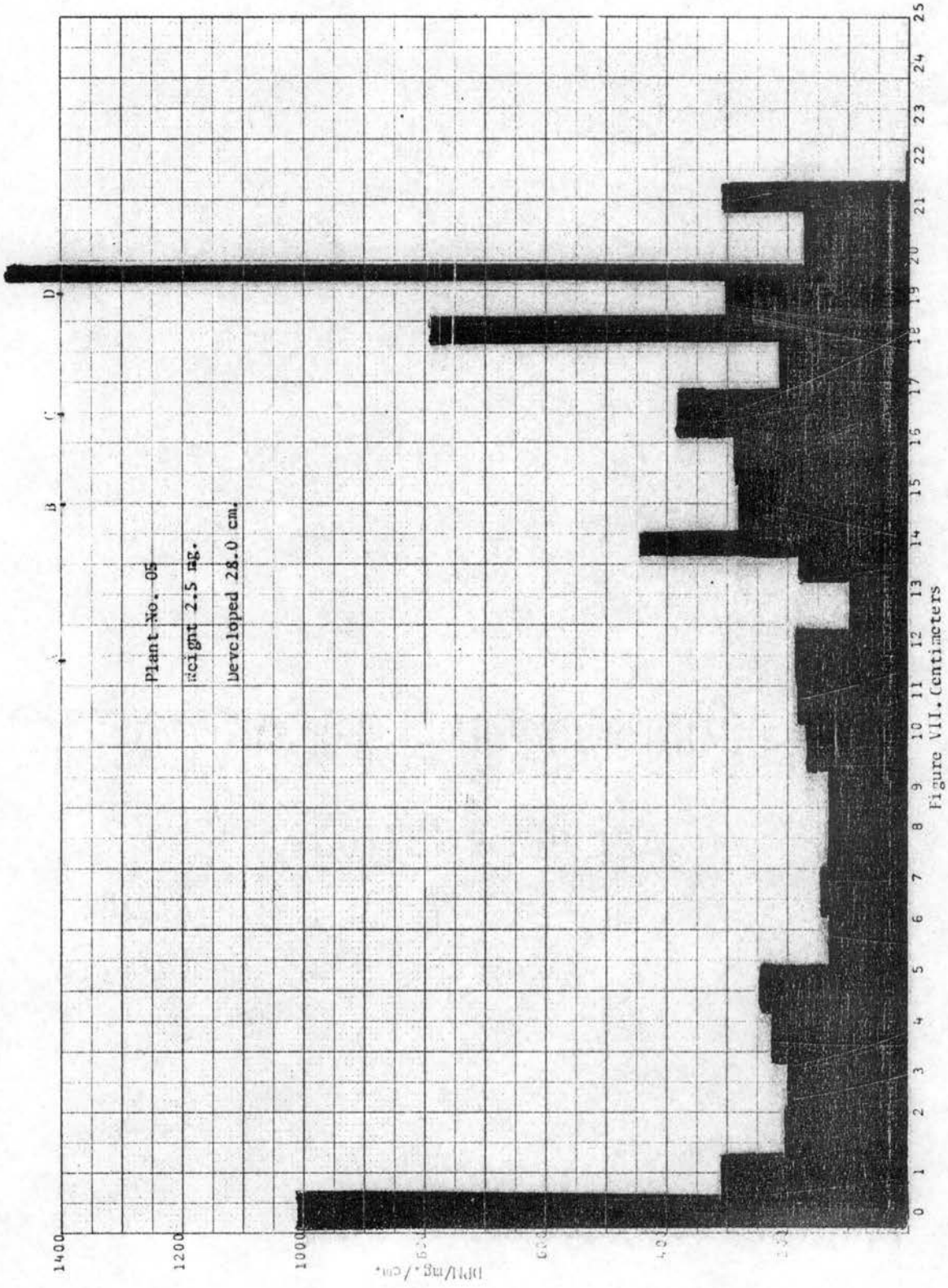


Figure VII. Centimeters

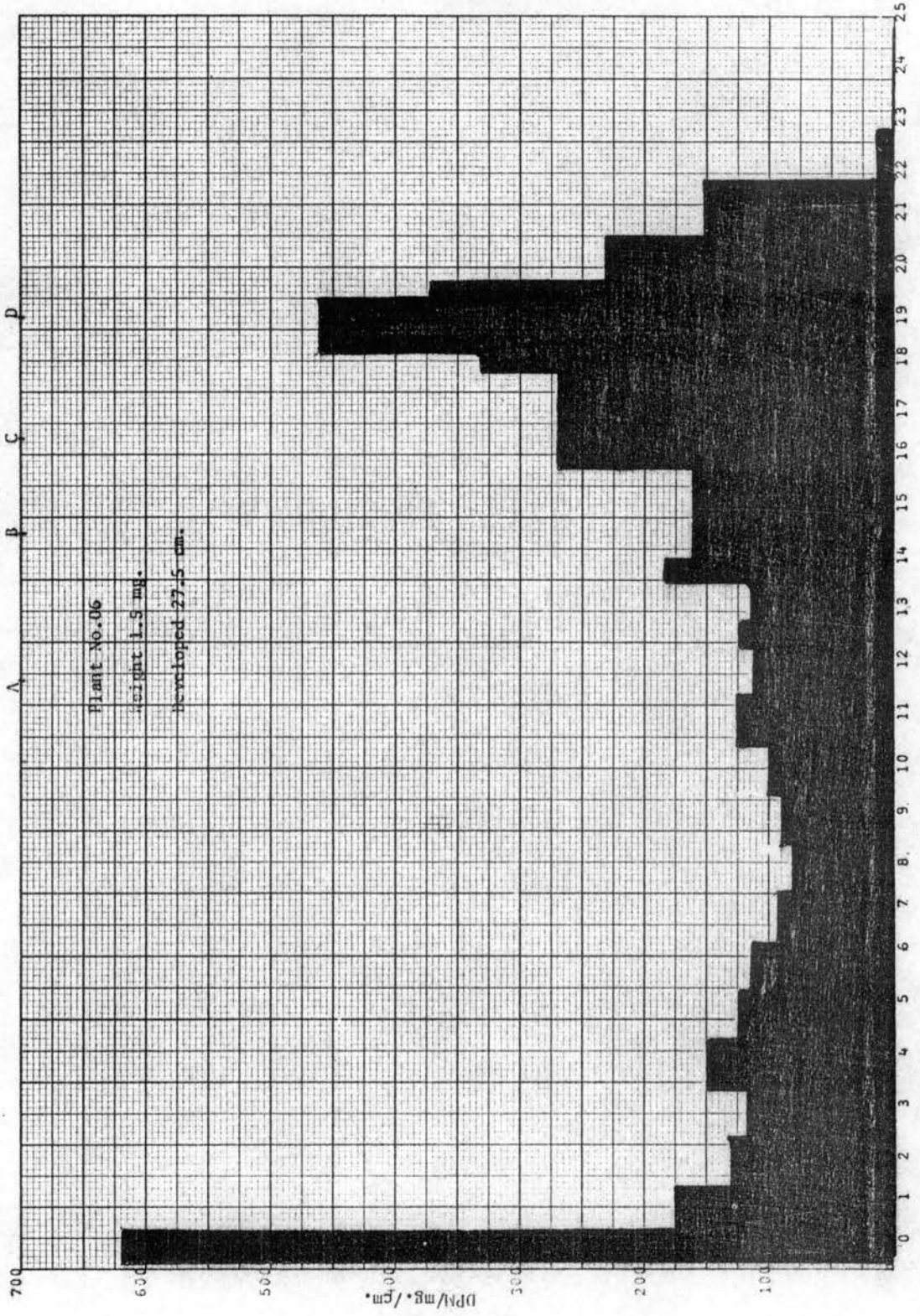
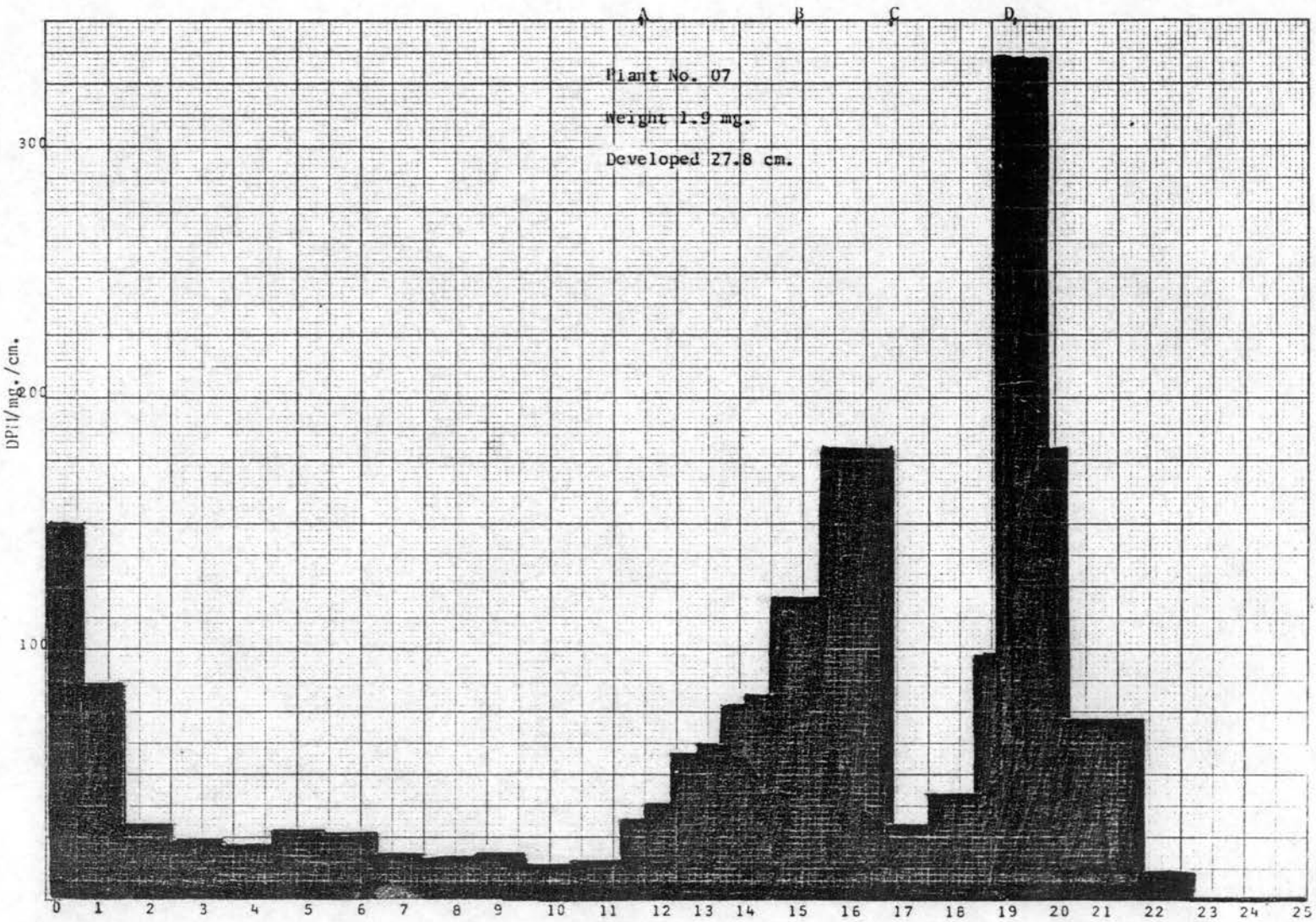


Figure VIII. Centimeters


A
B
C
D
Plant No. 07
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Developed 27.8 cm.
300
200
100
DPI/mg./cm.
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Centimeters

Figure IX.

