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Enhancement of growth and alkaloid production in tissue cultures of peyote, *Lophophora williamsii* (Lemaire) Coulter

Obermeyer, William Robert, Jr., Ph.D.

Philadelphia College of Pharmacy and Science, 1989

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ENHANCEMENT OF GROWTH AND ALKALOID PRODUCTION IN TISSUE CULTURES OF PEYOTE, LOPHOPHORA WILLIAMSII (LEMAIRE) COULTER

A Dissertation

Presented to the Faculty
of the Philadelphia College of Pharmacy & Science
in Partial Fulfillment of the Requirements for the
Degree, Doctor of Philosophy

by

William R. Obermeyer Jr.

January 1989

PHILADELPHIA COLLEGE OF PHARMACY AND SCIENCE

This is to certify that the Dissertation Prepared

BY

William Robert Obermeyer Jr.

Titled

EHANCEMENT OF GROWTH AND ALKALOID PRODUCTION IN TISSUE CULTURES OF PEYOTE, LOPHOPHORA WILLIAMSII (LEMAIRE) COULTER.

Complies with the Regulations of the Committee on Graduate Studies of the Philadelphia College of Pharmacy and Science and is Approved by the Advisory Committee as Fulfilling the Dissertation Requirements for the Degree of

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Abstract

Historically, higher plants have been and continue to be, essential for the biological and economical survival of mankind. Due to exploitation and rising production costs the once abundant supply of these plants has diminished (5-10). This has led to the development of plant tissue and cell culture (PTC) as a renewable source of food, phytochemicals, and other natural substances.

The peyote cactus, Lophophora williamsii (Lemaire) Coulter, is a perfect candidate for PTC because of its extremely slow growth and limited access in nature, along with its production of secondary metabolites of pharmacological importance. The PTC of the peyote also allows the study of environmental and biological factors on growth and secondary metabolite production in cells with simple organization.

A recent literature search revealed that the peyote cactus had not been successfully grown using traditional tissue culture techniques. After several experimental attempts to initiate callus tissues from the peyote cactus, the successful propagation of cells were propagated from the stem of the whole plant on a modified Murashige and Skoog medium. After several subcultures, the alkaloid production

did not diminish and the distribution pattern remained unchanged. The alkaloid pattern also remained similar to the parent plant, but the relative concentrations differed. Identification and quantitation of the accumulated phenolic and nonphenolic alkaloids were obtained by chromatographic and MS/MS procedures.

Growth and intra/extracellular alkaloid accumulations in the peyote cultures were found to be influenced by various factors such as growth regulators, light quality and quantity, specific aspects ("conditioned" medium additions, cell population, etc.) needed to establish and maintain liquid cell suspensions, and precursors to the phenethylamine alkaloid biosynthetic pathway. The individual factors showed complex interactions in growth, cellular differentiation and also influenced changes in the concentrations and distribution of the alkaloid pattern relative to the initial cultures.

Many of the phenethylamine compounds identified by other researchers in the whole plant were shown to also be accumulated in the cultured cells by MS/MS. Candicine, a quaternary amine, had not been previously confirmed in the whole plant, but was shown to be produced by the cultured cells.

These fundamental experiments designed to enhance growth and alkaloid production in the cultured cells of the peyote show much promise for the production of potentially

useful secondary metabolites. Further study of the isolated biosynthetic processes may lead to the production of aberrant alkaloids with promising pharmacological activity.

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List of Abbreviations

BA	benzyl adenine
CNS	central nervous system
2,4-D	2,4-dichlorophenoxyacetic acid
GC	gas chromatography
GC-MS	gas chromatography with mass spectrometry
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
KIN	kinetin; N^6-furfuryladenine
LCS	liquid cell suspension
L.E.	liquid endosperm or coconut water
MIKES	mass-analyzed ion kinetic energy spectrometer
MSk	Murashige and Skoog medium
MS/MS	tandem mass spectrometry
NAA	naphthaleneacetic acid
PCV	packed cell volume

PTC	plant tissue culture
RT	root(s)
SCV	settled cell volume
TLC	thin-layer chromatography
VIT	vitamin(s)

List of Terms and Synonyms

autotrophy	self-sufficiency, e.g. a photo-autotrophic organism synthesizes its own supply of glucose
auxin	plant growth regulator stimulating shoot cell elongation and resembling IAA in physiological activity
batch culture	cell suspension grown in a fixed volume of liquid medium
benzyl adenine	aminopurine; exhibits cytokinin activity in bud initiation
C_4 plants	plants having the C_4 pathway (Hatch-Slack) of carbon fixation
C_3 plants	plants fixing CO_2 directly through the reductive pentose phosphate pathway
callus	disorganized meristematic or tumorlike mass of plant cells under <u>in vitro</u> conditions
cytokinin	plant growthregulator stimulating cell division and resembles kinetin in physiological activity; mainly N^6-substituted aminopurine compounds
2,4-D	an auxin
explant	excised fragment of plant tissue or organ used to start a tissue culture

friable	crumbles or fragments readily
IAA	a naturally occurring auxin
inositol	*myo*-inositol; *meso*-inositol; *i*-inositol
liquid endosperm	the coconut water from young fruits of Cocos nucifera; used for formulation of initiation medium
ppm	number of milligrams of a constituent in 1000 cm^3 of solution
subculture	asceptic transfer of part of a culture (inoculum) to a fresh medium; passage
tissue culture	cellular mass grown in vitro with a solid or liquid medium

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Introduction

Higher plants have been and continue to be, essential for the biological and economic survival of mankind. Food, wood, fibers, oils, spices, and medicinal compounds are all botanical in origin (1-3). However, the once-abundant natural supply of these plant products has been diminishing due to exploitation and rising production costs. This has led to the development of hydroponics, plant tissue and cell culture (PTC) as potentially reliable and continuous sources of valuable foods, phytochemicals, and other natural substances (4-11).

The compounds currently produced by plant tissue and cell culture are diverse (12,13). Research is still in its early stages and more work is needed before industrial quantities of plant products are obtained (10,11,13-19). Studies have been directed to producing alkaloids (22-25), antitumor compounds (9,20), antibiotics (10,11,21), steroid precursors (8,26,27), ginsenosides (8,28,29), shikonin (10,11,30,31) and allergens (9,32). These chemicals are widely used in medicine and pharmacy as therapeutic or diagnostic agents, as drug precursors or for formulation and

flavoring agents (3,33).

To fundamentally enhance the production of compounds in plants or cultured tissues, one must first understand the underlying biosynthetic pathways. The specific pathways of interest can easily be studied by using cultured cells rather than intact plants because they lend themselves to experimental methods. The major experimental qualities of PTC are: 1) the relative ease of cultivation, 2) the ability to standardize nutritional and environmental conditions, 3) the avoidance of seasonal variations, 4) the ability to grow axenic cultures and to eliminate problems associated with contamination by microbes, 5) the use of isolated experimental systems which are simpler than the intact plant, 6) the ability to incorporate precursor material and potentially raise levels of desired compounds and, 7) the ability to use undifferentiated cells with simple organization. These advantages minimize problems such as translocation, permeability and segregation of metabolic pools such as those seen in rare and slow growing plants like the peyote cactus.

Recent studies have shown that the metabolites produced by PTC systems are largely influenced by environmental and biological factors. Tissue cultures are affected by such physical factors as light intensity and quality (8,10,11,13,16,22); chemical factors (such as the type and concentration of nutrients (8,10,11,13,21,26,

27,30,34)); or growth regulators (added to the medium for simulation of morphological differentiation and biosynthesis)(8,11,21,26,28,36); and biological factors such as the age of culture (8,10), growth rate (8,10,11,26,34), and the influence of genetic and epigenetic factors present in the parent plant used to establish the tissue culture, or those selected from a tissue culture (8,10,11,14). Other current approaches to increase secondary metabolite production and growth rates include genetic manipulation of cells by Agrobacterium transformation and phage induction (10,17-19,24,25) and cryopreservation (34) to maintain cell lines once they are produced. These are some of the many variables that must be explored in the frame-work of basic research to produce economically viable amounts of secondary metabolites in plant tissue cultures. Unfortunately, no two plants are optimally influenced by the exact same factors (8,12,13,27,30,35) and the empirical research required is, therefore, time consuming.

Ethnobotanical Studies of Lophophora williamsii

The peyote cactus, a popular name derived from the ancient Aztec word "peyotl" for Lophophora williamsii, is described as the prototype of the New World plant hallucinogens (38-40). The bulbous shaped shoot is sliced off and dried to form hard brown disks, known as mescal

buttons. The intoxicating alkaloids in the buttons may be kept almost indefinitely in this dried state until ingestion. The mescal buttons are usually softened in the mouth and swallowed directly without chewing (38,40,41). The peyote intoxication is very complex and variable due to the approximately 56 known *B*-phenethylamine and tetrahydroisoquinoline constituents found in the button (42,43). Its most vivid phase, consists of visual, auditory and tactile hallucinations, for which mescaline is chiefly responsible. The drug is taken mainly by the Indians and natives of Southwestern North America as a "religious medicine" and to induce visions leading to prophetic thoughts through which to direct their lives. The ingestion of the mescal buttons is also used to obtain a desired state of trance for ritual dances (38,44-46).

The peyote claims centuries of use as a narcotic and was described in pre-Columbian religious practices of the Aztec and other Mexican Indians (38,40,42,47). During the Spanish conquest of Mexico the missionaries tried to suppress peyote consumption for various social, political and religious reasons without much success. During the 19th century, the ritual use of peyote (peyotism) by the American Indians was driven "underground" and small cult groups were established (Peyote Church). The cult had increased in number and for protection against unjust persecution from missionary and political groups, was legally incorporated

into the Native American Church in 1922 (40,41). The Church members, who teach reverence, high moral values and abstinence from alcohol, conduct ceremonies every Saturday night by spending the evening in ritual singing, prayer and contemplation (41). There are currently some 300,000 (38) adherents in the United States. Widespread use has caused a serious depletion of the native plant population to the point of extinction. Therefore the tissue culture of this rare and endangered species is now of increased interest.

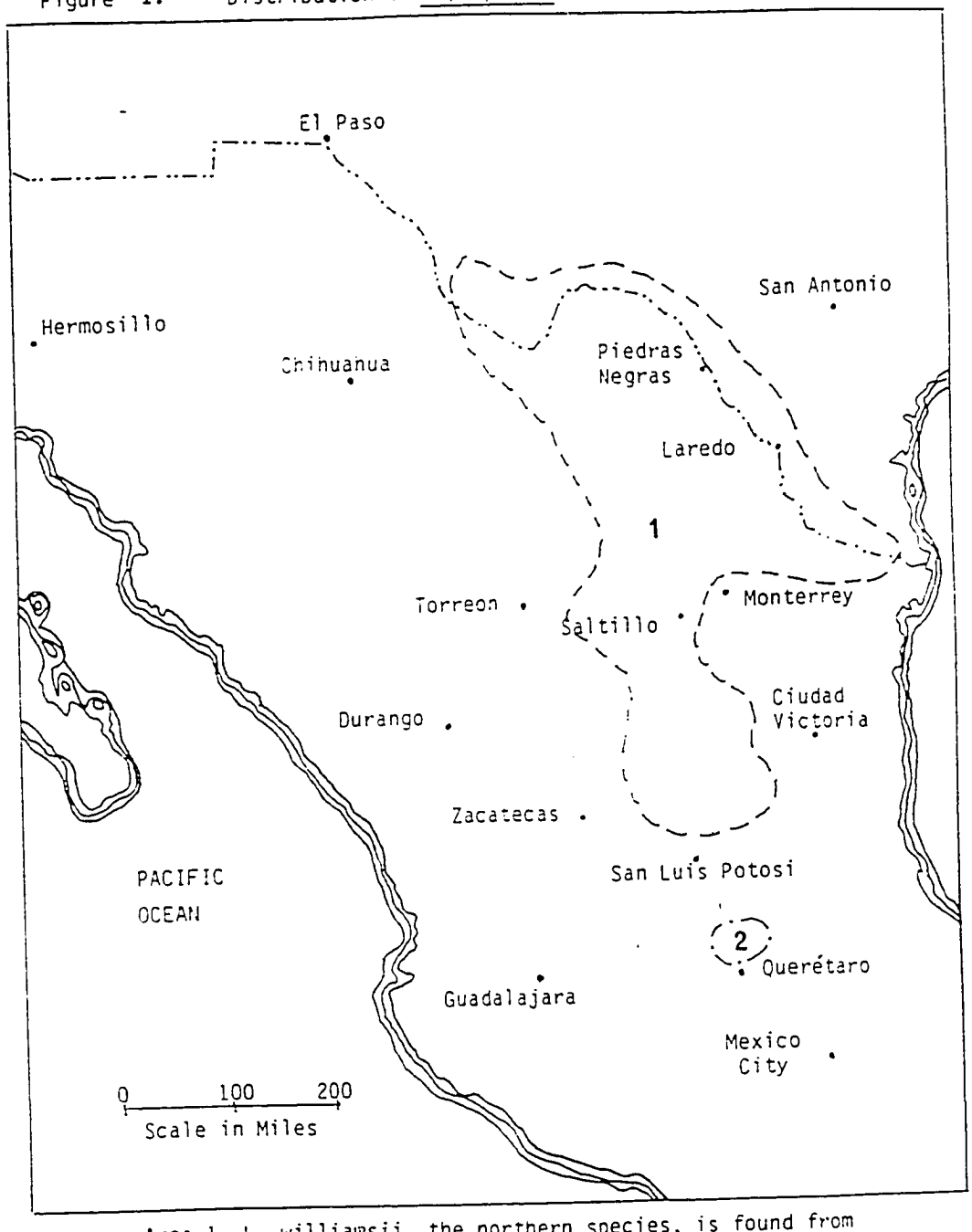
Other cactaceous plants, besides Lophophora spp., have been employed for their medicinal and hallucinogenic effects (38,44-47). Various species of Ariocarpus, Astrophytum, Aztekium, Dolichothele, Obregonia, Pelecyphora, and Solisia are classed as "peyote" in Mexico possibly due to their physical resemblance to Lophophora or because of use similar to that of the true peyote (38,40,41). The Tarahumare Indians use several species of Mammilaria and Echinocactus in place of Lophophora for special religious and medicinal purposes (45). A large columnar cactus found in Peru, Trichocerus pachanoi, also contains alkaloids similar to the peyote and forms the basis of a hallucinogenic drink taken by witch doctors for diagnosis, divination and to make the drinker the owner of another's identity (44). Complete ethnobotanical reviews of the cacti have been written by several authors (38-49).

Distribution and Habitat

The genus Lophophora, which contains two species (L. williamsii and L. diffusa), has a limited natural range within the Southwestern North America (Figure 1)(38,50). The region, located in the Chihuahuan Desert of Texas and Mexico, has considerable variation in both topography and vegetation. The genus tends to be restricted to a latitudinal distribution of about 1200 km between 20°54' to 29°47' North latitude at elevations of less than 50 meters to more than 1800 meters. Lophophora occurs in two characteristic vegetation zones described as a) the microphyllous desert scrub having shrubs with small leaves or leaflets such as Larrea divaricata, Prosopis julfilora and Flourensia cernua; and b) the "rosettophyllous" desert scrub with many plants bearing rosettes of leaves such as Agave lechequilla and Yucca spp. Although not uncommon in open, full sunlight, Lophophora will form clumps under the shade of associated plants in its "characteristic vegetation zones" and requires basic soils (pH 7.9-8.3) of limestone origin (38,50) for growth.

Reproduction is primarily sexual and generally many young plants can be found growing in close proximity to mature ones. Asexual reproduction can also occur by injury

Figure 1. Distribution of Lophophora



Area 1. L. williamsii, the northern species, is found from southern Texas southwards into Mexico. Area 2. L. diffusa is only found in a limited area in Querétaro. Redrawn with author's permission. (50)

or harvesting by man to induce new, multiple stem formations from a single root stock (38,41,50). These may become detached and root during the rainy season.

The age and size of the plant are two factors that determine the number of ribs (51). Young plants normally have five ribs, but mature individuals may have 5-14 or none at all. A range of rib variation can occur within the same clump and is apparently caused by environmental or physiological factors (49,51). This wide range of environmental morpholgical variation exhibited by Lophophora tends to make early systematic taxonomic conclusions inaccurate when based solely on gross morphological characteristics (38,49-52).

Classification

Plant taxonomy tends to be an ever-changing science with the goal being the arrangement of various kinds of plants into a scheme of classification that will show their true relationship to one another and the correct placement of the organism within the plant kingdom. The historical beginnings of taxonomy are well documented (53,54) and show general trends from purely artificial systems to phylogenetic systems to the "current system" that utilizes both structural and chemical characteristics to classify plants. Early artificial systems were based upon readily observed characteristics of plants, namely, a sexual system

based on the number of stamens and styles, (Linnaeus 1753), grouping into herbs, shrubs or trees, (Theophrastus 300 B.C.), without regard to their evolutionary or genetic significance.

The phylogenetic system contrasts with the artificial system in that it attempts to classify plants according to the evolutionary relationships inferred from indirect or direct genetic experimentation (53,54). This type of classification system has its own innate problems. Well-known taxonomists of the early to mid-1900's (Bessey, Hutchinson, Sporne) placed different emphasis on characters used to describe alleged primitive and advanced features and caused much confusion for the proper placement of the Cactaceae in phylogenetic trees (52,53). Due to this disorganization, the family, Cactaceae, has been placed in several orders, such as the Cactales, Chenopodiales, Caryophyllales, and Opuntiales (49,51-54). For the "peyote" cactus, structural differences among individuals may arise due to developmental and environmental variation. This led to the naming of different species and varieties (38,49-54), further adding to the confusion in this group. These examples demonstrate clearly that a new system was needed to clarify the conflicts in this classification system.

In the currently accepted taxonomic system, often called biosystematics, one attempts to study the complete biology (secondary metabolite, biochemical pathways,

structure, reproduction biology and environmental limits) of the plant and to utilize this data to clarify the taxonomic and evolutionary relationships of the taxon involved (53-58). This system is a compromise between the structural and chemotaxonomic classification systems. The two major groups of compounds that have been applied to resolving taxonomic problems are: a) macromolecules (proteins and nucleic acids) and b) secondary metabolic compounds (terpenes, flavonoids, alkaloids, amino acids, and various lipids)(53-56). Until recently, it was not feasible to analyze secondary constituents from single plants in naturally occurring populations. This is now possible through the use of sensitive separation techniques (HPLC, GC) and sensitive methods of instrumental analysis (IR, NMR, UV, and mass spectrometry). From the data evaluated by Cronquist (53,54), a new scheme for the Cactaceae has been formulated (Table 1). It can be seen from this table that the family Cactaceae, is now placed in the order Caryophyllales.

Description of Lophophora (as seen in ref; 49-51)

Lophophora Coulter, Contr. U.S. Nat. Herb. 3: 131. 1894

Type species: L. williamsii (Lemaire) Coulter.

Plants low, with long fusiform roots. Stems solitary

Table 1. Compromise classification of the family Cactaceae with evidence from pigment formation (53)

Structure classification	Chemical classification	Compromise classification
Centrospermae	Chenopodiales	Caryophyllales
Aizoaceae	Aizoaceae	Chenopodiineae
Amaranthaceae	Amaranthaceae	Aizoaceae
Basellaceae	Basellaceae	Amaranthaceae
Caryophyllaceae	Cactaceae	Basellaceae
Chenopodiaceae	Chenopodiaceae	Cactaceae
Didiereaceae	Didiereaceae	Chenopodiaceae
Molluginaceae	Nyctaginaceae	Didiereaceae
Nyctaginaceae	Phytolaccaceae	Nyctaginaceae
Phytolaccaceae	Portulacaceae	Phytolaccaceae
Portulacaceae		Portulacaceae
Cactales	Caryophyllales	Caryophyllineae
Cactaceae	Caryophyllaceae	Caryophllaceae
	Molluginaceae	Molluginaceae

or in clusters arising from the same root system, usually rounded above, depressed in the centers, blue-green, yellow-green, and occasionally appearing reddish green; 2-7 cm high, 4-12 cm in diameter. Areoles usually linearly arranged along ribs or at the apices of humplike tubercles or podaria, each bearing a tuft of soft, yellowish or whitish trichomes from which the flowers arise, 8-15 mm apart, 1-5 mm in diameter; spines absent except in seedlings and then only rudimentary. Flower 1-2.2 cm in diameter, 1-2.4 cm long; outer perianth segments with greenish midribs and greenish pink or whitish margins, the largest one elliptical, 3-12 mm long, 1-3 mm broad, mucronate, marginally minutely ciliate distally; inner perianth segments pink, white or rarely yellowish white, sometimes with greenish midribs, the largest ones elliptical, 8-22 mm long, 2-4 mm broad, mucronate or occasionally attenuate, margins ciliate or entire; filaments white; anthers yellow; pollen ± spheroidal, 0-18 colpate, 14.9-63 um diameter; style white, 5-14 mm long; stigmas 4-8 (rarely 3), 1-3 mm long, white though occasionally pinkish; ovary naked. Fruit pinkish red and fleshy at first, becoming brownish white and dry at maturity, naked, clavate, 15-20 mm long 2-3.5 mm in diameter, with the umbilicus large or the perianth parts persistent, emerging rapidly from within the trichomes at maturity. Seeds black, tuberculate (verrucose), pyriform, 1-1.5 mm long, 1 mm broad; hilum large and flattened;

cotyledons coalescent.

Flowering from March through September. Fruits mature 9-12 months after fertilization.

Key to the Species:

Plants blue-green, usually with well-defined ribs and furrows; tufts of trichomes usually equally spaced on the ribs; flowers pinkish or rarely whitish; not located in Queretaro, Mexico.
1. L. williamsii

Plants yellow-green, usually lacking well-defined ribs and furrows; tufts of trichomes usually unequally spaced on prominent podaria; flowers commonly whitish to yellowish white; located in Queretaro, Mexico.
2. L. diffusa

1. L. williamsii (Lemaire) Coulter, Contr. U.S. Nat. Herb. 3: 131. 1894.

Synonyms

Echinocactus williamsii Lemaire ex. Salm-Dyck in Otto Dietr., Allgem. Gartenzeit. 13: 385. 1845.

Anhalonium williamsii Rumpler in Forster, Handb. Cact. ed. 2. 233. 1886.

Anhalonium lewinii Hennings, Gartenflora 37: 410. 1886.

Mammillaria williamsii Coulter, Contr. U.S. Nat. Herb. 2: 129. 1891.

Lophophora williamsii Coulter, Contr. U.S. Nat. Herb. 3: 131. 1894.

Ariocarpus williamsii Voss, Vilmorin Illustrirte Blumengartneri 368. 1894.

Echinocactus lewinii K. Schumann, Nat. Pflanzenfam. 3. Abt.6a: 173. 1894.

Lophophora williamsii var. lewinii Coulter, Contr.

U.S. Nat. Herb. 3: 131. 1894.

Lophophora lewinii Rusby, Bull. Pharmacy 8: 306. 1894.

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Echinocactus williamsii var. lutea Rouhier, Trav. Labouret Mat. Med. Pharm. Galen 17: 65. 1926.

Lophophora echinata var. lutea Croizat, Desert Plant Life 16: 44. 1944.

Lophophora echinata Croizat, Desert Plant Life 16: 43. 1944.

Lophophora williamsii var. lutea Soulaire, Cactus et Medicine 121. 1947.

Lophophora lutea Backberg, Die Cactaceae 5: 2901. 1961.

Lophophora williamsii var. echinata H. Bravo, Cact. Sucul. Mex. 12(1): 12. 1967.

2. L. diffusa (Croizat) Bravo H., Cact. Sucul. Mex. 12: 13. 1967.

Synonyms

Lophophora echinata var. diffusa Croizat, Desert Plant Life 16: 44. 1944

Chemistry of the Peyote Alkaloids

Peyote Constituents

The peyote has been of long-standing interest because of its interesting medicinal and religious uses (38,42,43,59,60). According to the current literature the earliest chemical studies were made by Lewin in 1888, who

isolated the tetrahydroisoquinoline alkaloid, anhalonine (XXXVIII)(for structures see Table 2). It was later discovered that this crystalline isolate possessed no hallucinatory activity. In 1896, Heffter discovered three additional tetrahydroisoquinolines, pellotine (XXV), anhalonidine (XXX), and lophophorine (XLI), along with the active hallucinogenic principle of the drug, mescaline (XVII), a *B*-phenethylamine. Another alkaloid, anhalamine (XXI), was isolated by Kauder in 1899. Several years passed before Spath began work in 1919 on the structure elucidation and syntheses of all of these alkaloids (42,43,59-61,126). Spath was also credited with the isolation of five more peyote alkaloids (anhalinine (XXV), anhalidine (XXVII), *N*-methylmescaline (XVIII), *N*-acetylmescaline (XX), and *O*-methylanhalonidine (XXXIII)) and various alkaloid constituents from other Cactaceae (59-61).

Little additional work was done on the constituents of peyote and other cacti until the 1960's, when several factors caused a revitalization of interest. New, sensitive, separation and identification techniques (GC, mass spectrometry, etc.)(61) became available along with isotope tracers for biochemical studies. Also, this was the so called "hippie" era and interest in determining structure and pharmacological action of the hallucinogenic compounds was high. This resurgent interest started with the isolation of three *B*-phenethylamines (tyramine (I), *N*-methyltyramine

(II) and hordenine (III)) during 1966 from the peyote (62). Candicine (IV), a quaternary alkaloid, was also thought to be present in the peyote (by TLC evidence), but could not be confirmed by mass spectrometry (62). Dopa and dopamine (V) where isolated and shown to be biochemical intermediates to mescaline (63).

After 1967, progress was made on the minor constituents of the peyote by Kapadia and coworkers, when 26 additional compounds were identified (see Table 2).

The total alkaloid content in L. williamsii has been estimated by several authors to range between 3.4-3.7% for the dried upper slices (mescal buttons) and 0.4-0.41% for the whole fresh plant (38,40,59,60,126,138). The figures reported (Table 3) by various researchers are for the more abundant alkaloids (others not listed were considered trace elements) found in greenhouse grown specimens (59). Even though more consistent environmental conditions were achieved, seasonal variations in the alkaloidal composition were noted (59,138).