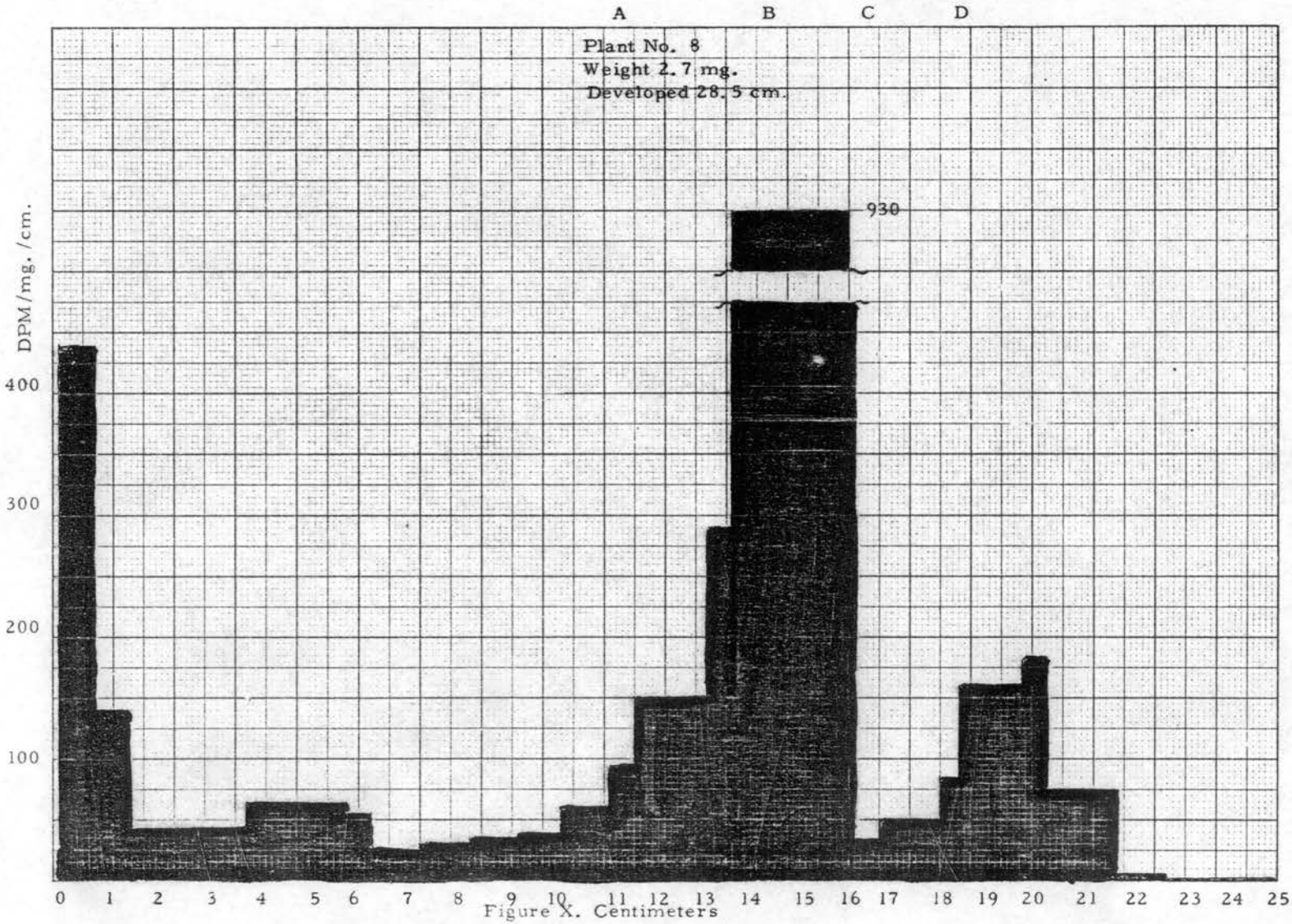

A
B
C
D
Plant No. 8
Weight 2.7 mg.
Developed 28.5 cm.
930
DPM/mg./cm.
400
300
200
100
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Centimeters

Figure X.

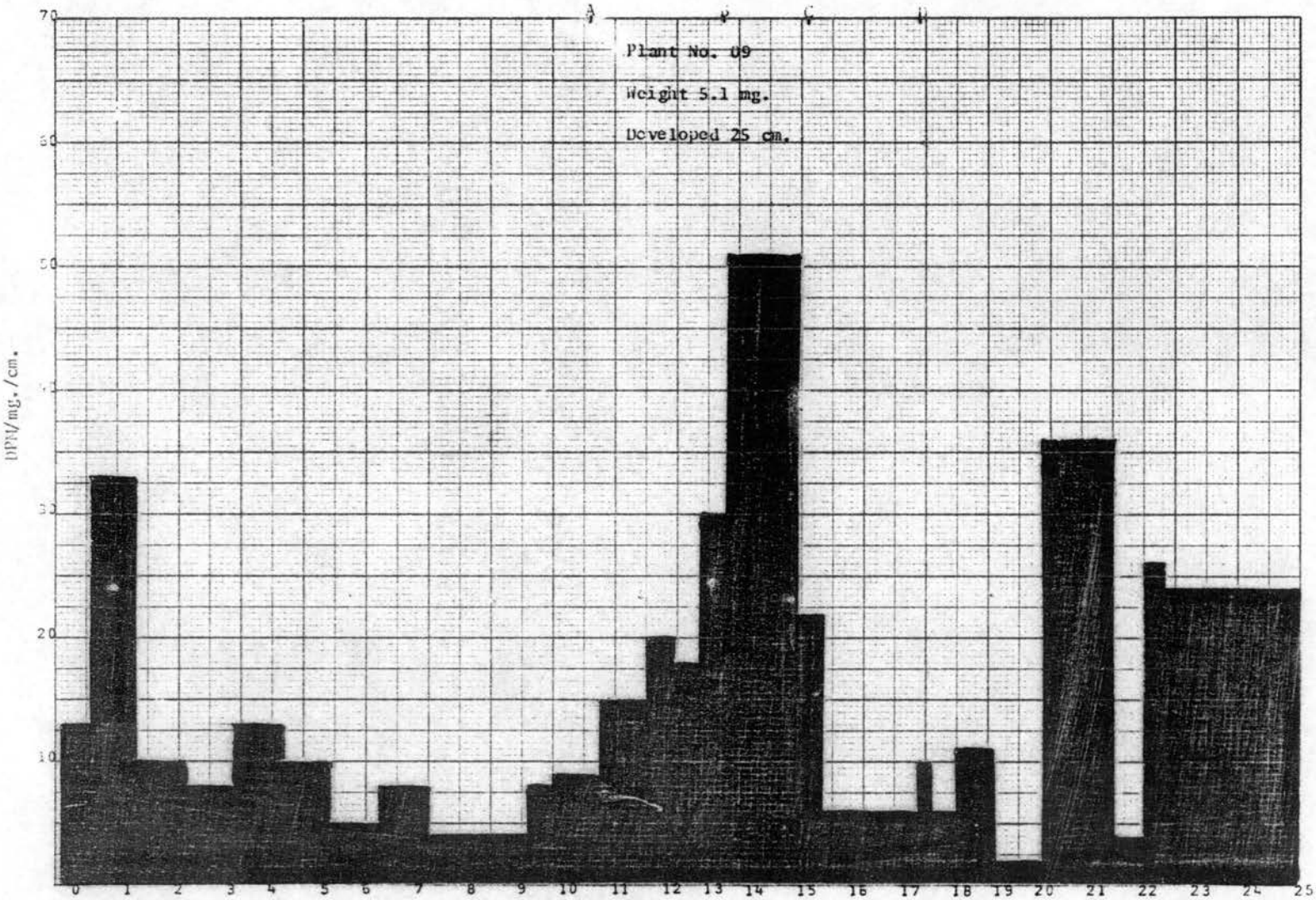

A
B
C
D
Plant No. 09
Weight 5.1 mg.
Developed 25 cm.
DPM/mg./cm.
70
60
50
40
30
20
10
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Centimeters

Figure XI.