Various reports have been completed on the species of peyote found in Queretaro, Mexico (L. diffusa) (38,42,43,74). This material contains some compounds similar to L. williamsii, but also some distinctly different in chemical composition and this species may contain a higher perentage of total alkaloids per dry weight (138). Large amounts of pellotine (XXXV)(>90% of total alkaloid content),

TABLE 2. PEYOTE CONSTITUENTS

A. MONO-OXYGENATED PHENETHYLAMINES

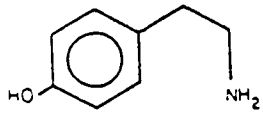
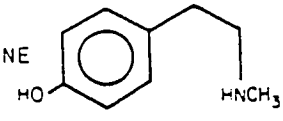
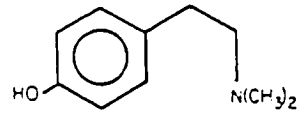
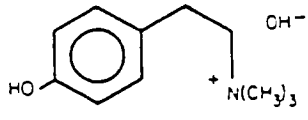
I. TYRAMINE		$C_8H_{11}ON$	MP= 161°C
II. N-METHYLTYRAMINE		$C_9H_{13}ON$	MP=127-128°C
III. HORDENINE		$C_{10}H_{15}ON$	MP=117-118°C
IV. CANDICINE		$C_{11}H_{19}O_2N$	MP=230-231°C (AS IODIDE)

TABLE 2. CONTINUED

B. DIOXYGENATED PHENETHYLAMINES

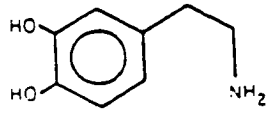
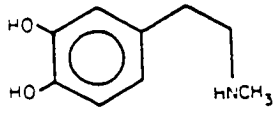
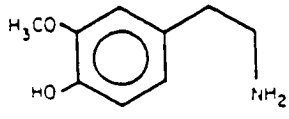
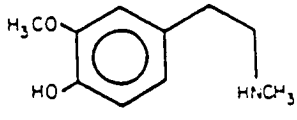
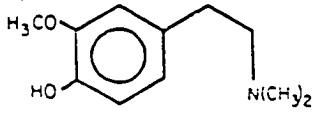
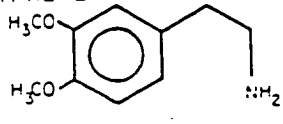
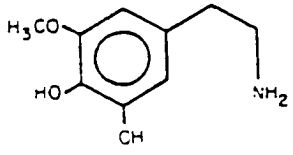
V. DOPAMINE		$C_8H_{11}O_2N$	MP=241°C (AS HCL)
VI. EPININE		$C_9H_{13}O_2N$	MP=188-189°C
VII. 4-HYDROXY-3-METHOXYPHENETHYLAMINE		$C_9H_{13}O_2N$	?
VIII. N-METHYL-4-HYDROXY-3-METHOXYPHENETHYLAMINE		$C_{10}H_{15}O_2N$	MP=154-155°C (AS HCL)
IX. N,N-DIMETHYL-4-HYDROXY-3-METHOXYPHENETHYLAMINE		$C_{11}H_{17}O_2N$	MP=190-191°C (AS HCL)
X. 3,4-DIMETHOXYPHENETHYLAMINE		$C_{10}H_{15}O_2N$	BP=188°/15 MM

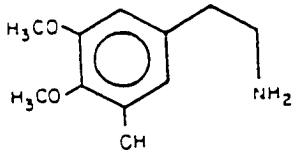
TABLE 2. (CONTINUED) C. TRIOXYGENATED PHENETHYLAMINES AND RELATED AMIDES

XI. 3,4-DIHYDROXY-5-METHOXYPHENETHYLAMINE



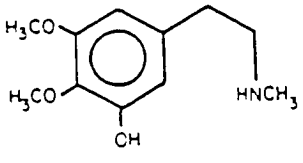
$C_9H_{13}O_3N$ MP=207°C

XII. 3-HYDROXY-4,5-DIMETHOXYPHENETHYLAMINE



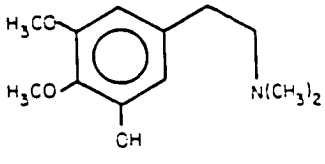
$C_{10}H_{15}O_3N$ MP=178-179°C (AS HCL)

XIII. N-METHYL-3-HYDROXY-4,5-DIMETHOXYPHENETHYLAMINE



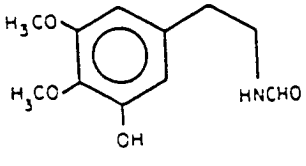
$C_{11}H_{17}O_3N$ MP=151-155°C (AS HCL)

XIV. N,N-DIMETHYL-3-HYDROXY-4,5-DIMETHOXYPHENETHYLAMINE



$C_{12}H_{19}O_3N$ MP=180-185°C (AS HCL)

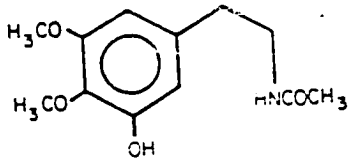
XV. N-FORMYL-3-HYDROXY-4,5-DIMETHOXYPHENETHYLAMINE



$C_{11}H_{15}O_4N$?

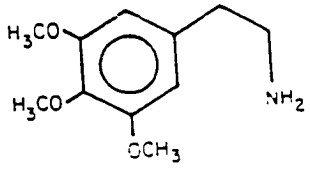
TABLE 2: (C: CONTINUED)

XVI. N-ACETYL-3-HYDROXY-4,5-DIMETHOXYPHENETHYLAMINE



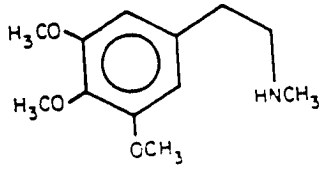
$C_{12}H_{17}O_4N$ MP=102-103°C

XVII. MESCALINE



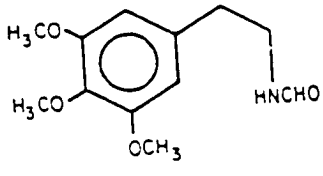
$C_{11}H_{17}O_3N$ MP=30-32°C BP=183-186°C

XVIII. N-METHYLMESCALINE



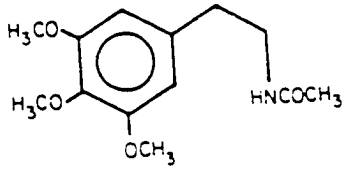
$C_{12}H_{19}O_3N$ MP=177-178°C (AS PICRATE)

XIX. N-FORMYLMESCALINE



$C_{12}H_{17}O_4N$ MP=68-69°C

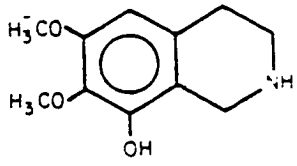
XX. N-ACETYLMESCALINE



$C_{13}H_{19}O_4N$ MP=93-94°C

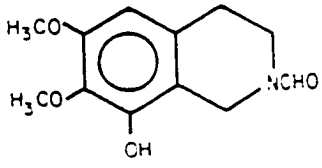
TABLE 2. D. TETRAHYDROISOQUINOLINES AND RELATED AMIDES
(CONTINUED)

XXI. ANHALAMINE



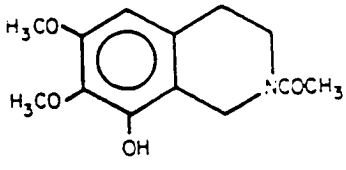
$C_{11}H_{15}O_3N$ MP=189-191°C

XXII. N-FORMYLANHALAMINE



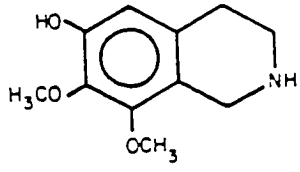
$C_{12}H_{15}O_4N$?

XXIII. N-ACETYLANHALAMINE



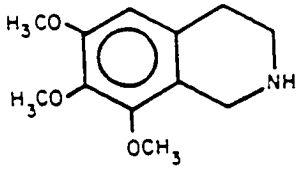
$C_{13}H_{17}O_4N$?

XXIV. ISOANHALAMINE



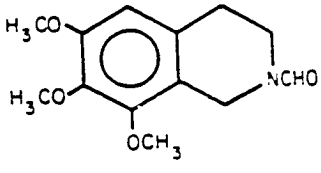
$C_{11}H_{15}O_3N$ MP=213-215°C (AS HBR)

XXV. ANHALININE



$C_{12}H_{17}O_3N$ MP=61-63°C

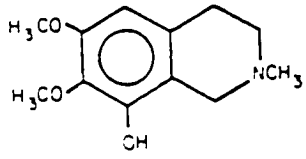
XXVI. N-FORMYLANHALININE



$C_{13}H_{17}O_4N$?

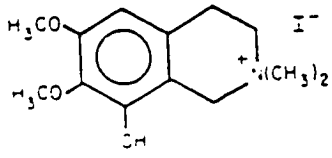
TABLE 2: (D. CONTINUED)

XXVII. ANHALIDINE



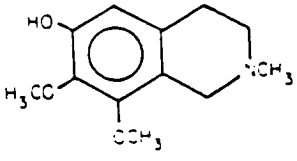
$C_{12}H_{17}O_3N$ MP=131-133°C

XXVIII. ANHALOTINE (AS AN IODIDE)



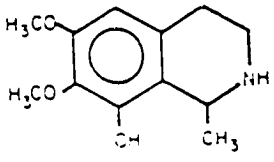
$C_{13}H_{20}O_3NI$ MP=219-220°C

XXIX. ISOANHALIDINE



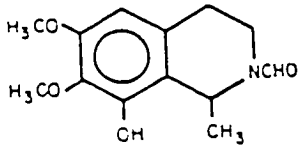
$C_{12}H_{17}O_3N$ MP=215-218°C (AS HCL)

XXX. ANHALONIDINE



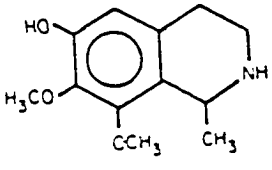
$C_{12}H_{17}O_3N$ MP=160-161°C

XXXI. N-FORMYLANHALONIDINE



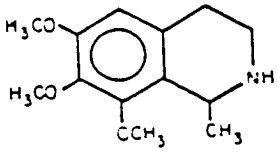
$C_{13}H_{17}O_4N$?

XXXII. ISOANHALONIDINE



$C_{12}H_{17}O_3N$ MP=209-211°C

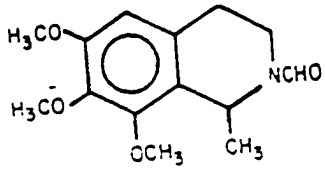
XXXIII. S-(+)-O-METHYLANHALONIDINE



$C_{13}H_{19}O_3N$ BP=140°C

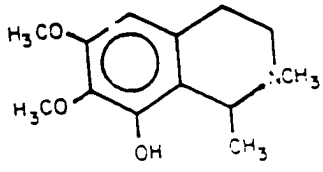
TABLE 2. (D: CONTINUED)

XXXIV. N-FORMYL-O-METHYLANHALONIDINE



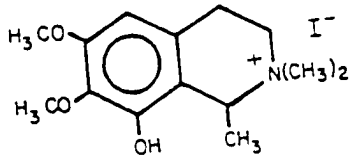
$C_{14}H_{19}O_4N$?

XXXV. PELLOTINE



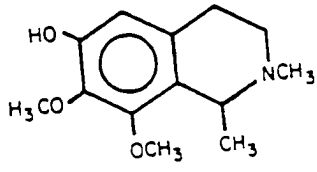
$C_{13}H_{19}O_3N$ MP=111-112°C

XXXVI. PEYOTINE (AS AN IODIDE)



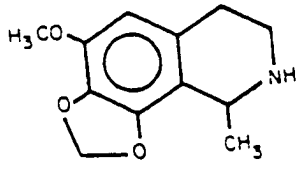
$C_{14}H_{22}O_3NI$ MP=185-186°C

XXXVII. ISOPELLOTINE



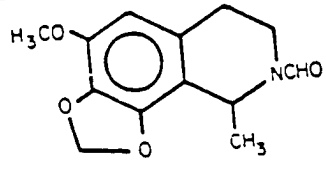
$C_{13}H_{19}O_3N$ MP=212-222°C (AS HCL)

XXXVIII. S-(-)-ANHALONINE



$C_{12}H_{15}O_3N$ MP=85.5°C

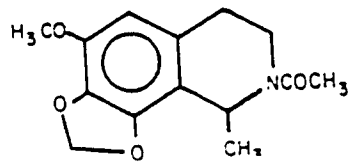
XXXIX. N-FORMYLANHALONINE



$C_{13}H_{15}O_4N$?

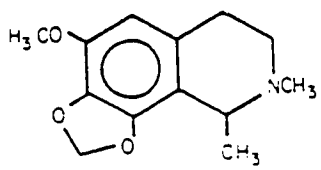
TABLE 2. (D; CONTINUED)

XL. N-ACETYLANHALONINE



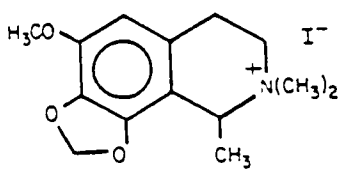
$C_{14}H_{17}O_4N$?

XLI. S-(-)-LOPHOPHORINE



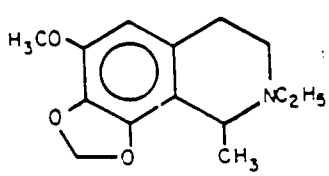
$C_{13}H_{17}O_3N$ BP=140-145°C/ 0.05 MM HG

XLII. LOPHOTINE (AS AN IODIDE)



$C_{14}H_{20}O_3NI$ MP=240-242°C

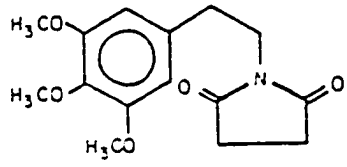
XLIII. PEYOPHORINE



$C_{14}H_{19}O_3N$ MP=155-156°C (AS PICRATE)

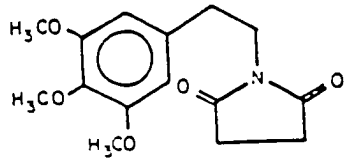
TABLE 2. (CONTINUED) E. CONJUGATES WITH KREBS ACIDS

XLIV. MESCALINE SUCCINIMIDE



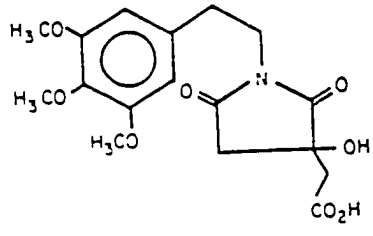
$C_{15}H_{19}O_5N$ MP=125-126°C

XLV. MESCALINE MALIMIDE



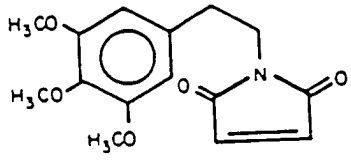
$C_{15}H_{19}O_6N$?

XLVI. MESCALINE CITRIMIDE



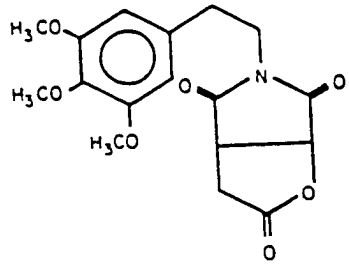
$C_{17}H_{21}O_8N$?

XLVII. MESCALINE MALEIMIDE



$C_{15}H_{17}O_5N$?

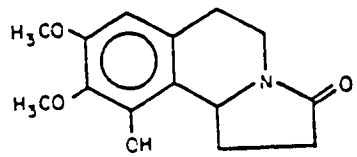
XLVIII. MESCALINE ISOCITRIMIDE LACTONE



$C_{17}H_{19}O_7N$?

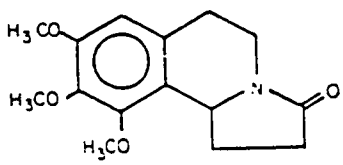
TABLE 2. (E. CONTINUED)

XLIX. PEYOGLUTAM



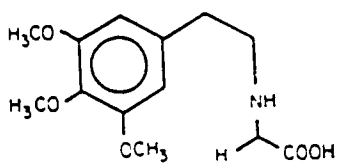
$C_{14}H_{17}O_4N$ MP=217-219°C

L. MESCALOTAM



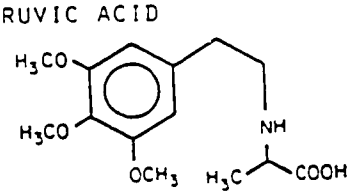
$C_{15}H_{19}O_4N$?

LI. MESCALOXYLIC ACID



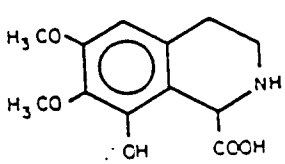
$C_{13}H_{19}O_5N$ MP=187-189°C

LII. MESCALORUVIC ACID



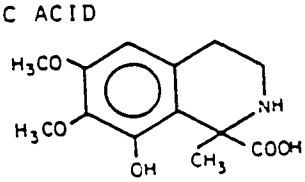
$C_{14}H_{21}O_5N$ MP=235-236°C

LIII. PEYOXYLIC ACID



$C_{12}H_{15}O_5N$ MP=237-238°C

LIV. PEYORUVIC ACID

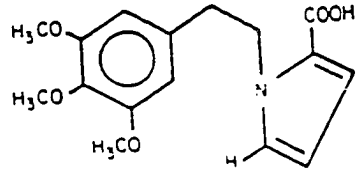


$C_{13}H_{17}O_5N$ MP=233-234°C

TABLE 2. (CONTINUED)

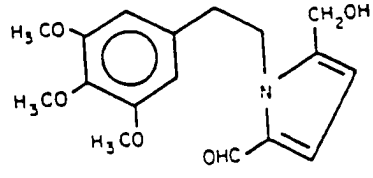
F: PYRROLE DERIVATIVES

LV. PEYONINE



$C_{16}H_{19}O_5N$ MP=131-133°C

LVI. PEYOGLUNAL



$C_{17}H_{21}O_5N$?

Table 3. Alkaloid composition in the peyote (L. williamsii) (59)

Alkaloid	Content (%) of total alkaloids in: Alkaloid fraction of fresh plant	Dried plant
Hordenine	8	
N,N-Dimethyl-4-hydroxy-3-methoxyphenethylamine	0.5-2	
3-Demethylmescaline	1-5	
Mescaline	30	6
N-Methylmescaline	3	
Anhalamine	8	0.1
Anhalinine	0.5	0.01
Anhalidine	2	0.001
Anhalonidine	14	5
O-Methylanhalonidine	0.5	
Pellotine	17	0.74
Isopellotine	0.5	
Anhalonine	3	3
Lophophorine	5	0.5
Peyophorine	0.5	

small amounts of anhalamine (XXI) and lophophorine (XLI), traces of anhalonidine (XXX), *N*-methylmescaline (XVIII), and hordenine (III), and a new alkaloid, *O*-methylpellotine were found.

Botanical Occurence of Phenethylamines and Simple Isoquinoline Alkaloids In Nature

Other Cacti

Much work has been done on the alkaloid constituents of giant cacti that belong to the subtribe Cereanae (Cactaceae). Several species belonging to the genera Lophocereus (59), Carnegiea (75,76) and Pachycereus (76-80) have been shown to contain 6,7-dioxygenated and 6,7,8-trioxygenated tetrahydroisoquinoline alkaloids along with lesser amounts of 7,8-di and 5,6,7-trioxygenated analogs.

Another genus, Dolichothele, has long been used ethnobotanicaly as a "peyote" for its narcotic principles. This genus contains many of the common peyote alkaloid constituents as well as five new tetrahydroisoquinoline alkaloids (81-84).

Other cacti of ethnobotanical importance from the genera Ariocarpus (85-90), Backebergia (109-110), Coryphantha (91-101), Lepidocoryphantha (102), Mammillaria

(103), Obregonia (104), Opuntia (101,106-108), Pterocereus (105), and Trichocereus (111-115) have been studied with respect to their alkaloids. These studies have yielded the "normal" phenethylamine and tetrahydroisoquinoline alkaloids. Anaylses of Echinocereus (117) and Pilosocereus (118) have identified salsoline and *N*-methylheliamine, respectively, for the first time in the Cactaceae.

Other Higher Plants

The phenethylamines are not limited to the Cactaceae, but have also been found in other families in the plant kingdom (62-70). In several genera of the Cannabinaceae, Euphorbiaceae, Gramineae, Leguminosae, Magnoliaceae, Passifloraceae, Plumbaginaceae, Rosaceae, and Rutaceae, investigations have yielded many examples of phenethylamine accumulation (120-123,126-128).

As the phenethylamines are not limited to the Cactaceae, the simple isoquinoline alkaloids can also be found in many other families. Isoquinoline compounds have been identified in the following plant families: Alangiaceae, Annonaceae, Berberidaceae, Chenopodiaceae, Euphorbiaceae, Fumariaceae, Leguminosae, Monimiaceae (Antherospermataceae), Musaceae, Nymphaceae, Papaveraceae, Rununculaceae, and Sterculiaceae (59,121,122,127,128).

Fungi

Very little work has been done to date to establish the presence of phenethylamines or tetrahydroisoquinoline alkaloids in fungi. The higher fungi have been screened for specific phenethylamines (hordenine (III), tyramine (I), and *N*-methyltyramine (II)) and these compounds have been identified in some species of Boletus, Fomes, and Polyporus (125). Hordenine and other tyramine analogs may be associated with other lignicolous fungi or infectious fungi of crop plants and could be of chemotaxonomic importance (124).

Biosynthesis of Major Alkaloids

Site of Biosynthesis

It is generally recognized that some of the most intensively studied alkaloids (nicotine, hyoscyamine, etc.) are produced in quantity in the the roots of intact plants, while the leaves and shoots in other plants may be the site of formation for other alkaloids (morphine, steroid alkaloids, lupinine, etc.) (60,128,129). Various experiments using isolated root organ cultures, under aseptic conditions, often yield no alkaloid accumulation (5,6,8,12).

This indicates that the isolation of organs prevents migration of metabolic by-products to and from the roots and that other active growing parts may play an active role in alkaloid formation (60,131). Some researchers have shown that translocation, distribution and accumulation of alkaloids are very complicated in plants and are sometimes important for completion of the secondary constituents into their "final" products (56,60,126,128,129).

To date, little work has been done to locate the site of biosynthesis in cacti. Early work with peyote, by Janot and Bernier, showed that alkaloid accumulation (though not necessarily the site of formation) was almost exclusively located in the internal cells of the cortical parenchyma at the top of the plant (60). Other early experiments on another mescaline-producing genus of cacti, Trichocereus, provided various conclusions for the area of alkaloid accumulation. Niedfeld (38) situated the alkaloids in the chlorophyllaceous cortical parenchyma, while Reti (126) found 0.29% total alkaloids in the green epidermal layer and the central parts (including cortical parenchyma) contained 0.45%. The results of Todd (74)(Table 4) and other researchers (42,43) have inferred that *N*-methyl derivatives of tyramine (*N*-methyltyramine, hordenine, candicine (II-IV), etc.) occur only in the roots of peyote where active *N*-methylation enzymes exist. The same roots and tops of the L. williamsii examined by Todd also contained mescaline,

Table 4. Distribution of alkaloids in tops and roots of Lophophora. (74)

	L. williamsii		L. diffusa	
	tops	roots	tops	roots
Nonphenolic alkaloids				
anhalinine (2ug)[a]	3[b]	2-3	0	0
anhalonine (2ug)	3	3	0	0
lophophorine (10ug)	3	2-3	2	2
mescaline (4ug)	3-4	1-3	1	1
Phenolic alkaloids				
anhalamine (4ug)	3	3	2	0
anhalonidine (4ug)	3	3	1	1
hordenine (4ug)	0	3	0	0
pellotine (4ug)	2-3	2-3	5	5

[a]Amount of standard used for reference spot during TLC.

[b]Estimation of amount relative to reference spot: absent (0), trace (1), definitely present but less than reference compound (2), same amount as reference compound (3), larger amount than reference compound (4), much larger amount than reference compound (5).

anhalonine, anhalamine and other alkaloids that are not *N*-methylated. Recent experiments (131) with grafted cacti (Trichocereus) resulted in the detection of mescaline in the aerial portions of the alkaloidiferous cacti but not in the roots. The experiment also showed no vertical translocation of mescaline from the alkaloidiferous aerial parts to the non-alkaloid producing cacti sections. This supports the hypothesis that alkaloids are made and stored (in situ) in the aerial parts near the epidermis.

Review of Precursor Experiments in Whole Plants

General comments

Between the late 1960's and the mid-1970's, several groups of researchers performed experiments pertaining to the biosynthesis of alkaloids in the peyote and other cacti, because of their hallucinogenic effects. In general, the biosynthetic pathways for the peyote alkaloids have been extensively described in the literature (126-154), but some slight discrepancies exist between these main groups of researchers concerning the exact pathways and precursor incorporation necessary to achieve the major peyote alkaloids. Since the mid 1970's efforts in this area of research have been limited and the study of the complex systems which yield alkaloids have yet to be competely

elucidated in the whole plant. Prior to this study, no one had successfully tissue cultured peyote to aid in the elucidation of Lophophora williamsii chemotaxonomy.

Phenethylamine Alkaloids

The phenethylamine constituents of the peyote may be classsified according to their oxygenation pattern into derivatives of tyramine, dopamine, and 3,4,5-trihydroxy or 3,4,5-trimethoxyphenethylamines. The occurrence of these compounds concurrently in the same plant tissues suggests that they are biogenetically interrelated. In the generally accepted biosynthetic route to mescaline (Figure 2), some controversy has arisen over whether phenylalanine is a precursor to tyrosine (42,43,63). It is well documented that tyrosine is incorporated into alkaloids and is hydroxylated to give DOPA which may be further hydroxylated to dopamine (V) (63,139-141). Tyrosine also may be decarboxylated to form tyramine (I) and then transformed to dopamine (V). The dopamine may be transformed to an intermediate, 3,4,5-trihydroxyphenethylamine (134), which is then converted to 3-hydroxy-4-methoxyphenethylamine (VII) and then to 3,4-dihydroxy-5-methoxyphenethylamine (XI). *O*-Methylation at position 3 and 4 forms the mescaline intermediate, 4-hydroxy-3,5-dimethoxyphenethylamine, before completion to mescaline (XVII)(147). An alternative path for

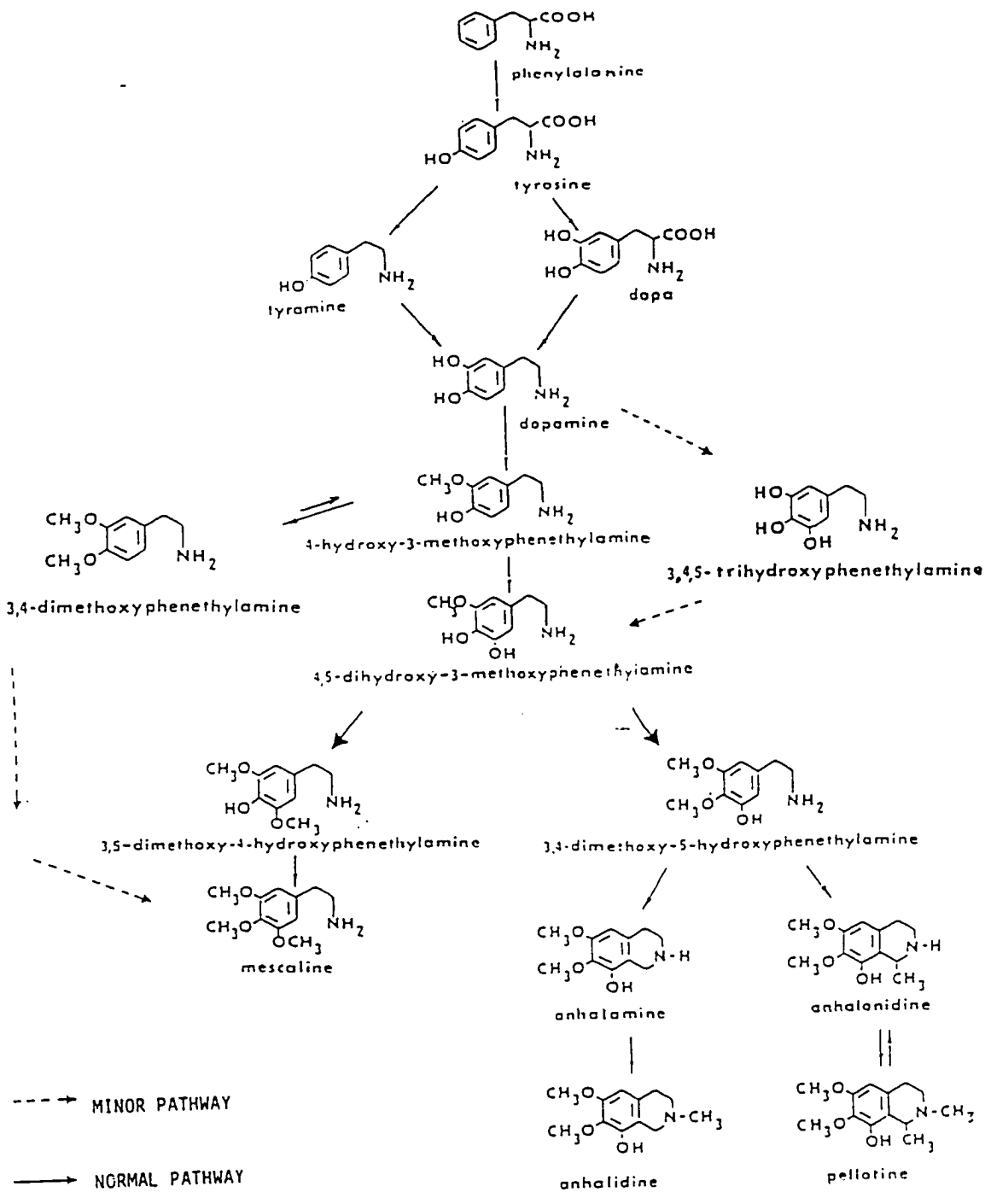


Figure 2. BIOSYNTHETIC PATHWAYS FOR MAJOR ALKALOIDS IN *Lophophora williamsii*

compound XI is *O*-methylation at position 4 to give 3-hydroxy-4,5-dimethoxyphenthylamine (XII) (3-demethylmescaline)(145), the precursor to the tetrahydroisoquinoline alkaloids (146). The methylation is catalyzed by a well-characterized (135,137,138,147) *O*-methyltransferase with L-methionine or S-adenosylmethionine as the source of the methyl groups.