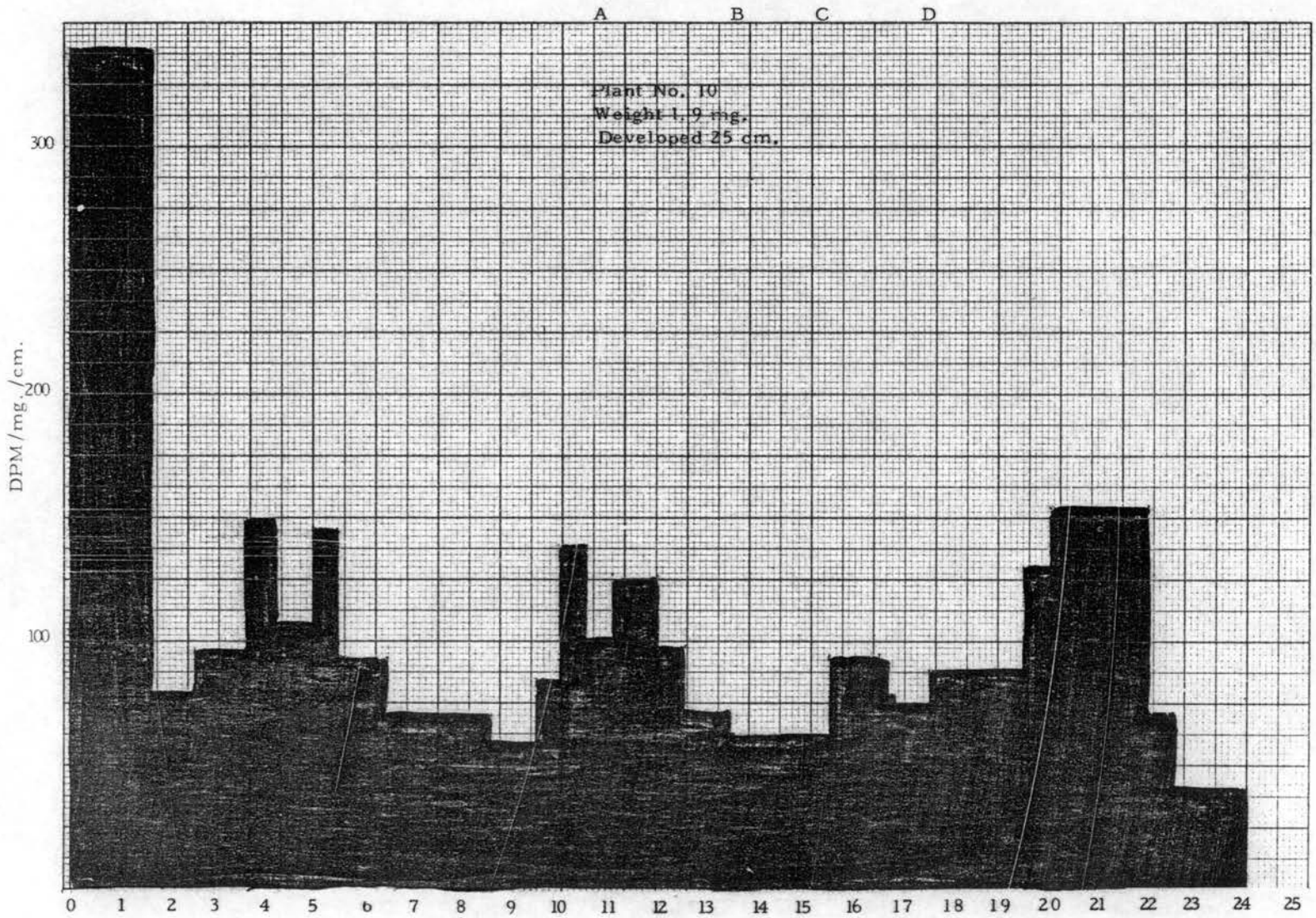


Figure XII. Centimeters

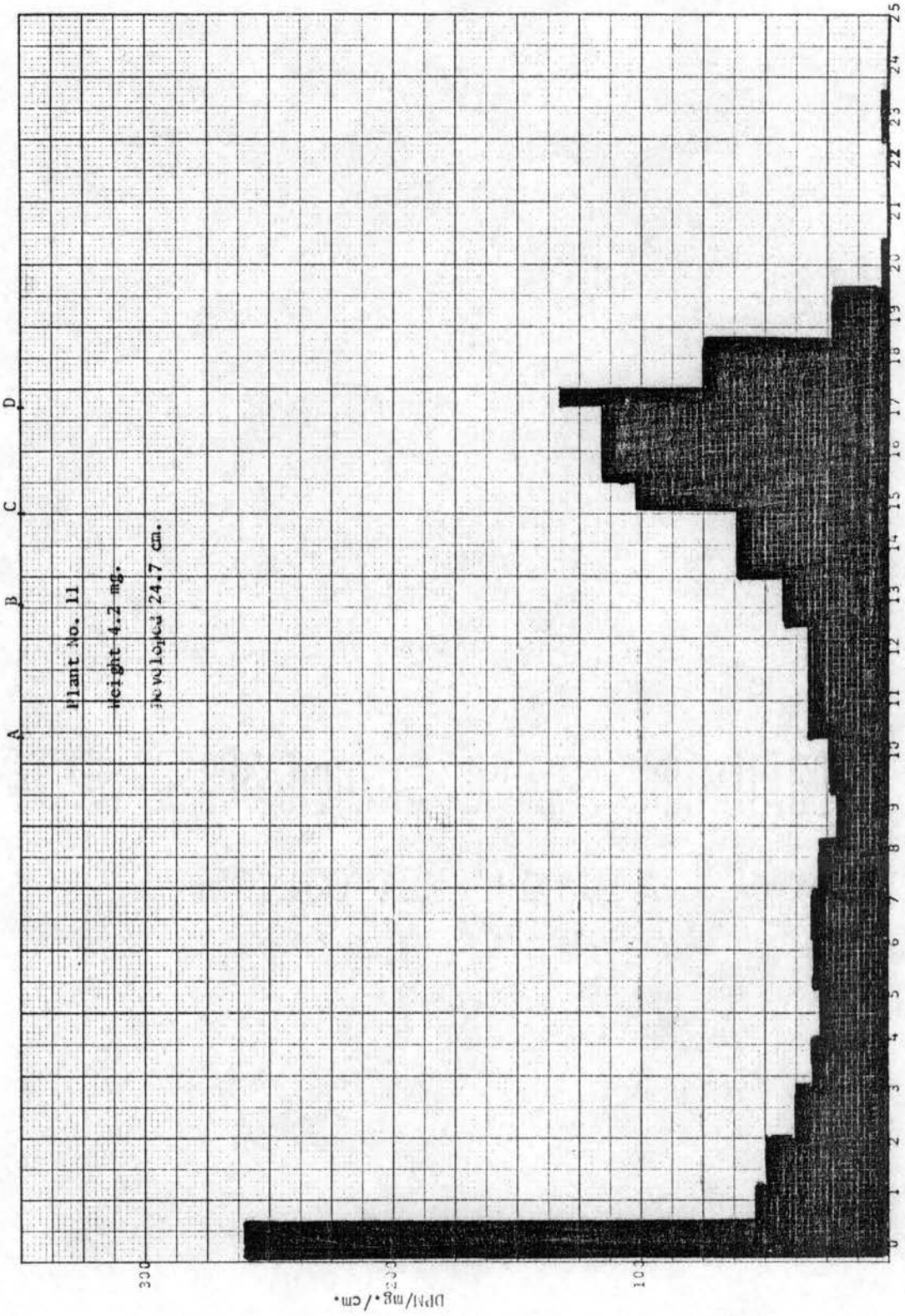


Figure XIII. Centimeters

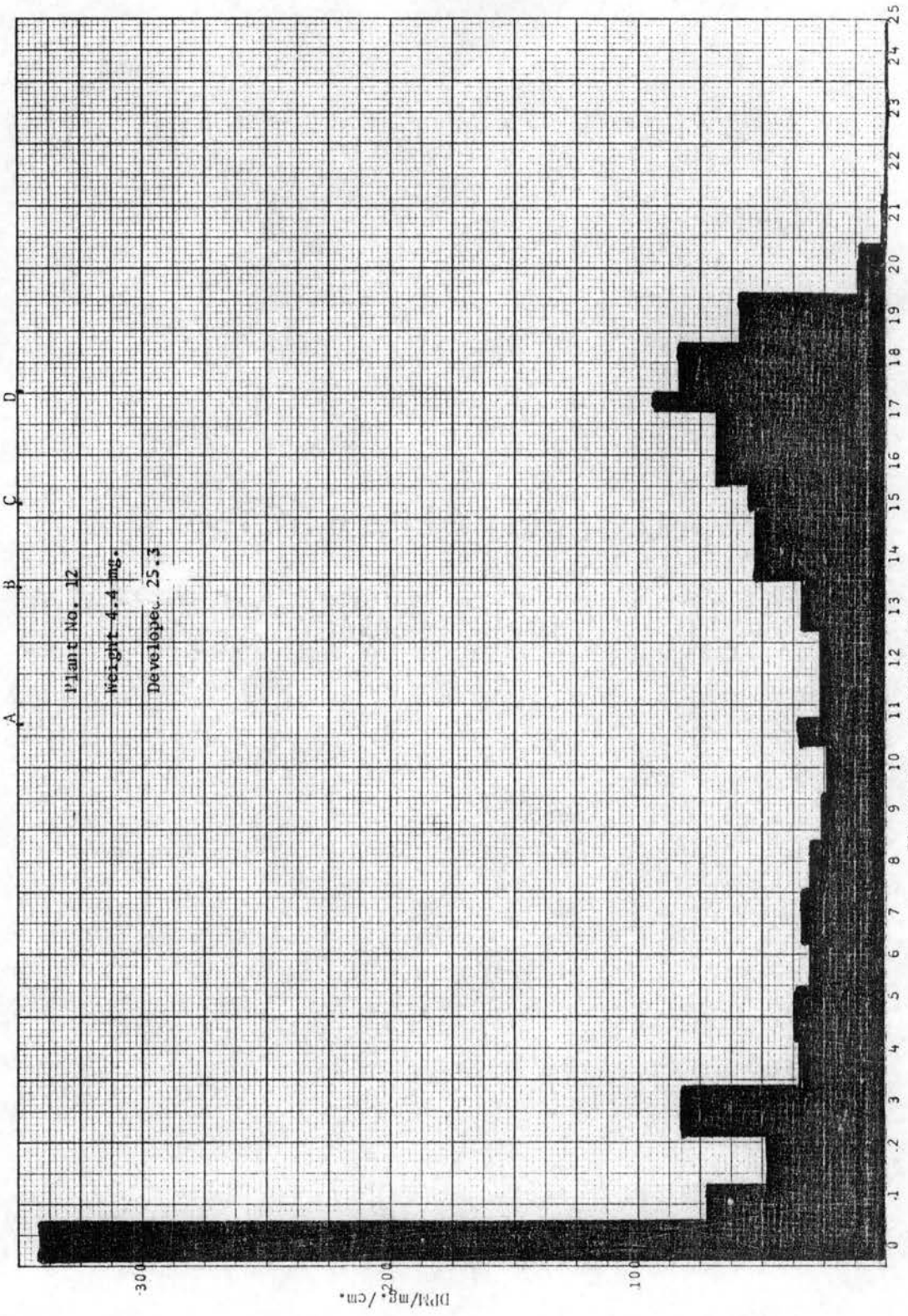


Figure XIV. Centimeters