Some controversy exists as to the extent of incorporation of 3,4-dimethoxyphenethylamine (X) into the general biosynthetic pathway leading to mescaline. Paul et al (134), working exclusively with L. williamsii, reported a high incorporation (3.75%) of 3,4-dimethoxyphenethylamine into mescaline (Table 5), while Agurell (112) showed the compound to be a dead-end product in a pathway found in Trichocereus pachanoi (Figure 2). These studies strongly support the hypothesis that 3,4-dimethoxyphenethylamine is a direct precursor to mescaline in L. williamsii by a slightly different biosynthetic route than that found in T. panchanoi.

The *N*-methylated derivatives of tyramine (*N*-methyltyramine, hordenine, epinine (VI), etc.) follow a similar route to that described above (Figure 3) (62,154). The source of the methyl groups were again shown to be methionine via catalysis by an *N*-methyltransferase. The conversion of both tyrosine and tyramine to hordenine (III)(133) and the presence of *N*-methyltyramine (II) and

Table 5. Radioactive Precursors to Mescaline (134)

Percent injected radioactivity recovered as mescaline HCl.	
DL-phenylalanine	0.03%
DL-tyrosine	0.32%
tyramine	1.53%
DL-dopa	1.58%
dopamine	1.90%
4-hydroxy-3-methoxyphenethylamine	1.45%
3-hydroxy-4-methoxyphenethylamine	0.10%
3,4-dimethoxyphenethylamine	3.75%
3,4,5-trihydroxyphenethylamine	0.72%
4,5-dihydroxy-3-methoxyphenethylamine	2.49%
3,4-dimethoxy-5-hydroxyphenethylamine	0.21%
3,5-dimethoxy-4-hydroxyphenethylamine	15.43%

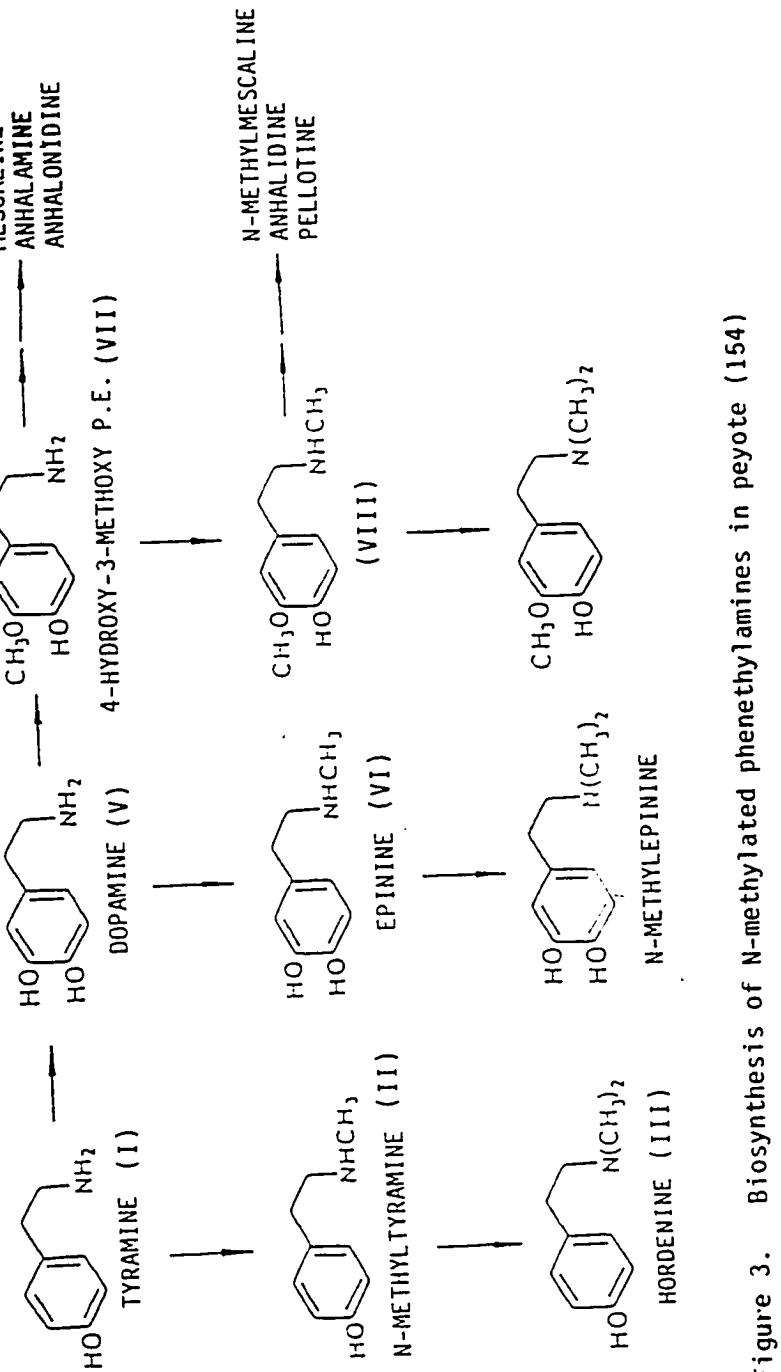


Figure 3. Biosynthesis of N-methylated phenethylamines in peyote (154)

tyramine (I) indicates that the biosynthesis of hordenine in peyote is similar to the biosynthesis of hordenine found in barley rootlets (63,139-141).

The metabolism of aromatic amino acids is closely related to the biosynthesis of alkaloids in many plants, including the peyote cactus. Previous studies on the biosynthetic pathway leading to mescaline using D,L-phenylalanine and D,L-tyrosine reported inconsistent incorporations of the labelled precursors (133-141). Generally only L-isomers of amino acids are biochemically active. Due to the low amounts of incorporation of these D,L mixtures, it would appear that peyote does not possess a phenylalanine or tyrosine racemase or a nonspecific decarboxylase (42,43, 128,141).

Tetrahydroisoquinoline Alkaloids

Even though many detailed experiments have been performed, the complete picture of the biosynthetic pathways leading to the different tetrahydroisoquinoline alkaloids is not fully developed (146-156). The main phenolic tetrahydroisoquinolines present in the peyote are anhalonidine, anhalamine and the *N*-methylated compounds pellotine and anhalidine (59,146,153,154). Several experiments (148-150,152-154) have shown that anhalonidine and anhalamine are derived from tyrosine via dopamine along

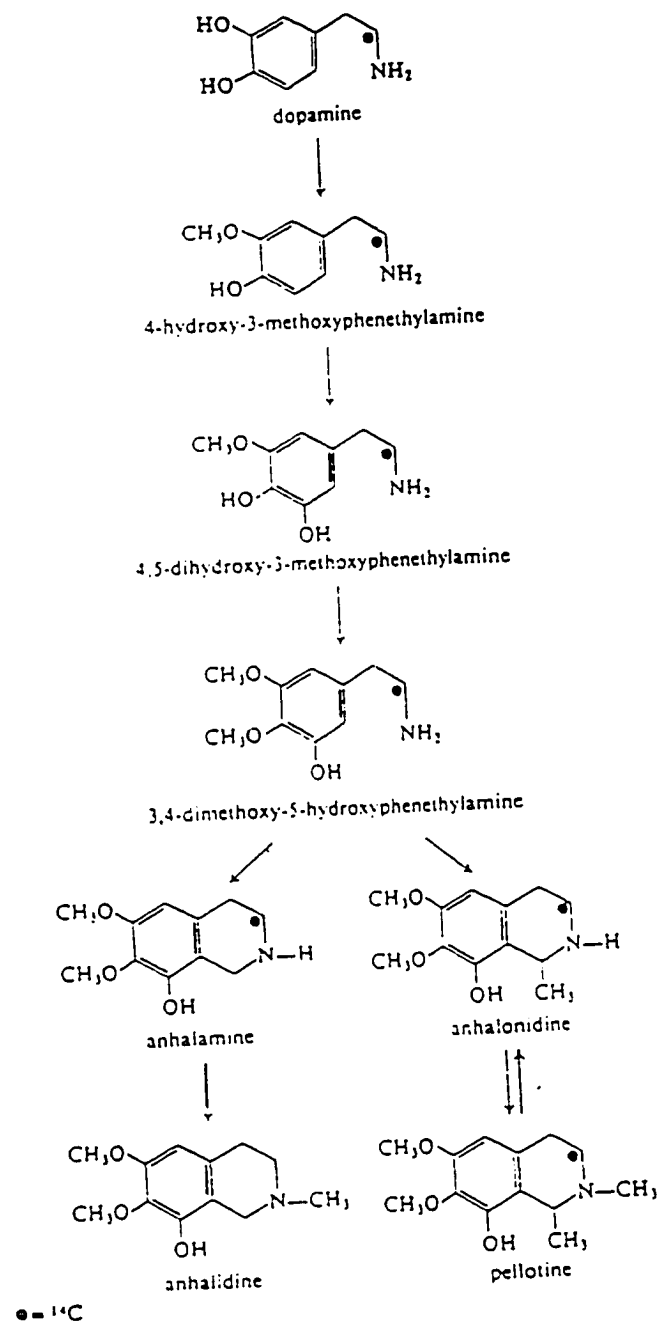


Figure 4. Biosynthesis of phenolic tetrahydroisoquinoline alkaloids in peyote (153)

a pathway similar to that leading to mescaline (Figure 4) (59,144-146,152,154). The precursor from the phenethylamine pathway that is directly involved in the ring closure has been shown to be 3-hydroxy-4,5-dimethoxyphenethylamine (XII) (3-demethylmescaline), but this was not incorporated into the *N*-methylated derivatives. Pellotine and anhalidine are derived through an independent tetrahydroisoquinoline pathway that involves *N*-methylated phenethylamine derivatives (Figure 3).

The origin of the one-carbon (C-1) and two-carbon (C-1 and C-2) ring closing units was assumed to be from formate and acetate respectively, because of early experiments of Battersby (136). In later work, Leete and Braunstein (148) found that pyruvate was specifically incorporated into the two-carbon unit of anhalonidine and it was suggested that the methyl group at the C-1 was formed through acetyl coenzyme A and the *N*-acetyl substituted 3-demethylmescaline. Another researcher (149) has collected results strongly suggesting that the simple tetrahydroisoquinoline (anhalamine, anhalonidine) ring-closure mechanism is the result of a condensation reaction of glyoxylic or pyruvic acid to give peyoxylic or peyoruvic acid. The presence of dihydroisoquinolines in peyote supports such a pathway.

Miscellaneous

Studies of biosynthesis in higher plants have been generally elucidated by means of labelled precursor feeding experiments (63,126-155). These experiments depend on the natural precursor being incorporated efficiently into the alkaloid of interest. There are many published accounts that show that non-physiologic precursors may be processed into naturally occurring alkaloids (59,127,128,130). Other researchers (155) have also been able to incorporate synthetic or non-physiologic precursors to form unnatural or aberrant alkaloids. Such investigations could be important for several reasons: 1) few examples exist in the formation of unique alkaloids, 2) series of unnatural precursors could reveal enzyme(s) specificity in the pathway, and 3) the formation of novel alkaloids (*in vivo*) has potential application for preparation of biologically active analogs in bioreactor-type systems.

Functions of Alkaloids in Plants

The proposed role of alkaloids in plant metabolism, catabolism, or plant physiology is an area that is highly speculative and is not really known. Some general concepts of the role of alkaloids in plants are 1) end products of metabolism or waste products, 2) storage reservoirs of

nitrogen, 3) protective agents for the plant against herbivores, 4) antimicrobial or antiviral agents to protect against plant pathogens, or 5) plant growth regulators.

The structural similarity of some alkaloid structures to several plant growth hormones provided the idea that alkaloids could act as plant growth regulators. Many examples of alkaloids causing inhibition or stimulation of plant growth have been cited (128,129,161,167). Certain alkaloid precursors such as indole derivatives, purines and nicotinic acid are powerful growth stimulators, while phenylalanine-tyrosine derivatives are inhibitory for some plants. The phenethylamines and tetrahydroisoquinolines (and their derivatives) that occur in the peyote and other cacti have been shown to cause growth inhibitory effects comparable to that of abscisic acid on other plants. These compounds, if leached from the cacti, may also play an allelopathic role in the arid environment (157).

One of the most feasible roles proposed by researchers of the alkaloids in plants is their function as a plant protection device. It has been suggested that the compounds serve to protect the plant from invading pathogens (10,158-161). It is well documented that the wound sites of cacti show a marked increase of phenolic substances (chiefly dopamine) and that these inhibit fungal or bacterial invasion into the wound. The antimicrobial effects have also been well documented during topical application to human

wounds and in screening experiments for antimicrobial principles in plants (163-166).