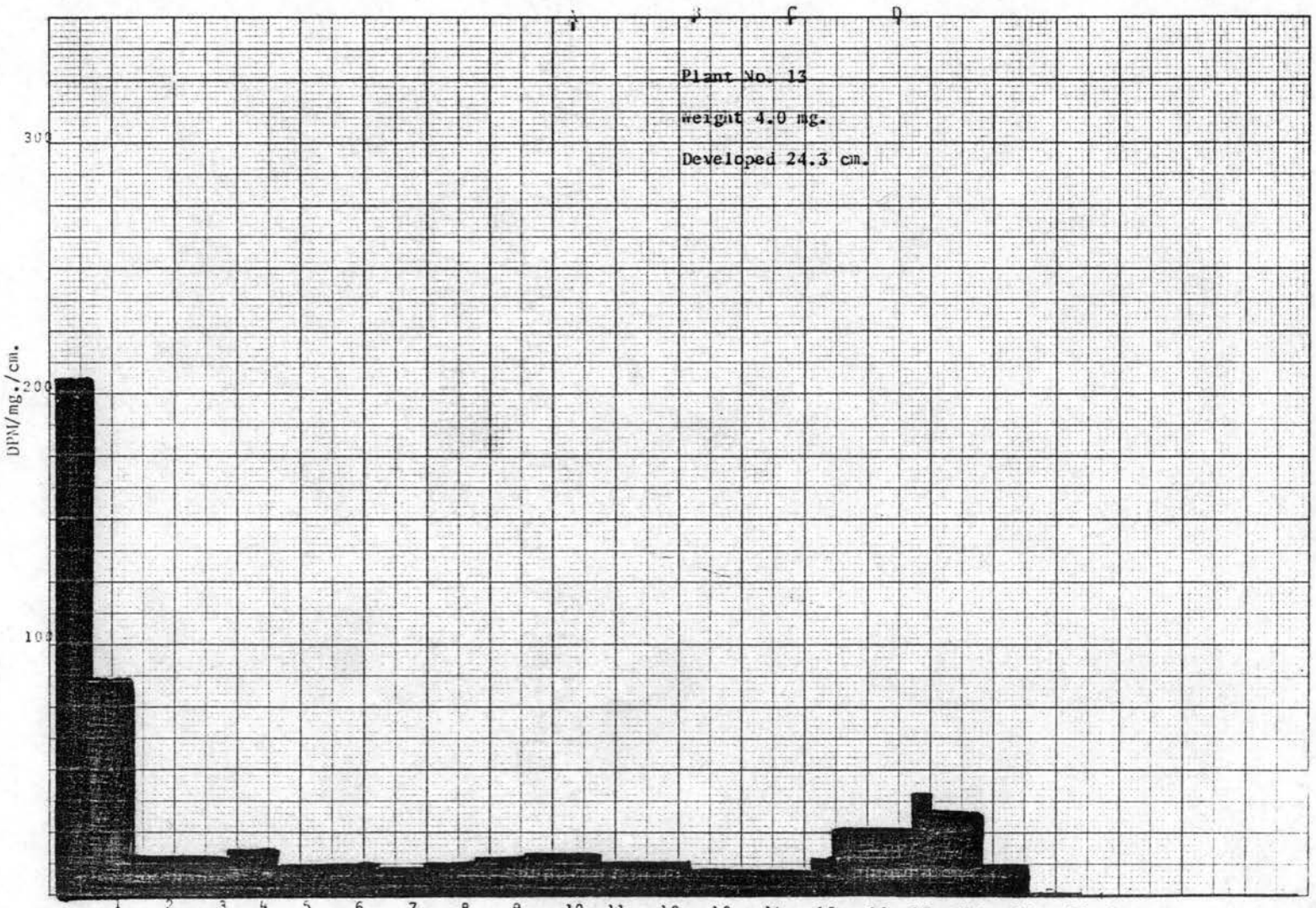


Figure XV. Centimeters

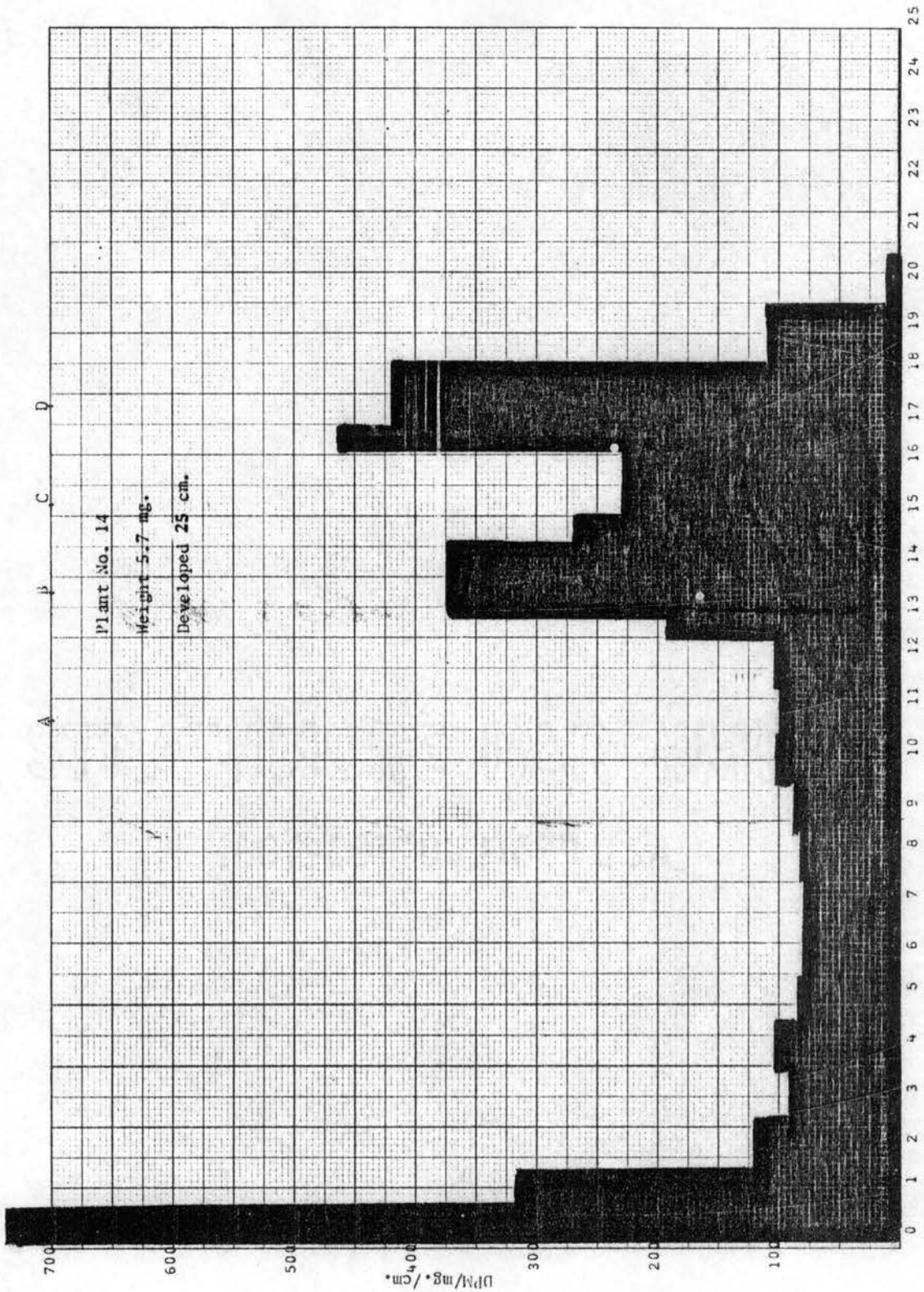


Figure XVI. Centimeters

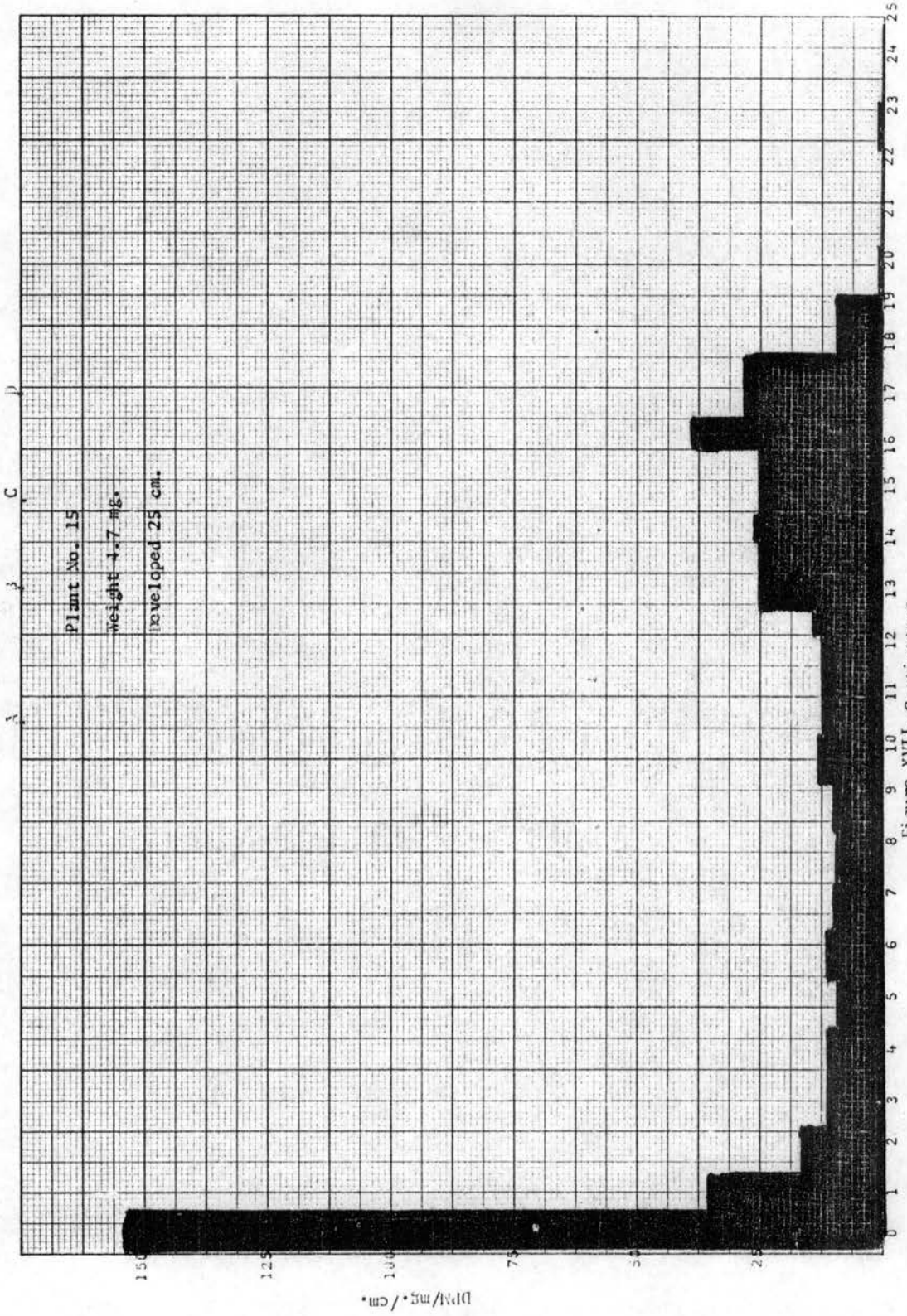
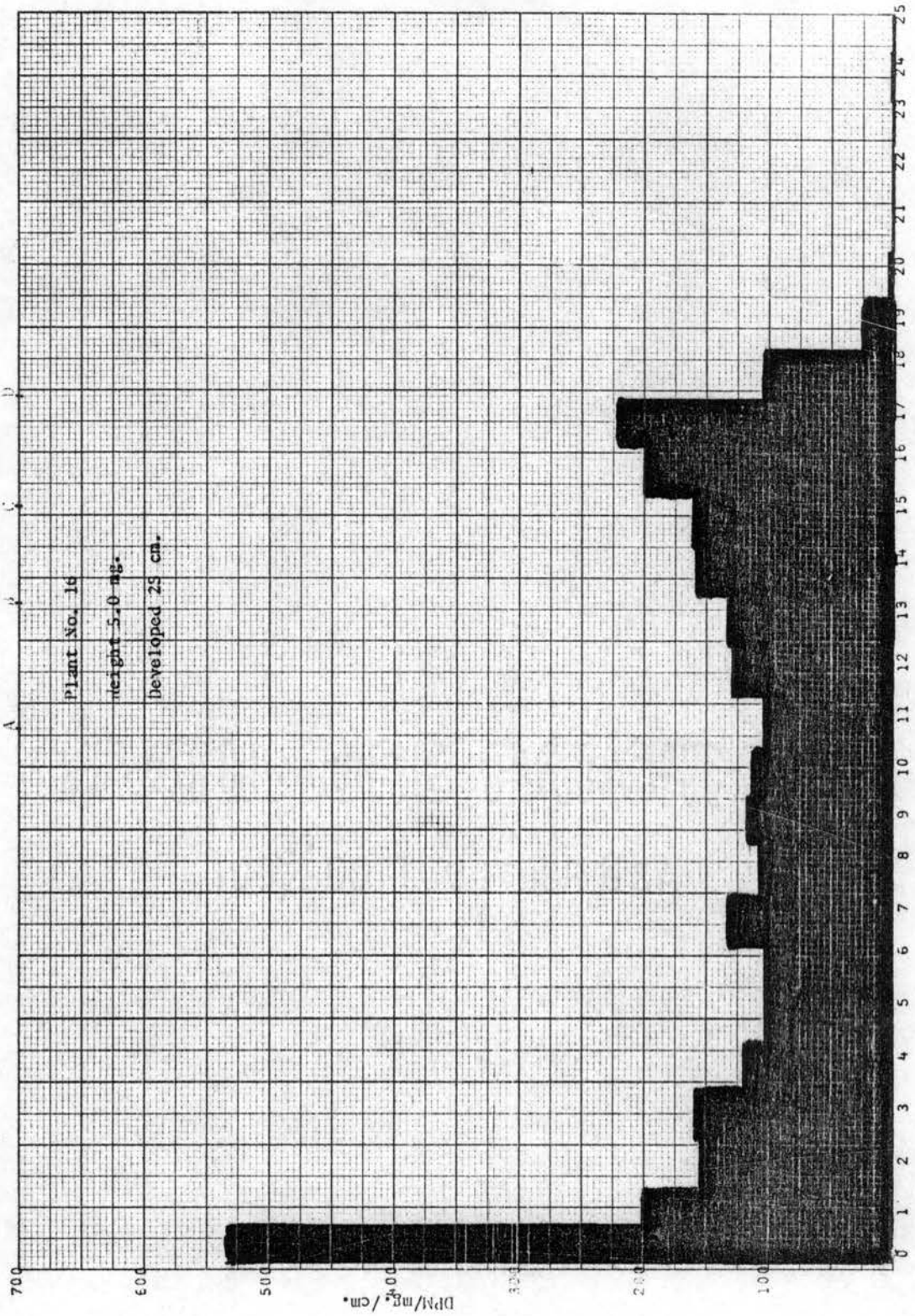