An older proposed role of alkaloid formation to prevent insects or vertebrates from feeding on them may not be true (127-130). Mammals do not generally feed on any one particular plant and will only threaten specific local plant populations in time of drought. The bitter taste of the alkaloids, thought to be perceived by most mammals, usually does not deter feeding and sometimes may enhance grazing for the extra "spice". The insects on the other hand tend to be monophagous or oliphagous, damaging large areas of a specific plant when feeding. Generally the alkaloids provide little protection against the long range effects of insect feeding due to rapid tolerance or coevolutionary changes producing metabolic pathways to detoxify the compound (127-130).

The other general proposed idea for the role of alkaloids is to act as end products of metabolism or as a means to store nitrogen (130,167). It is clear that alkaloids are not inert end products but rather active constituents in the metabolic process of the plant. Fluctuations of alkaloid concentrations have been observed with seasonal and environmental changes as well as diurnal and developmental variations (8,12,15,127-130,167). Although there is little evidence that alkaloids are utilized as a nitrogen source in times of nitrogen deficiency, they still

may provide the plant with a nitrogen pool during specific turn over periods (127-130).

Current Analytical Methods to Separate and Identify Peyote Alkaloids

Thin-Layer Chromatography (TLC)

Until the paper by McLaughlin and Paul (62), no thin-layer or paper chromatographic procedures for the rapid identification of Lophophora bases had been published. Soon after this, many other systems were developed to specifically separate the phenolic, nonphenolic and the quaternary alkaloids (62,65,74,81,86,92,95,168-170). More sophisticated spray reagents were eventually needed to visually differentiate between the primary and secondary amines (42,43,171). The use of fluorescent reagents (fluorescamine and dansyl chloride) gave distinguishing color reactions for primary and secondary amines and also did not interfere with the subsequent use of additional spray reagents (170-172). Further separation of the fluorescent conjugates could then be analyzed by HPLC or mass spectrometry (171,172).

High-Performance Liquid Chromatography (HPLC)

The period between 1974-1978 showed the most dramatic increase in the scope and versatility of high-performance liquid chromatography (HPLC), due to improvements in the availability and efficency of column packing materials. The technique has been widely accepted for the separation and identification of natural products, but little work has been done on simple basic plant natural products (173). Studies on the separation of simple amines have been dominated by examination of clinically important biogenic amines and drugs (173). To date, only two HPLC procedures have been reported which attempt to completely separate isomeric pairs of the major alkaloid groups found in the Cactaceae (174,175). Both of these separation methods are very complicated and involve interconnecting two columns and the need for two different solvent systems to elute the various alkaloids from each of the tandem column sets.

Gas Chromatography (GC)

Gas chromatographic (GC) procedures surpass other methods in sensitivity for the analysis of drugs and biological amines. Many investigators (42,43,70,117,176) have used GC for cacti alkaloid separation, identification

and estimation of their precursors. The combination of gas chromatography with mass spectrometry (GC-MS) appears to be the method of choice by many workers for final positive identification of alkaloids and intermediates found in minute amounts (75,102,112,113).

Mass Spectrometry (MS) and Multiple Stage Mass Spectrometry (MS/MS)

When commercial mass spectrometers were developed in the 1940's the analysis of complex mixtures by MS by itself was of little use. Only when the combination of GC/MS became available for separating the components of complex mixtures, such as those that occur in most natural products, able to be analyzed (75,102,112,113). The time-consuming nature and the limitations of chromatography gave rise to more sophisticated MS equipment (178,179). Early experiments using a mass-analyzed ion kinetic energy spectrometer (MIKES) made it possible to make positive identification of specific mixture components in crude extracts and intact plant material (42,43,115,179). The current use of MIKES and tandem MS/MS (59,76,110,180-186) has a limited ability to separate and distinguish extremely complex mixtures and isomeric molecules, but has distinct advantages over chromatographic procedures. Decreased analysis times, continuous availability of all mixture components for examination, improved limits of detection and selection of

the ionization method to maximize information obtainable from compounds of particular types are advantages that may be applied when analyzing natural products. Many review articles have been written on the use of MS/MS in analyzing complex natural products (177,185,186).

Pharmacology of Major Peyote Alkaloids

General

The pharmacological effects of the tetrahydroisoquinolines in peyote and other simple isoquinoline alkaloids found in natural sources have been the subject of much research (42,188-190). Although these may be good initial models, none of the naturally-occurring compounds appear to possess enough activity to stimulate more research for their use as a pharmacological tool or as a drug (48,191).

Studies with the main peyote tetrahydroisoquinolines shows no hallucinogenic activity in man such as their phenethylamine counterparts (mescaline) possess (42,43,48,59,188,191). They have been shown to have pharmacological effects on both the central and peripheral nervous systems (59,60). Effects encountered in the central nervous system (CNS) have been reported as a depressant, stimulant and also possess convulsant properties (59,60).

Other significant reports on the activities of the tetrahydroisoquinoline alkaloids have shown that they produce vasopressor or depressor actions and various effects on the smooth muscle (59,60,192). Simple dihydroisoquinolines have been shown to be potent inhibitors of cyclic nucleotide phosphodiesterases (192), while catecholamine-derived compounds can act as false neurotransmittors (193).

The phenethylamines possess the main biological activity found in the peyote and have been the primary target compounds because of their hallucinogenic properties (126,190,191,194). The phenethylamines also act as sympathetic stimulants or adrenergic agents (42,190,196), potential antitumor agents (193), and enzyme inhibitors (197,198). The specific actions of the phenethylamines and their analogs will be explained in the following sections.

Adrenergic (Sympathomimetic) Amines

The compounds that resemble catecholamines (tyramine, *N*-methyltyramine, dopamine, hordenine, candicine, epinine, etc.) have a general pharmacological action that includes a rise of arterial blood pressure, stimulation of the heart muscle, constriction of arterioles, stimulation of the CNS, and either stimulation or inhibition of smooth muscle

(42,48,126,188,195,199).

Mescaline

The main hallucinogenic compound in L. williamsii also has been shown to have effects on the cardiovascular system and as a depressant in the respiratory system (42,126). Other miscellaneous actions of mescaline which have been reported are vasoconstriction of the umbilical vessels of the human placenta and hyperthermia in rabbits (188).

Hallucinogenic dosages for humans range from 350 to 400 mg. The LD_{50} of mescaline in rats are 330-410 mg/kg i.p., 157 mg/kg i.v., and 534 mg/kg s.c.. Flexor convulsions and respiratory arrest are terminal events (200).

Anhalonidine

This compound has shown little to no significant effects in mammals (60). In early experiments by Heffter, the only observed reactions were in frogs, producing a narcotic effect at low doses followed by a curare-like paralysis at high doses (as seen in 42).

Pellotine

Doses of 5-10 mg caused temporary convulsions in frogs, dogs and cats (60). Early researchers (Joachimogula and Keeser, 1924; as seen in 42) believed pellotine could be used by man as a relatively safe narcotic.

Lophophorine

Heffter had shown this base to be the most toxic in peyote; 0.25-1 mg produced extended tetany in the frog followed by excitability for several days. In rabbits hyperexcitability, accelerated respiration and increased blood pressure were noted. Progressively higher doses induced hypotension, tetany and eventually led to death (48,60).

Structure-Activity Relationships

Phenethylamine Hallucinogens

Orientation of aromatic substituents has shown that the 2,4,5-trisubstitution pattern yields optimally active compounds over other substitution patterns (191,194). Within the 3,4,5 trisubstituted series, replacement of the 4-methoxy (mescaline) with bromine, alkyl, alkylthio group leads to other compounds with high activity (194).

In general for ring substitutions, the most active compounds possess 4,5-disubstitution with a methoxy group at either the 2-position (2,4,5-substitution) or at the

3-position (3,4,5-substitution). Little work has been reported with 2,4,6-substitutions, but 2,4,6-trimethoxy-amphetamine possessed about 10 times the activity of mescaline in humans (188,194).

It has been demonstrated with several compounds that *N*-alkylation abolishes or dramatically attenuates in vivo activity (194) and in vitro receptor affinity (201). Other side chain modifications have shown an increase in activity in vivo with methylation of the alpha-side chain of 2,4,5-trisubstituted compounds (188). Very little potency increase is noted in the 3,4,5-substituted compounds after alpha-side chain methylation. Expansion of the alpha-methyl group to ethyl or higher homologs abolishes activity (188,194,195). Addition of a *B*-methyl group to the side chain dramatically attenuates in vivo activity in animals (201).

Antitumor Activity

Many of the phenethylamine compounds have been shown to inhibit or retard the growth of lymphocytic leukemia tumors (193). A series of dopamine analogs confirmed that the ortho-aromatic hydroxyl groups were required for activity and the aminoethyl side chain could be replaced by aminomethyl groups with retention of activity. *N*-Methylation did not decrease activity, but *O*-alkylation eliminated all

activity.

Miscellaneous Activity

Several researchers have proposed that phenethylamines and analogs can function as specific enzyme inhibitors in humans having potential involvement in schizophreniform behaviors (197) as well as anorectic effects (198).

Experimental Objectives

Initiation Growth of Peyote Callus Tissue

To date, no published work has been done with the peyote in tissue culture. Therefore, it was necessary to determine empirically the medium composition so that callus growth from seeds or parts of the whole plant could be established. Previously successful hormone combinations and medium types used to establish callus tissues on other slow growing plants, were employed to initiate growth in the peyote cactus. Another aim, was to determine the specific tissue area in the whole plant that would provide actively growing cells to initiate callus growth.

Growth Response of Callus Tissues to Individual Growth Regulators

Several initial experiments using undefined medium led to successful establishment of the peyote callus tissue from stem sections of the whole plant. To prepare a defined medium, a series of individual growth regulators (2,4-D, IAA, kinetin, or BA) were added to the medium. The effect of specific concentrations were observed to characterize the growth response of the callus tissues to these regulators.

Growth and Alkaloid Accumulation in Response to Combinations of Growth Regulators

It is known that combinations of growth regulators can be used to stimulate growth and encourage callus tissues to produce secondary products associated with root, shoot, or undifferentiated tissues. An increase of auxins (2,4-D, IAA) over the cytokinin (BA, KIN) concentration will generally simulate a root environment, while a increasing the concentration of cytokinin greater than the auxin will provide a hormonal condition similar to shoots. Equal proportions of auxin and cytokinin will generally promote undifferentiated callus growth. This regulation of the hormonal environment will give an indication to the general organizational needs of the tissues to accumulate the most alkaloids and enhance callus growth.

Effects of Light Intensity and Quantity on Callus Development

The characteristics of light radiation (intensity, spectral quality and photoperiod) that influence plant development are also those that will effect plant tissues in culture. Some experiments have shown that callus tissues can produce photoautotrophic responses to enhance their growth and secondary metabolite production by variation of light duration and the quality of the radiation (10-12,35-37). These sets of experiments were designed to test the various

effects of light intensities and qualities to promote "photomorphogenesis" in the peyote callus tissues. Indications of a photoresponse in the callus tissues were suggested by enhancement of cellular differentiation and increases in alkaloid accumulation.

Enhancement of Growth and Alkaloid Production of Callus Tissues by Auxin and Cytokinin Manipulation on Solid Media

Results from simple growth regulator combinations (Expt 3) suggested that 2,4-D and BA produced the best growth response and alkaloid accumulation. A more finite set of concentration combinations of the two growth regulators was statistically designed enabling the generation of a response surface graph of tissue growth to 2,4-D and BA concentrations. This type of factorial design enabled a better prediction of growth and alkaloid trends in the callus tissues with various 2,4-D and BA concentrations and proportions.

Measurements of Growth in Liquid Cell Suspensions (LCS)

There are several ways to determine growth in LCS, but all have their own inherent errors. The techniques generally used are settled cell volume, fresh and dry matter increase, and measurement of optical density. These methods were tested to provide a reliable, reproducible and nondestructive method to measure cell growth in liquid cell

suspensions.

Effect of "Conditioned" Medium Additions to LCS

Many researchers have noted that "conditioned" medium (from previously growing cell cultures) or a high concentration of cells must be added to LCS to inhibit retardation of the initial growth phase or death of the cells. During the beginning of these studies, retardation of growth in the liquid medium was noted, necessitating a medium containing an appropriate level of "conditioned" medium which could promote good cellular growth characteristics.

Enhancement of Growth and Alkaloid Production of Suspended Cells by Auxin and Cytokinin Manipulation in Liquid Media

With the increased cellular contact of the components in the liquid medium, a new set of experiments was needed to determine the best proportions of the two growth regulators (2,4-D, BA) to achieve good growth and alkaloid accumulations in both LCS and callus-clump additions of peyote cells.

Effect of Varied Concentrations of M8k Salt Base in the Liquid Medium

Researchers have shown that there are essential components in most media, when found in low concentrations, can limit growth (233-244). Another potential limiting

growth factor can be produced by the normal salt base in the liquid medium. If the osmotic strength is too high it will create a water stress situation and inhibition of proper cellular growth will occur. The objective was to find the proper concentration of a salt base of fixed relative composition to insure proper growth without imposing extreme osmotic effects.

Precursor Addition to LCS

The biosynthetic pathways leading to the phenethylamine and tetrahydroisoquinoline alkaloids in the whole peyote cacti have been fairly well characterized by previous investigators using direct injection of radioactive precursors. However, there are still a few minor conflicts. The reason for these experiments is to attempt to increase alkaloid production by addition of known precursors of mescaline, creating a bioreactor system which will efficiently incorporate relatively cheap, nonlabelled materials.

Confirmation of Phenethylamine Alkaloids by Mass Spectrometry (MS/MS)

The identification of the phenethylamine alkaloids has been tentatively established in all experiments by TLC, HPLC and GC methods. Although these techniques are specific for quantitation and have good resolution of individual alkaloid

constituents, positive confirmation of these constituents by MS/MS was necessary.

Materials and Methods

Experiment 1

Initiation of the Peyote Callus Tissue

Plant Material

Whole specimens of peyote cactus were grown and collected from the Penick Experimental Greenhouse located at the Philadelphia College of Pharmacy and Science. Plants obtained from the University of Michigan were previously vouchered as Lophophora williamsii (Lemaire) Coulter. Several small plants (1.0 - 1.5 cm dia.) were collected, rinsed with distilled water to remove excess dirt and dipped in 95% ethanol for one minute, then rinsed in sterile distilled water. The prepared plants were transferred to a solution of 15% Clorox for ten minutes, then rinsed in sterile distilled water. The surface sterilized plants were subdivided, with the epidermis left intact, into root and shoot pieces, then sliced (1 mm thick) and placed on several types of media to initiate callus tissue growth.

Medium Composition

The foundation of the medium consisted of a commercial Murashige and Skoog salt base (Carolina Biological Supply Co.)(204), vitamins, dextrose, and agar (MSk)(Table 6). In

addition, various concentrations of 2,4-dichlorophenoxy-acetic acid (2,4-D)(1 ppm and 5 ppm) were combined with the liquid endosperm from green coconuts (0, 5, 10 and 15 %, v/v). After mixing the individual medium types, the different solutions (30 mL) were transferred to separate containers and then autoclaved for twenty minutes at 125°C and 18 psi.

Growth Conditions

The excised plant parts were maintained under low light conditions (Sylvania 40W cool white fluorescent tubes at 9.9 uE s^{-1} m^{-2}, light intensities were measured by a LI-1000 Data Logger with a Q-8030 quantum sensor; LI-COR, Lincoln, NE) in the lab under a 10/14 (day/night) regimen at ambient temperature. After callus initiation, the callus tissues were maintained under similar light conditions and subcultured every 30 - 45 days.

Experiment 2

Growth Response of Callus Tissue to Individual Growth Regulators

Plant Material

The callus tissues derived from the stem sections of the peyote cactus were divided and maintained on solid medium containing MSk + 1 ppm 2,4-D + 10 % liquid endosperm. After subculturing for several months, enough callus tissue was achieved to start other experiments and also limit the

TABLE 6: FORMULATION FOR MURASHIGE AND SKOOG MEDIUM (MSK)

SALT BASE (204)

COMPONENTS	MG/LITER
NH_4NO_3	1650.000
KNO_3	1900.000
$CaCl_2$ (ANHYDROUS)	333.000
$MgSO_4$ (ANHYDROUS)	181.000
KH_2PO_4	170.000
FeNaEDTA	36.700
H_3BO_3	6.200
$MnSO_4 \cdot H_2O$	16.900
$ZnSO_4 \cdot 7H_2O$	8.600
KI	0.830
$Na_2MoO_4 \cdot 2H_2O$	0.250
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
TOTAL	4303.530

VITAMINS (STOCK SOLUTION)

COMPONENTS (MG/100.00 ML)		
NIACIN	500	50
THIAMINE HCL		50
PYRIDOXINE HCL		50
I-INOSTITOL		1000

ADD 1.000 ML/ OF STOCK/LITER

DEXTROSE 30.00 GM/LITER

AGAR (SOLID MEDIUM ONLY) 10.00 GM/LITER

amount of original explant tissue. The callus tissues were used to inoculate the separate medium types and were then grown as in Experiment 1.

Medium Composition

The basic MSk solid medium was modified by the addition of individual growth regulators (auxins; IAA, 2,4-D and cytokinins; BA, KIN) at various concentrations (0, 1, 3, 5, 10, and 20 ppm).

Growth Conditions

The callus tissues were grown as described in Experiment 1.

Measurement of Growth Response

The callus tissues were placed in tared sterile tubes and weighed before transplantation to determine their initial fresh weights. A series of ten samples were taken to determine the average percentage dry weight per fresh weight of callus tissue. This corrected for water fluctuations in the cells that may occur during the initial transplantion and final collection and also allows for initial dry weight estimations. After thirty days, the callus tissues were collected and placed in tared tubes to be weighed for final fresh weights. The tissues were frozen and lyophilized after which they were quickly weighed for the final dry weights. Calculations were made to determine dry matter and water percentages of the final fresh weight and the growth index (G.I.) for both fresh and dry callus tissues. The formula

used for determination of growth index was as follows:

$$G.I. = \frac{\text{Final Weight - Initial Weight}}{\text{Initial Weight}}$$

The G.I. corresponds to a measure of the relative growth increase during the course of the experiment (e.g. a G.I. of 0.00 indicates no growth, while a value of 1.00 indicates a 100 % increase of growth over the initial callus tissue added.)

Experiment 3

Growth and Alkaloid Accumulation in Response to Combinations of Growth Regulators

Plant Material

The callus tissues used for transplantation were continuously subcultured on MSk + 1 ppm 2,4-D + 10 % liquid endosperm as previously described.

Medium Composition

The general MSk solid medium described previously was used with addition of auxin (IAA or 2,4-D): cytokinin (BA or KIN) at 1:1, 1:5, 1:10, 5:1, and 10:1 ppm.

Growth Conditions

The callus tissues were grown as decribed in Experiment 1 for thirty days.

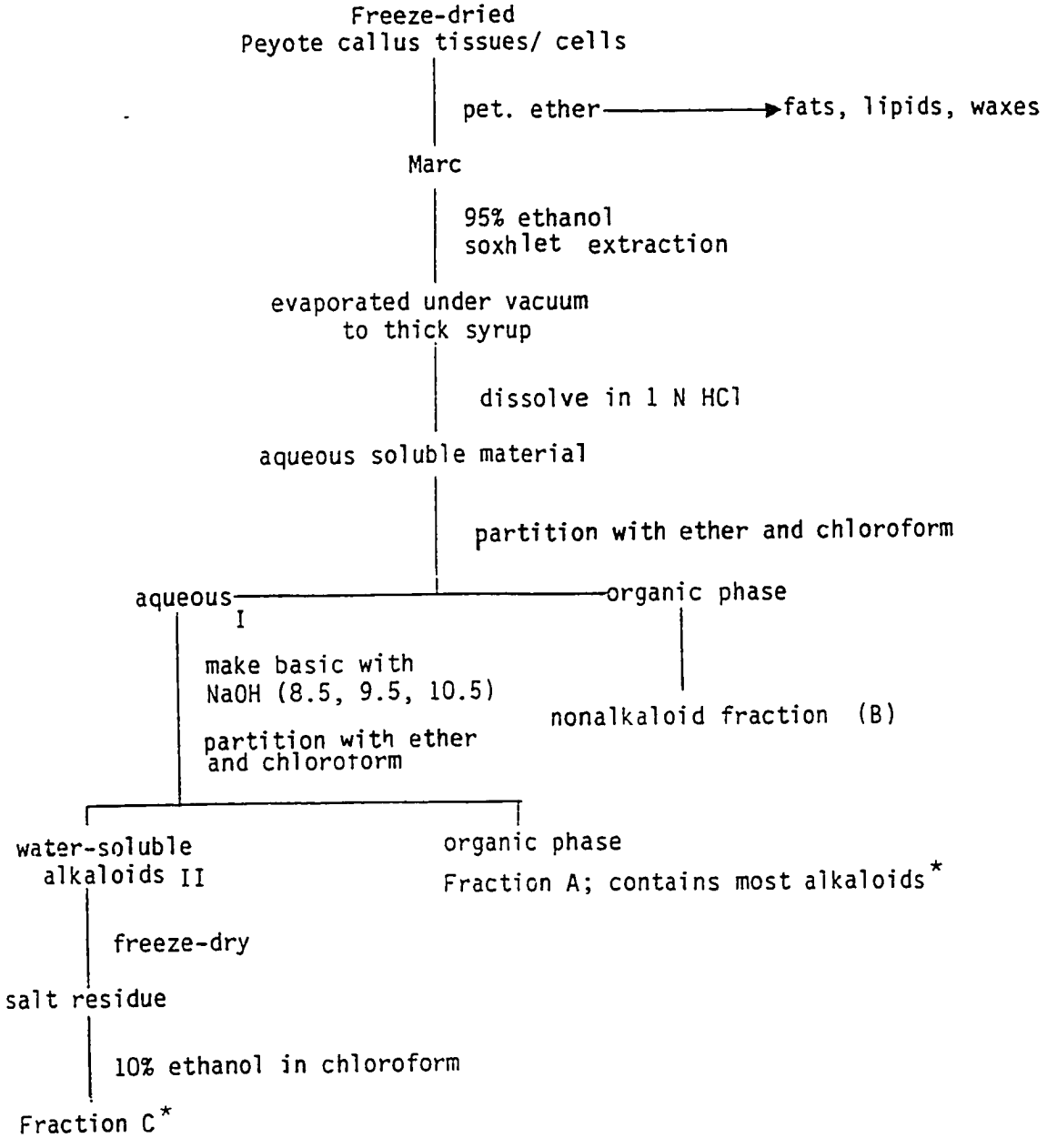
Measurement of Growth Response

Eight samples were taken to determine the average percentage dry weight per freshweight of callus tissue. Following weight determination in sterile tared tubes, the procedure and calculations for fresh and dry weight G.I. of the calli were calculated as described in Experiment 2.

Alkaloid Determination

Alkaloid Extraction- The lyophilized peyote callus tissues from each growth regulator combination in this experiment were analyzed for alkaloid production. The tissue was initially defatted with petroleum ether to remove waxes, lipids and other nonpolar components. The marc was then extracted several times with 95 % ethanol, combined and evaporated under vacuum, leaving a thick brownish syrup. The syrupy residue was redissolved in 10 mL of 1.0 N HCl to remove aqueous soluble material. The aqueous extracts were filtered, combined, and then partitioned several times with equal volumes of both ether and chloroform. These organic extracts were combined, dried over anhydrous sodium sulfate, and concentrated under vacuum to a residue containing mainly nonalkaloidal material (Fraction B)(Figure 5). The aqueous portion was saved for further extraction.

Following basification of the aqueous solution I to pH 8.5 with sodium hydroxide, the solution was extracted with 3 x 20 mL each of ether and chloroform. Repeated extractions at pH 9.5 and 10.5 were combined and concentrated under



*Combine alkaloid fractions A and C for analysis

Figure 5. Preparation of crude alkaloid fraction

vacuum. The residue which remained contained the bulk of the alkaloids (Fraction A).

After removing traces of organic solvent from the aqueous solution II by placing it under vacuum at 40°C for 30 min the solution was freeze-dried. The remaining salt residue was extracted with portions of 10 % (v/v) ethanol in chloroform until the eluates were colorless. The combined eluates were filtered and condensed under vacuum to yield the water-soluble alkaloids (Fraction C).

After analysis by thin-layer chromatography (TLC), fractions A and C were combined for analysis by semiquantitative TLC and gas chromatography (GC).

TLC - The TLC methods of Ludstrum and Agurell (170) for peyote alkaloids were used to provide tentative identification of alkaloids in the extracts. Analytical TLC plates prepared with a 250 uM layer of silica gel G were used (Analtech, Newark, DE).

Analytical separations were achieved by the use of the following solvent systems: System A (phenolic alkaloids) chloroform - ethanol - diethylamine (85:10:5, v/v/v); System B (nonphenolic alkaloids) chloroform - *n*-butanol - conc. ammonia hydroxide (50:50:2.5, v/v/v)(170).

Alkaloids were visualized by the use of *o*-dianisidine reagent (equal volumes of 0.5 % *o*-dianisidine in 1 N HCl and 10 % sodium nitrite in water)(170) or acidified iodoplatinate reagent (202).

GC - Initial attempts to separate the peyote alkaloids followed the procedures of Ludstrom and Agurell (70) and Kapadia and Rao (176), but were then modified to take advantage of better (more selective) stationary phases and the availability of temperature programming instrumentation.

All separations were carried out with a Varian Model 3700 gas chromatograph using a packed six foot Alltech OV-17 (3 %) column with KOH (0.01 %) on Chromosorb W-HP 100/120 mesh. Improved resolution was obtained with a temperature program starting at 165°C (5 min) increasing to 185°C (4°C/min) for 30 minutes. The injector and flame ionization detector (FID) were set at 170°C and 240°C respectively. Nitrogen was used as the carrier gas at a flow rate of 40 mL/min and the detector sensitivity was 128 x 10^{-12}.

Experiment 4

Effects of Light Intensity and Quality on Callus Development

Plant Material

The callus tissues from previous subcultures were divided and maintained on MSk medium containing 1 ppm 2,4-D + 10 % liquid endosperm. The callus tissues were transferred to a MSk medium containing 1 ppm 2,4-D + 5 ppm BA and were subcultured until a consistent growth rate and appearance

were obtained on the new maintenance medium.

Medium Composition

It was determined from the previous experiment that the combination of 1 ppm 2,4-D and 5 ppm BA stimulated the best growth and alkaloid production and so was used in this study.

Photoperiod Effects

The callus tissues were grown in sterile glass canning jars (1 qt) laid on their sides containing 100 mL of the MSk media with 1 ppm 2,4-D and 5 ppm BA for 30 days. The callus tissues were grown in a Norlake environmental chamber (Sylvania 25W incandescent bulbs and Sylvania 215W fluorescent tubes model FR96T12/CW/VHO/135; 450 uE s^{-1} m^{-2}, 50 % humidity, 24/17°C) under 0/24, 8/16, 16/8, and 24/0 day/night conditions.