Figure XVII. Centimeters



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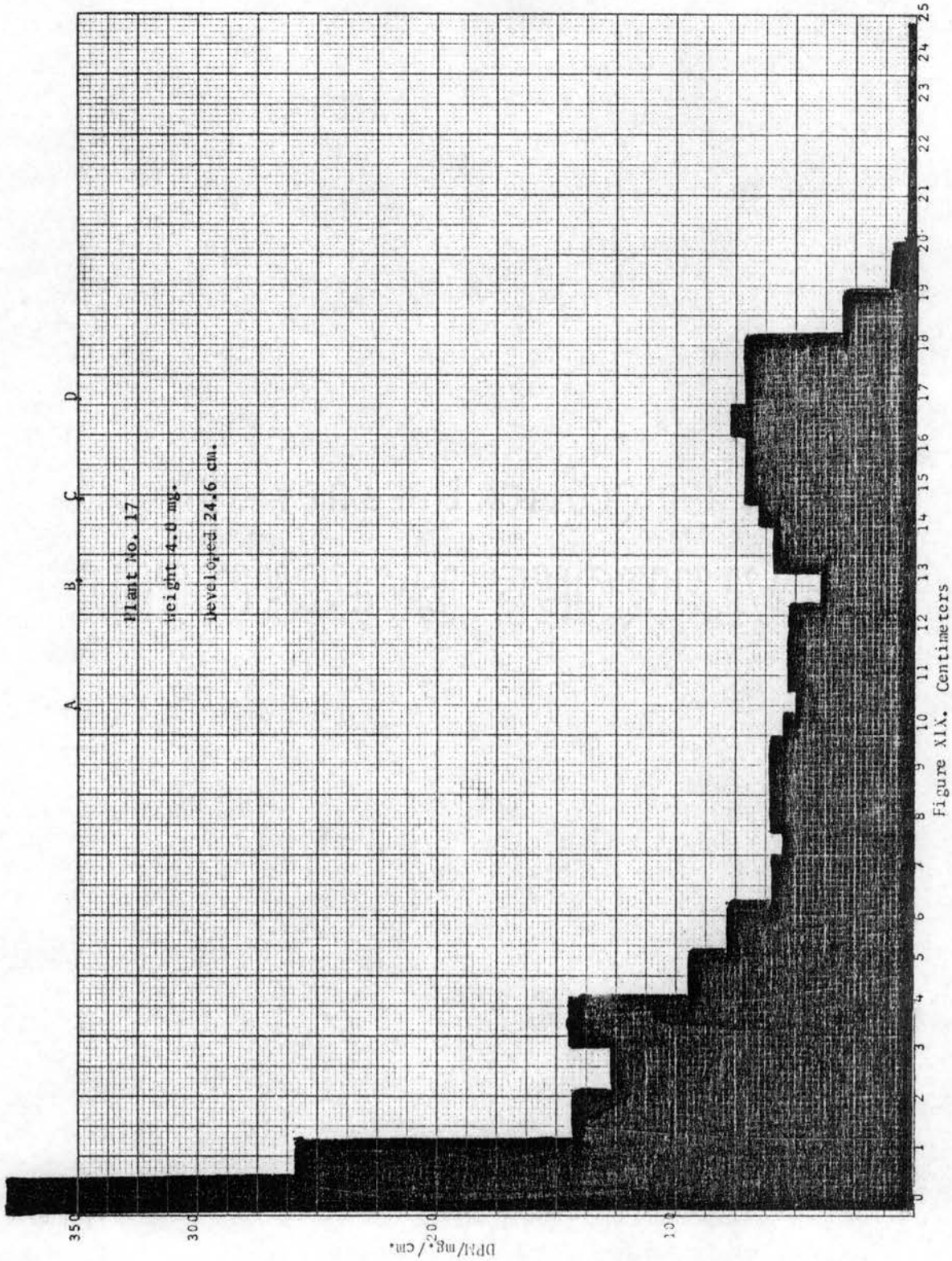


Figure XIX. Centimeters

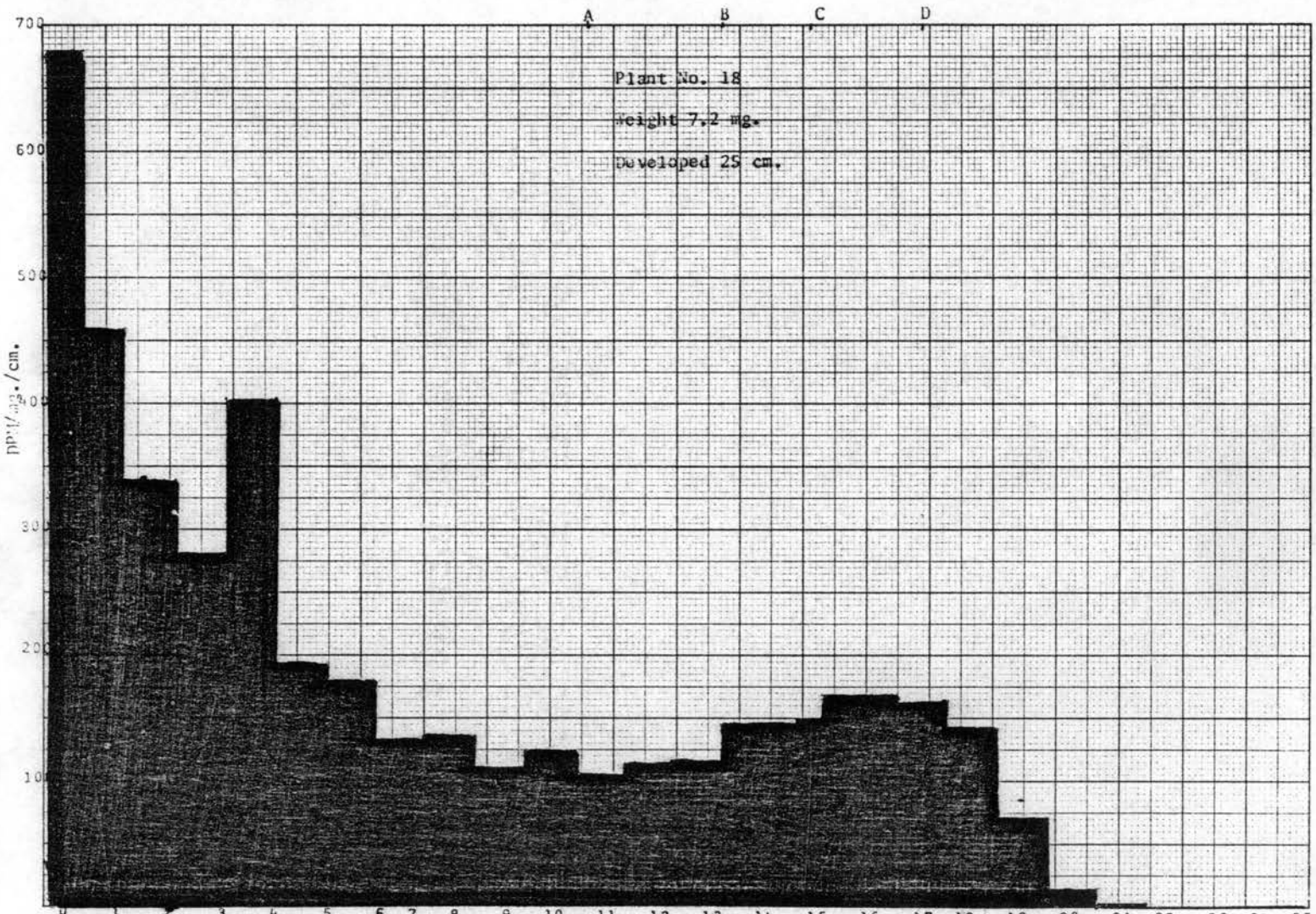

A
B
C
D
Plant No. 18
Weight 7.2 mg.
Developed 25 cm.
700
600
500
400
300
200
100
mμg./cm.
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Centimeters

Figure XX.

Plate I

Infrared Spectrum of Ajaconine

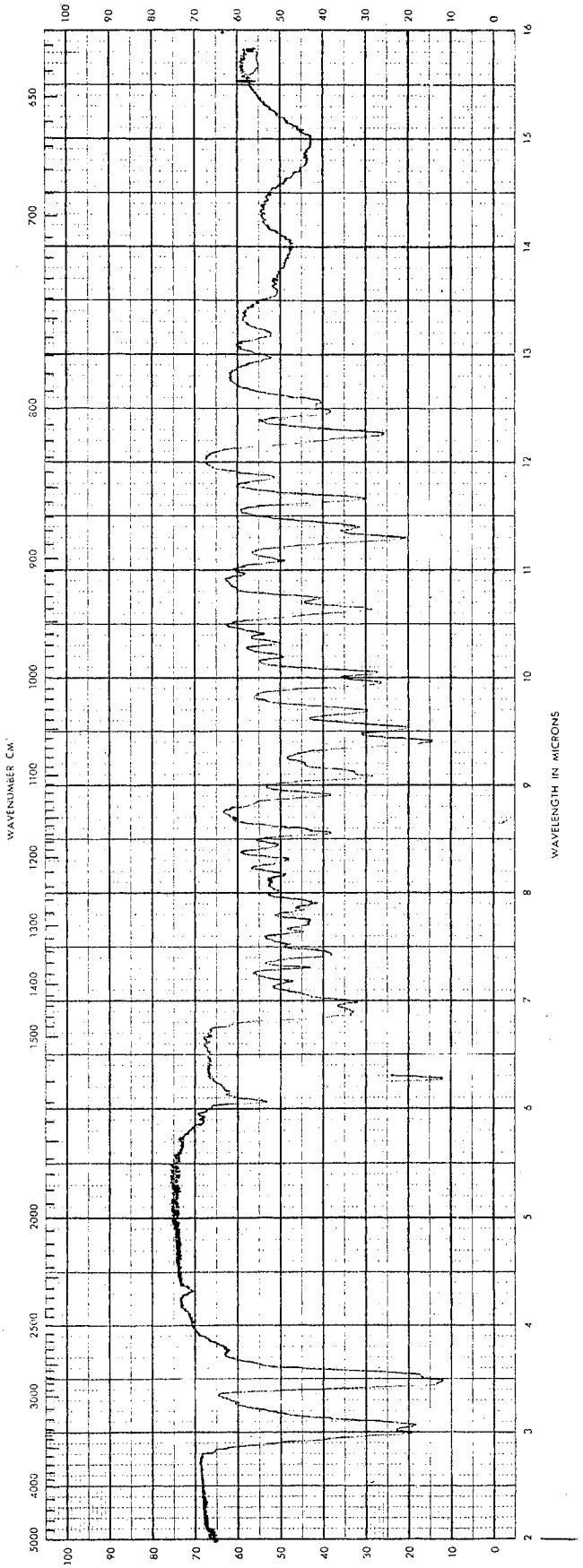


Plate II

Infrared Spectrum of Delcosine

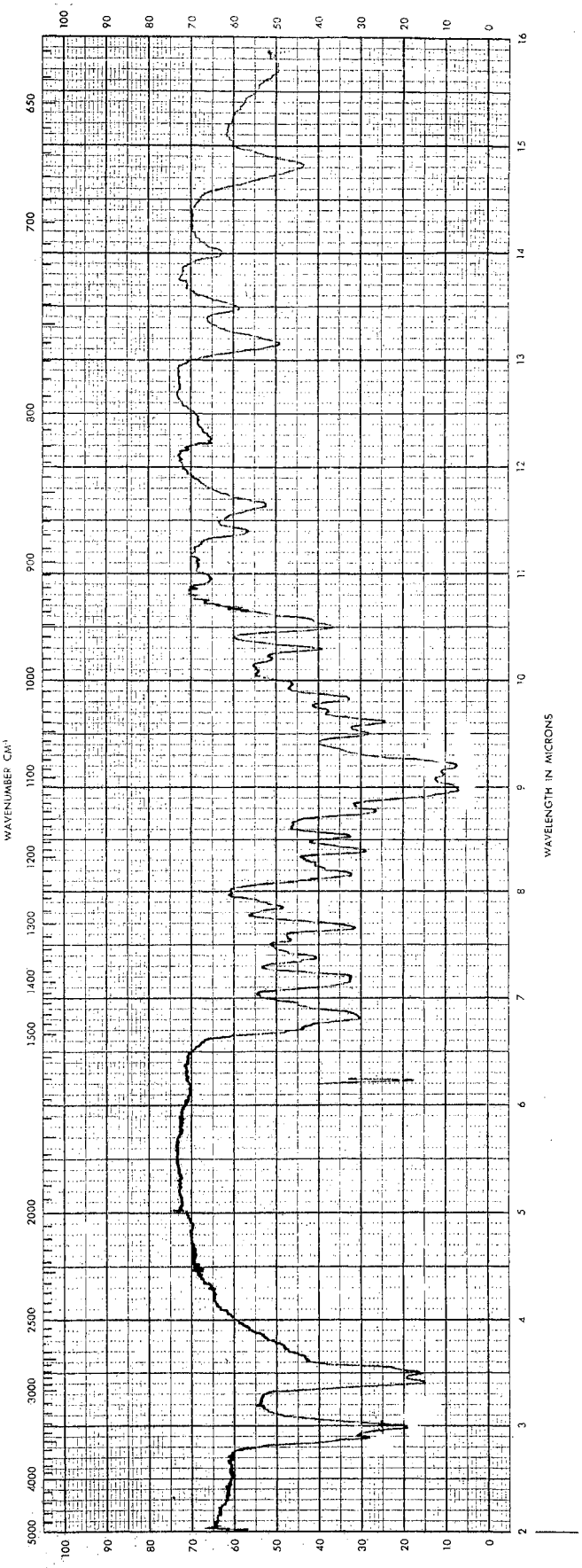


Plate III

Infrared Spectrum of O-Acetyldelcosine

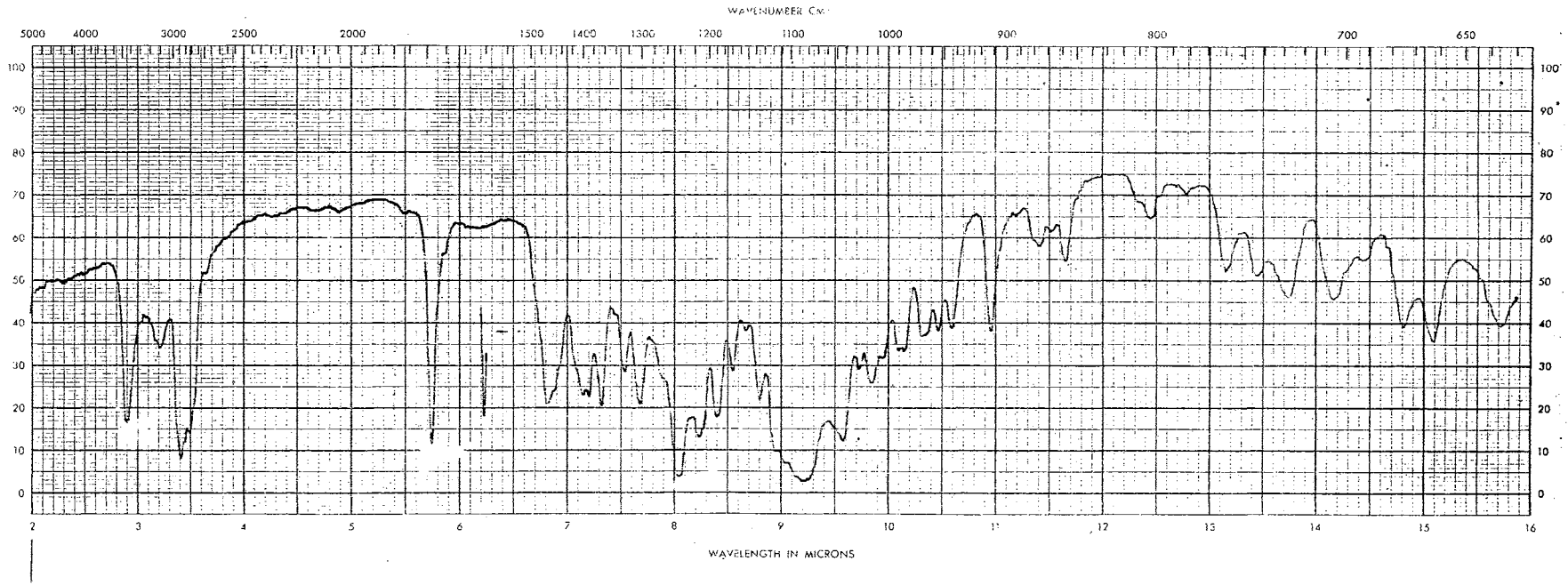


Plate IV

Infrared Spectrum of LBA-3

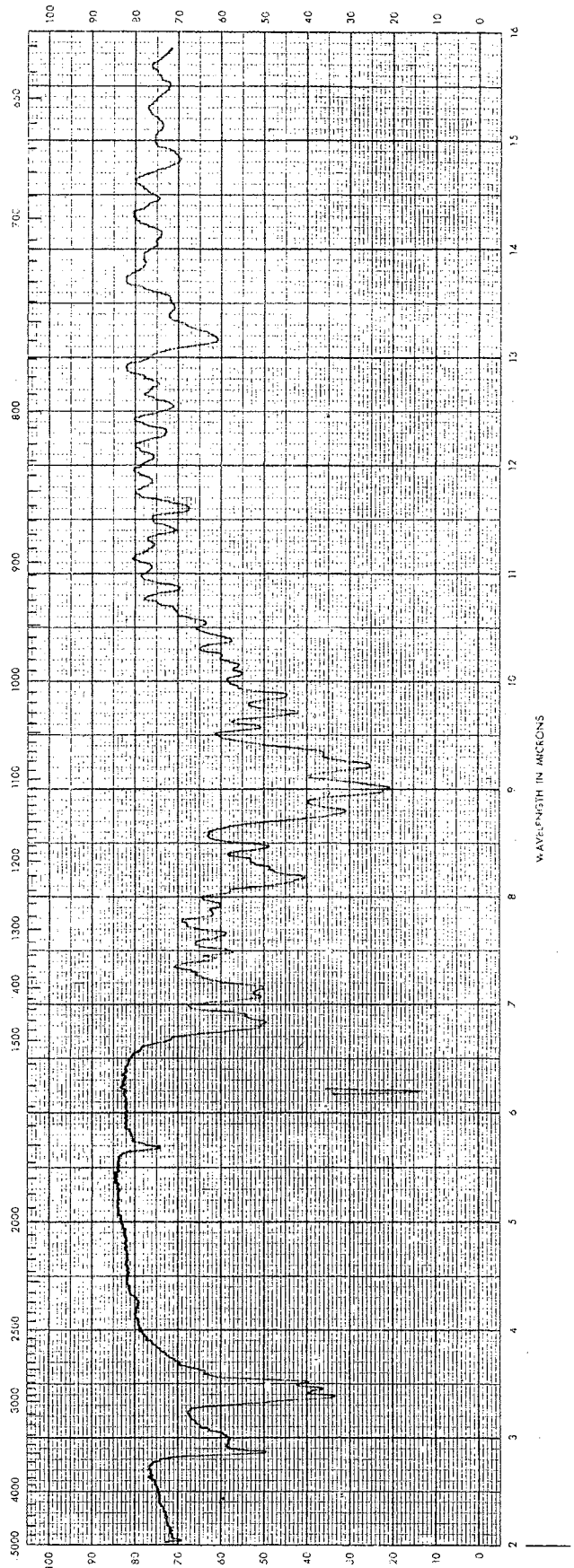


Plate V

Nuclear Magnetic Resonance Spectrum of Ajaconine

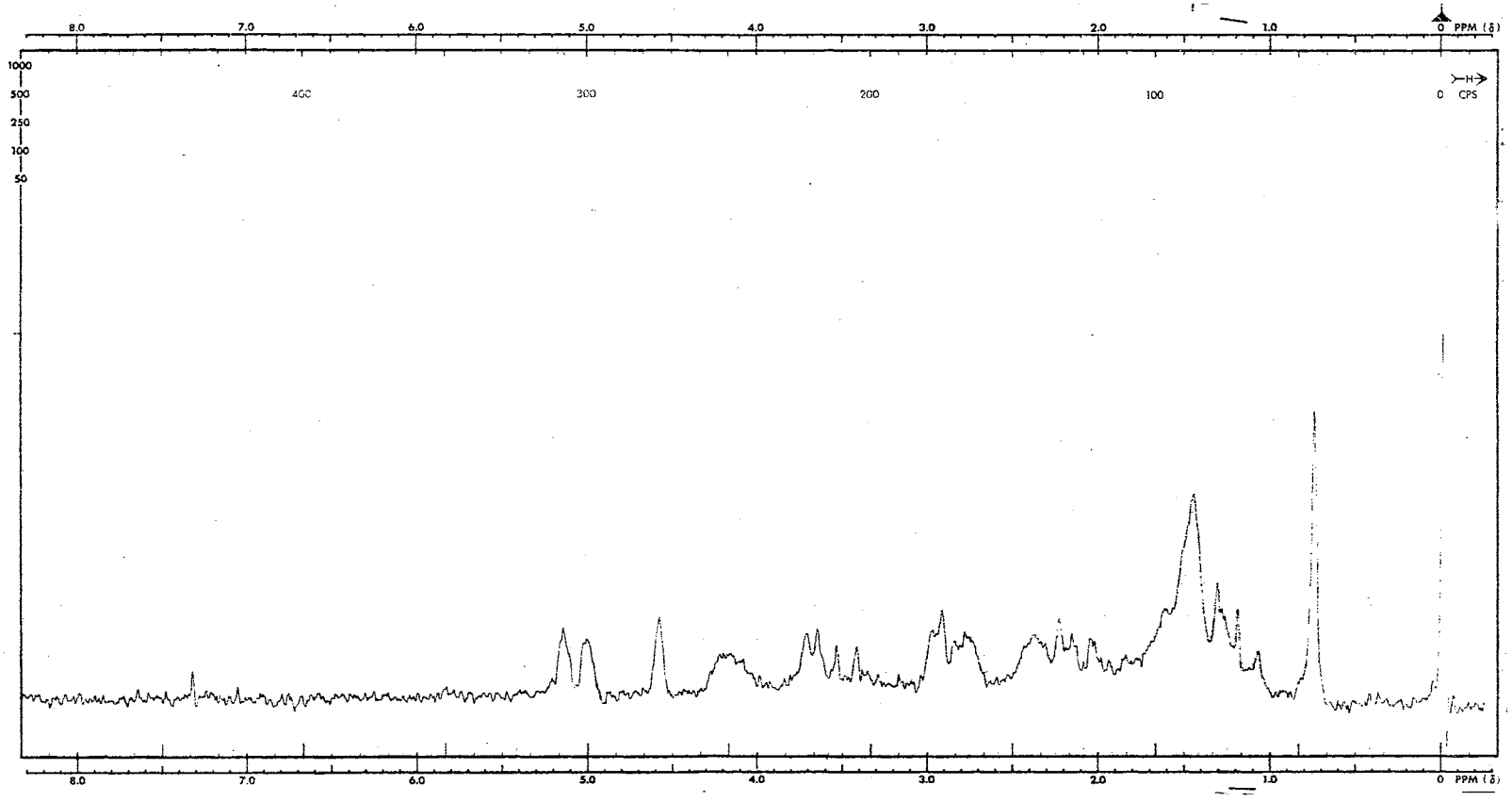


Plate VI

Nuclear Magnetic Resonance Spectrum of Delcosine

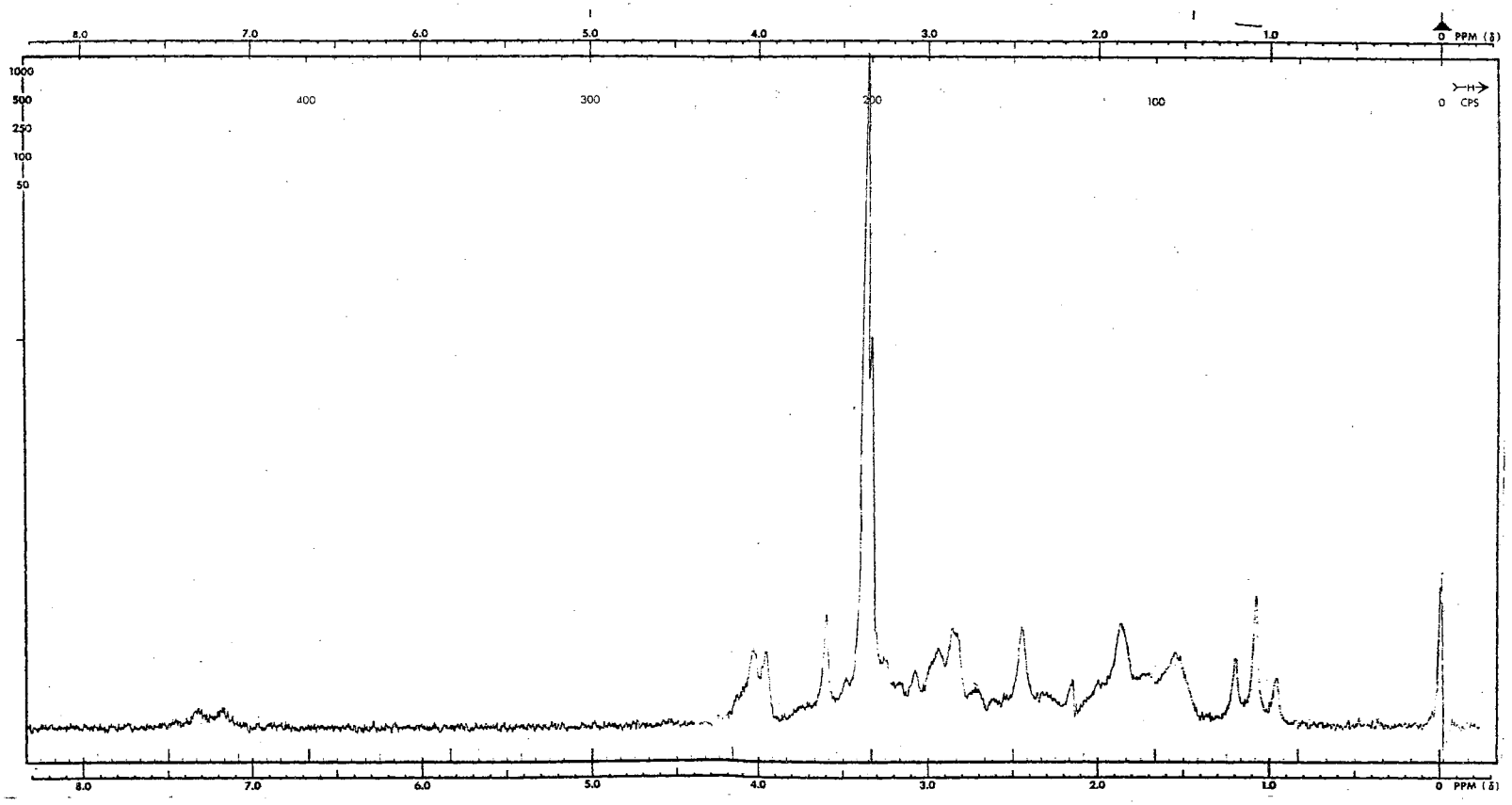


Plate VII

Nuclear Magnetic Resonance Spectrum of O-Acetyldelcosine

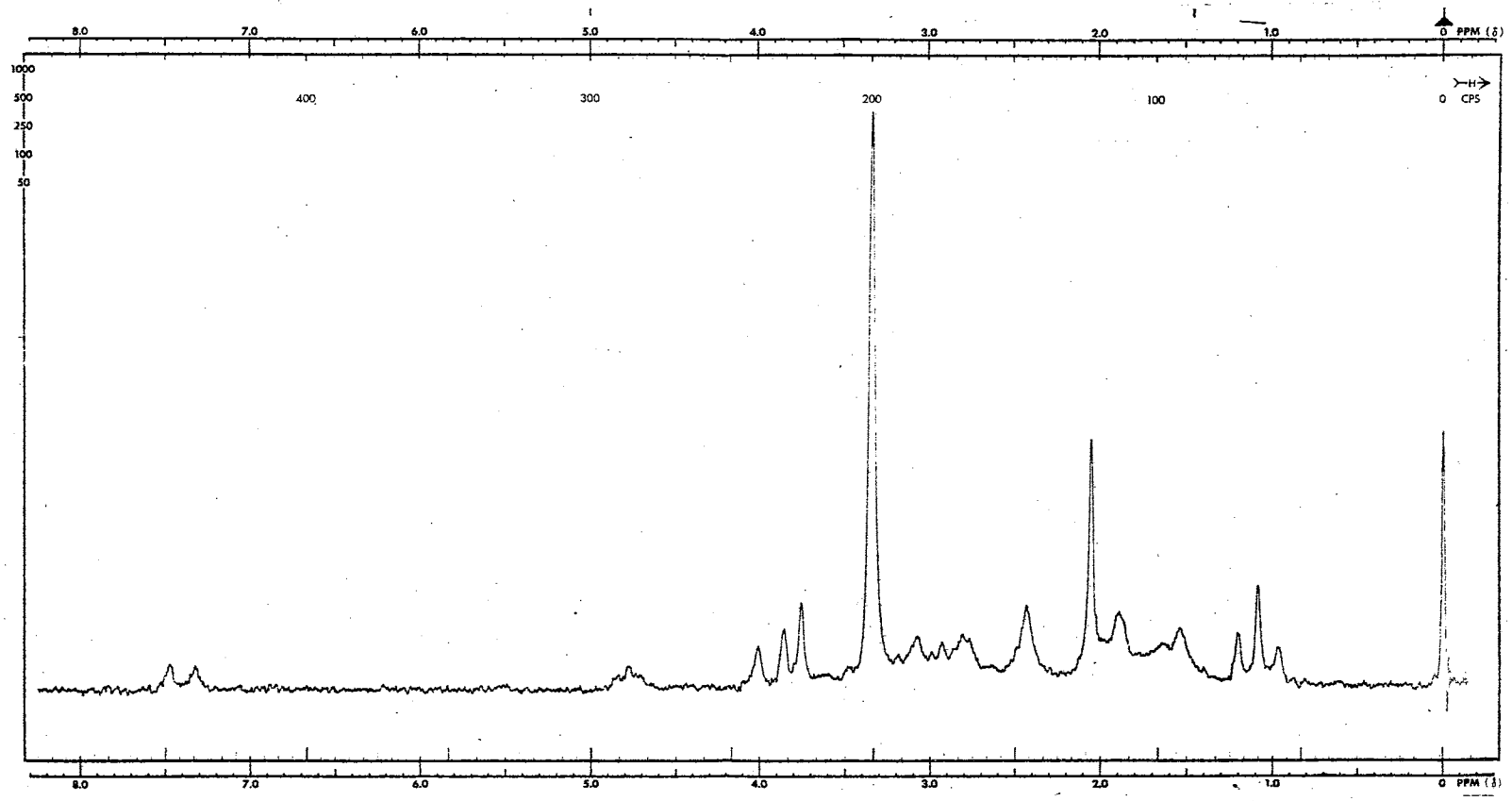


Plate VIII

Nuclear Magnetic Resonance Spectrum of LBA-3

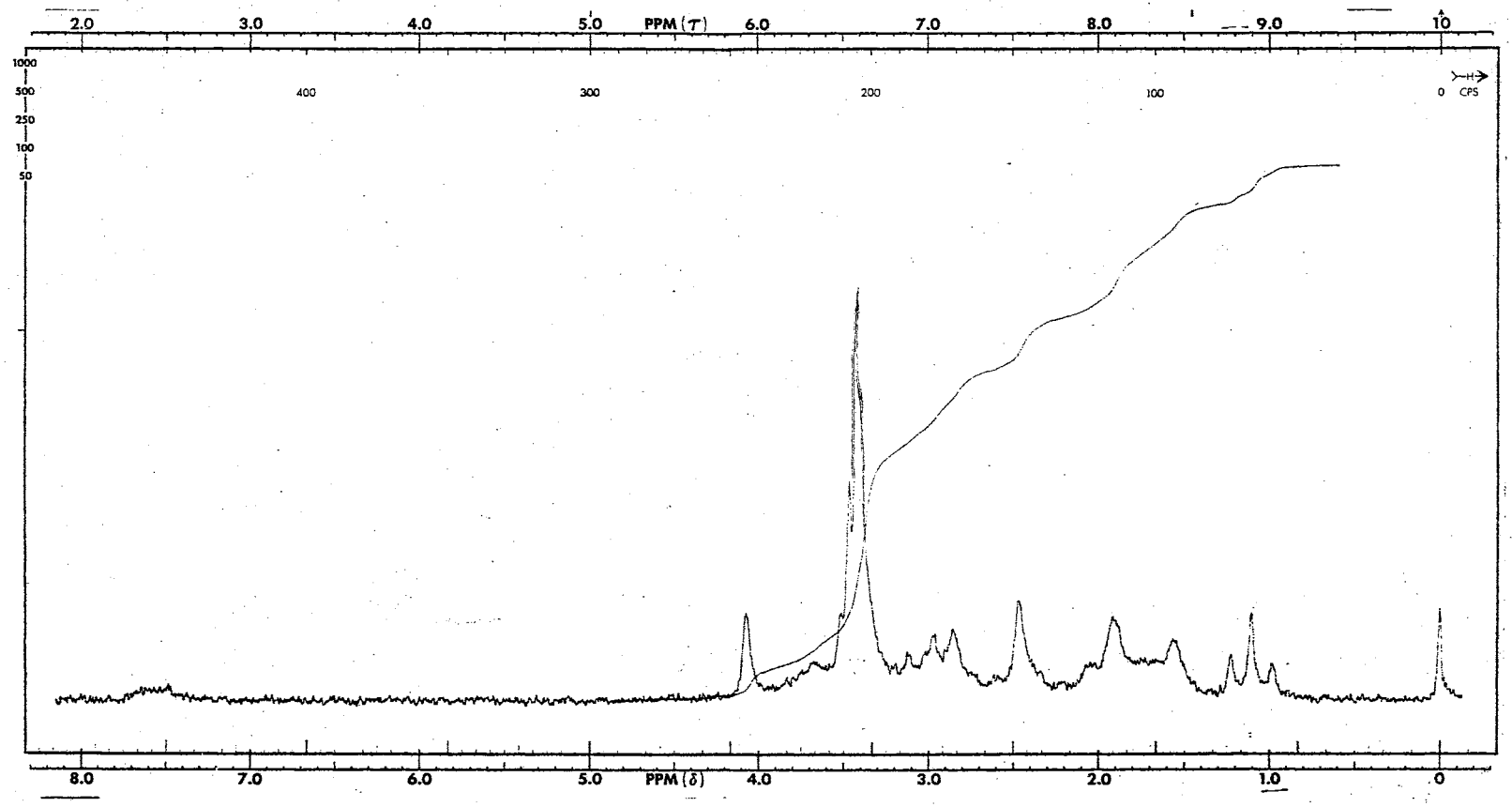


Plate IX

Mass Spectrum of Ajaconine

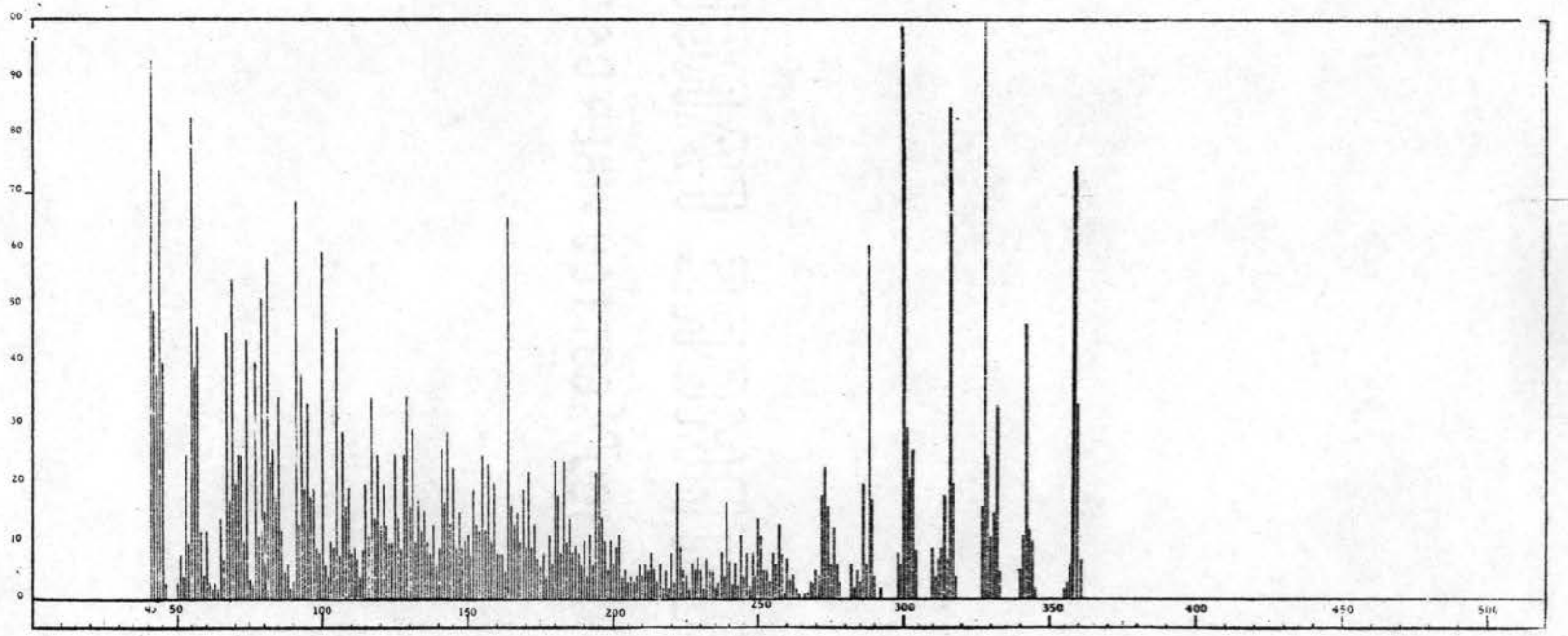


Plate X

Mass Spectrum of Delcosine

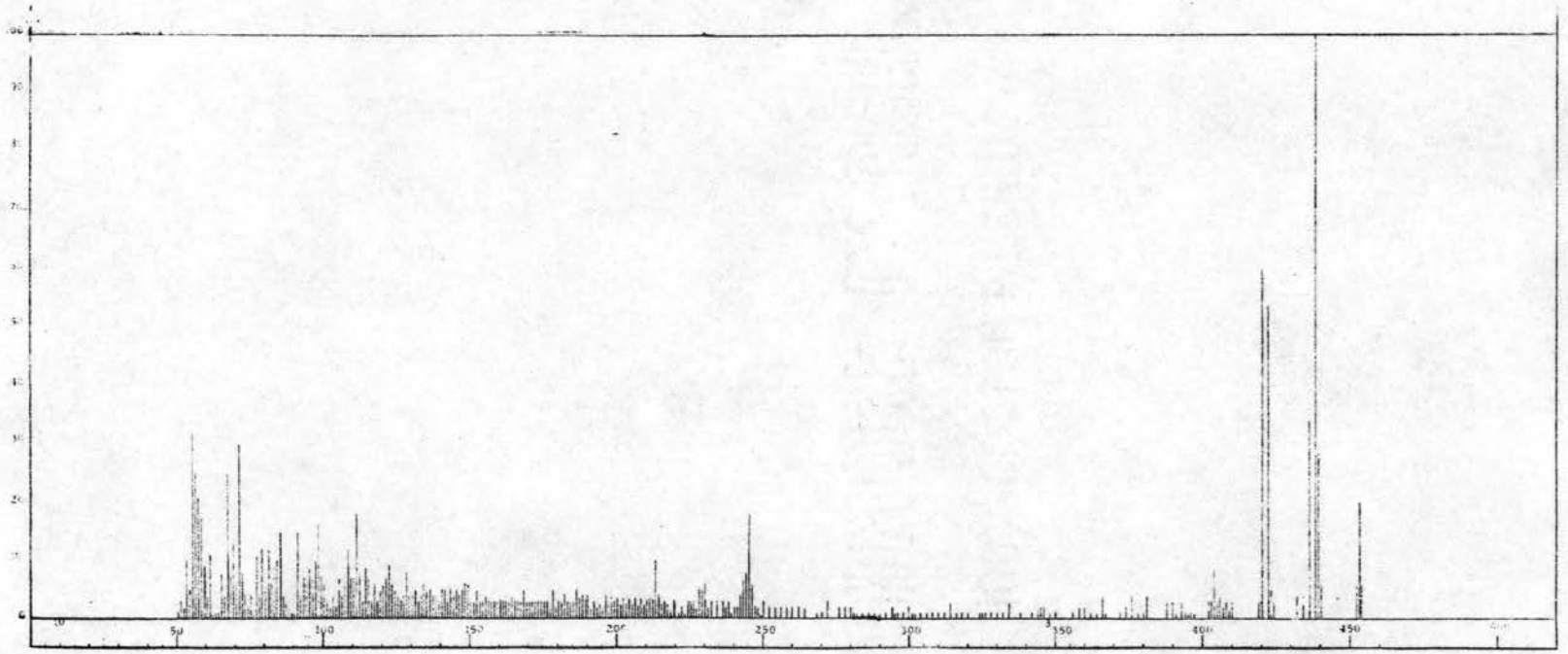
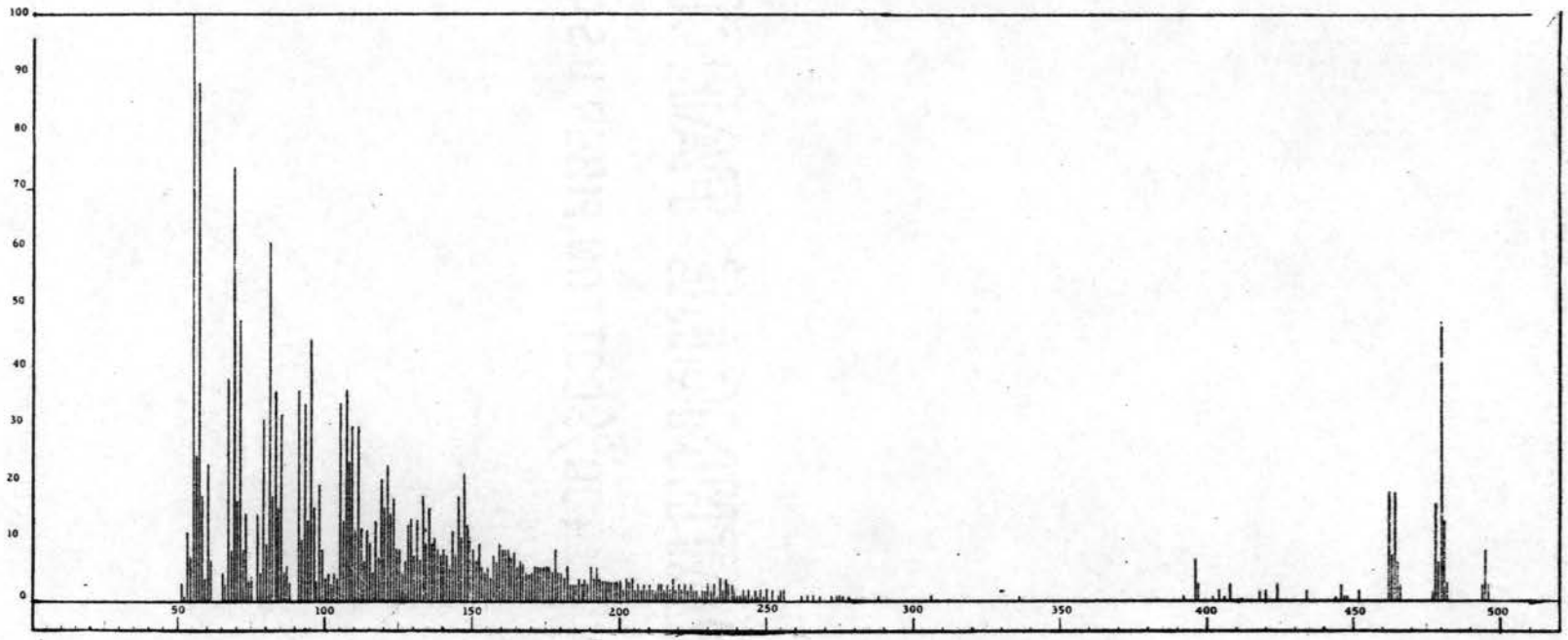


Plate XI

Mass Spectrum of O-Acetyldelcosine



VITA

Ronnie Lee Hale

Candidate for the Degree of

Master of Science

Thesis: STUDIES IN THE BIOSYNTHESIS OF DITERPENOID ALKALOIDS

Major Field: Chemistry

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Education: Graduated from Maud High School, Maud, Oklahoma in May, 1960. Received the Associate of Science degree from Murray State College in June, 1962; received the Bachelor of Science degree from Oklahoma State University, with a major in chemistry, in June, 1964; completed the requirements for Master of Science degree, with a major in chemistry, in January, 1966.

Professional Experience: Undergraduate Laboratory Assistant, Murray State College, 1960-1962; National Science Foundation Undergraduate Summer Research Participant, Oklahoma State University, 1963; Research Assistant in Organic Chemistry Oklahoma State University, 1964; National Science Foundation Co-Operative Graduate Fellow, Oklahoma State University, 1964-66.

Professional Societies: Phi Lambda Upsilon.