Light Quality Effects

The light quality experiment involved similar growth conditions as described above with the exception of the wavelength of light available to the cultures. Light filters (Carolina Biological Supply Co.) provided a light range centered on 450 nm, 545 nm, 650 nm, or 750 nm (Figure 6)(203) at an intensity of 0.35 uE s^{-1} m^{-2}. Height adjustment of filters provided similar intensities. The photoperiod of 16 hours per day was chosen from the previous experiment since it provided the greatest growth index for the regimens tested. After a growth period of 30 days, the

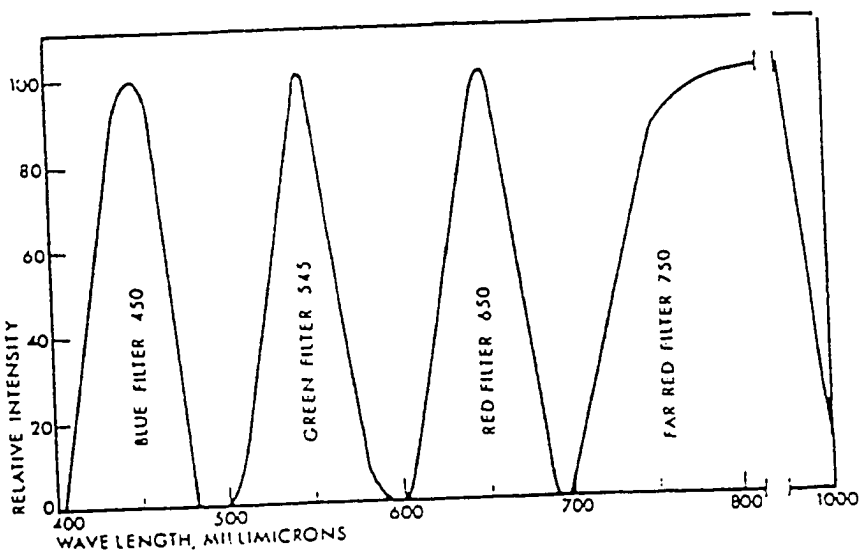


Figure 6. Spectral evaluation of monochromatic filters (203)

growth response, quantity and quality of the alkaloids in the callus tissues were determined as previously described.

Experiment 5

Enhancement of Growth and Alkaloid Production of Callus Tissues By Auxin and Cytokinin Manipulation on Solid Media

Plant Material

The callus tissues were subcultured and maintained on solid MSk medium containing 1 ppm 2,4-D + 5 ppm BA as described in Experiment 4.

Medium Composition and Growth Conditions

In Experiment 4 it was determined that combinations of 2,4-D and BA stimulated the best growth and alkaloid production. In order to further enhance growth and investigate the qualitative and quantitative influence of the growth regulators on alkaloid production, a finer gradation of concentrations was needed. A response surface experiment (205) was designed to test 2,4-D and BA in combinations at concentrations of 0, 0.5, 1.0, 3.0, 5.0, 7.0, and 10.0 ppm.

Callus tissue was transferred to glass test tubes (18 x 150 mm) containing 10 mL slants of MSk media with the various concentrations and proportions of 2,4-D and BA. They were grown for thirty days.

The G.I. of the tissue was determined by the procedure

previously described. Alkaloid fractions were isolated as before, but were analyzed qualitatively by an improved TLC procedure prior to determination by GC.

Alkaloid Determination

TLC - The TLC methods of Ranieri and McLaughlin (171) were employed to provide tentative identification of alkaloids in the extracts using Analtech analytical TLC plates. This method increases detection sensitivity and compound selectivity by employing an improved spray reagent.

Following development of the plates using diethyl ether - methanol - conc. ammonium hydroxide (85:10:5, v/v/v) for 15 cm, the alkaloids were located by a series of spray reagents giving both fluorescent and visible conjugates. The first spray used in the series was fluorescamine (0.02 % in acetone, w/v) to visualize primary amines (aquamarine or yellow fluorescence) and secondary amines (quenched, dark purple). Overspraying the plate with 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl; 0.05 % in acetone, w/v) converts secondary amines from fluorescamine conjugates (dark purple) to fluorescent Dns conjugates (yellow). A subsequent spray with iodoplatinate then visualizes the tertiary amines (purple) with visible light (171).

Two-Dimensional TLC - Complete separation of the complex mixture of the phenethylamine and tetrahydro-

isoquinoline compounds produced by the whole plant and the peyote callus tissues was achieved by the use of the following solvent systems: system 1, diethyl ether - methanol - conc. ammonium hydroxide (85:10:5, v/v/v) and system 2, chloroform - n-butanol - conc. ammonium hydroxide (50:50:2.5, v/v/v). The plates were allowed to run for 15 cm from the origin for both solvent systems. The plate was air dried for 10 minutes before development in the second system at a 90° angle to the direction of the first development. The plate was removed, air dried for several minutes, and then sprayed with the series of three reagents mentioned above.

GC - The procedure was as previously described (Exper. 3).

Experiment 6

Measurements of Growth in Liquid Cell Suspension (LCS)

Plant Material

The callus tissues used were obtained from sources already described. In order to initiate growth of peyote cells and cell masses in liquid medium, large clumps of callus tissue grown on the M8k medium. They were then physically broken apart into smaller pieces (clump addition method). A cell suspension consisting of single cells and very small cell aggregates was obtained using the clump

addition method in a large rotary shaker flask, followed by growth for thirty days. The single cells and small aggregates were then collected by passing the heterogeneous mixture through a metal screen (1.0 mm mesh size) and removing the larger cell clumps. The filtrate was then passed through another metal filter (140 um mesh size; Carolina Biological Supply Co.), which removed the single cells and aggregates from the liquid. The cells were resuspended with some of the filtrate to provide a LCS such that equal aliquots could be easily pipetted into the individual treatments.

Medium Composition

The foundation of the liquid media was similar to that of the solid media, but contained no agar. The growth regulators added initially were the same as for the solid media (1 ppm 2,4-D and 5 ppm BA), but were later changed (0.5 ppm 2,4-D and 0.5 ppm BA) following the experiments which utilized various proportions and combinations of 2,4-D and BA.

Growth Conditions

After inoculation (clump or single cell), the cells were grown in 500 mL Erlenmeyer flasks containing 50 mL of MSk medium with 0.5 ppm 2,4-D and 0.5 ppm BA while being swirled on a rotary platform shaker (60 Hz). Single cell suspensions for Klett meter measurements were maintained under similar conditions, but were initially inoculated into

500 mL Nephelo flasks (Crown Glass Co. and Wheaton Glass Co.) The cultures were grown under the low light conditions described previously (Exper. 1) and the final collection was made after 28 - 30 days.

Measurements of Growth

Klett-Summerson Method - A Klett-Summerson photoelectric colorimeter fitted with a blue filter (400 - 465 nm) was used to measure the relative growth rate of the single cell suspensions (SCS). After inoculation (zero time) into the Nephelo flasks, the SCS were analyzed by filling and emptying the side arm several times before reading in the Klett meter. The readings were made every three days following the inoculation and on the final day just prior to collection. The collection of the cells was made by vacuum filtration through a lyophilized and tared millipore filter (0.45 um) so that the Klett meter readings could be correlated with actual weights.

Fresh and Dry Growth Index - For the clump tissue addition method, the callus tissues were placed in sterile tared tubes and weighed before inoculation to determine the initial fresh weight. Several samples (6-8 samples) were taken to determine the average percentage fresh and dry weight of the callus tissue and to correct for any water fluctuations in the cells that may occur during the experiment. After 28 - 30 days the callus tissue clumps amd

small aggregates were collected by vacuum filtration with tared millipore filters (0.45 um) and placed in tared tubes to determine the final fresh weights. The tissues were frozen and lyophilized. The tissues were weighed and calculations were made for the final percentage dry weight per final fresh weight and the growth index (G.I.) for both fresh and dry weights.

The SCS were inoculated, after isolation and dilution, with approximately the same number of cells and small aggregates (based on Klett readings). Six samples of these aliquots were taken and collected on tared millipore filters (0.45 um) to determine initial fresh and dry weights of the cells. The filters with the collected cells were placed in petri dishes, frozen, and lyophilized until all the free water was removed.

Settled Cell Volume - Growth with the clump tissue method was tested by placing the fragmented clumps in a large, graduated, conical tube to allow relative amounts of tissues to be measured. Weighed tissues were broken apart after being added to the liquid medium and measured immediately in a sterile conical tube for the initial settled cell volume (SCV). The clumps were placed into their culture flasks to continue the growth cycle on the rotary shaker and the SCV was determined every 4 days. After 28 - 30 days of growth, the clumps were collected by vacuum filtration on tared millipore filters (0.45 um),

lyophilized, and the fresh and dry G.I. calculated.

Experiment 7

Effect of "Conditioned" Medium Addition to LCS

Plant Material

The callus tissues were grown on solid media as described previously for clump addition to the liquid medium.

Medium Composition

The liquid medium was the same as that decribed in Experiment 6. Old or "conditioned" medium was obtained by filtering thirty day old LCS through a Nalgene sterilization filter unit (type S; 115 mL; 0.20 um pore size). Aliquots of the conditioned medium were placed in the Erlenmeyer flasks along with various quantities (25 mL total volume) of freshly prepared media of the same initial composition to yield percentages of conditioned medium 0, 25, 50, 75, and 100 %.

Growth Conditions and Growth Measurements

After inoculation, the cells were grown in 125 mL Erlenmeyer flasks on a rotary platform shaker (60 Hz).

Growth was for 28 - 30 days under light and temperature conditions described previously. Cultures were analyzed for growth characteristics as described (excluding settled cell volume) in Experiment 6.

Experiment 8

Enhancement of Growth and Alkaloid Production of Suspended Cells by Auxin and Cytokinin Manipulation in Liquid Media

Plant Material

The callus tissues were obtained through previously described sources and conditions for the clump and single cell additions to the liquid medium.

Medium Composition

From Experiment 4, it was determined that combinations of 2,4-D and BA stimulated the best growth and alkaloid production. In order to enhance growth and investigate the qualitative and quantitative influence of the growth regulators on alkaloid production in the liquid media, a finer gradation of these two growth substances was needed. Similar to the solid medium experiments, a response surface experiment (205) was designed to test 2,4-D and BA in combinations at concentrations of 0, 0.5, 1.0, 3.0, 5.0, 7.0, and 10.0 ppm.

In addition to the new media composition, 50 % of the final volume (50 mL total) was supplemented with conditioned

media (MSk + 0.5 ppm 2,4-D + 0.5 ppm BA). The addition of the "conditioned" medium was to prevent inhibition of growth or death of the cells as suggested by the results of the previous experiment.

Growth Conditions and Growth Measurements

After inoculation, the cells were grown in 500 mL Erlenmeyer flasks on a rotary platform shaker (60 Hz). Growth was for 28 - 30 days under light and temperature conditions as described previously. Samples were analyzed for growth characteristics as described (excluding settled cell volume) in Experiment 6.

Alkaloid Determination

The extraction procedure, TLC identification and GC quantitation were as described in Experiment 5.

Experiment 9

Effect of Varied Concentrations of MSk Salt Base in the Liquid Medium

Plant Material

The callus tissues were grown as described previously and used for the clump and single cell additions to liquid medium.

Medium Composition

From the results of Experiment 8, it was suggested that the combination of MSk with 0.5 ppm 2,4-D and 0.5 ppm

BA stimulated the best growth and alkaloid production in liquid medium (50 mL total volume). This experiment was designed to vary the relative MSk salt base concentrations (0.05, 0.25, 0.50, 0.75, 1.00, and 1.25; relative to 1.00 MSk = 4.303 g/liter) in order to investigate the influence on growth.

Growth Conditions and Growth Measurements

After inoculation, the cells were grown in 500 mL Erlenmeyer flasks on a rotary platform shaker (60 Hz). Growth was for 28 - 30 days under light and temperature conditions described previously. Cultures were analyzed for growth characteristics as described (excluding settled cell volume) in Experiment 6.

Experiment 10

Precursor Addition to LCS

Plant Material

The callus tissues were grown as described previously and used for the clump and single cell additions to liquid medium.

Medium Composition

From the results of Experiment 8, it was suggested that the combination of MSk with 0.5 ppm 2,4-D and 0.5 ppm BA stimulated the best growth and alkaloid production in liquid medium (50 mL total volume). This experiment used

precursors (D and L-phenylalanine, D and L-tyrosine, 3,4-dimethoxyphenethylamine, and methionine) shown to be incorporated in the alkaloid biosynthetic pathway found in the whole cactus. Glycine was used as a control compound. After dissolving in water, the precursor solutions were millipore filtered and added to the liquid medium providing 2.5, 5.0, 10.0, or 20 mg of precursor per flask.

In addition to the fresh medium, 50 % of the final medium volume of each flask consisted of old medium (MSk + 0.5 ppm 2,4-D + 0.5 ppm BA) to prevent death or initial inhibition of growth ("conditioned" medium) of the cells.

Growth Conditions and Growth Measurements

After inoculation, the cells were grown in 500 mL Erlenmeyer flasks on a rotary platform shaker (60 Hz). Growth was for 28 - 30 days under light and temperature conditions as described previously. Cultures were analyzed for growth characteristics as described (excluding settled cell volume) in Experiment 6.

Alkaloid Determination

The extraction procedure and TLC identification were as previously described in Experiment 5. The GC methods were modified from previous descriptions to take advantage of a more suitable stationary phase and the increased resolution capabilities of the megabore capillary column.

GC - All separations were carried out with a Varian Model 3700 gas chromatograph on a ten meter (i.d. 0.53 mm)

Alltech bonded FSOT RSL-300 column with a liquid stationary phase (1.2 um film thickness) of polyphenylmethylsiloxane. Higher resolution was obtained with temperature programming starting at 120°C (7 min) increasing to 165°C (7°C/min) for 30 minutes. The injector and FID temperatures of 140°C and 190°C respectively. Nitrogen was used as the carrier gas at a flow rate of 2.0 mL/min and the detector sensitivity was at 8 x 10^{-10} amps/mV. The data were collected and processed with a Varian 4270 integrator.

Experiment 11

Comfirmation of Phenethylamine Alkaloids by Mass Spectrometry (MS/MS)

Alkaloid Determination

The extraction and TLC procedure were as described in Experiment 5.

Various fractions obtained from the extraction of the callus tissues and isolated bands from the analytical TLC were analyzed for phenethylamine alkaloids.

MS/MS Intrumentation

Tandem mass spectrometry was carried out using a Finnigan TSQ (Model 4500) triple quadrupole spectrometer at E. R. Squibb and Sons (Princeton, NJ). This system consists of three coaxially arranged quadrupole rod assemblies. The first and third quadrupoles are conventional mass analyzers,

and the second quadrupole is used as a focusing collision cell to generate daughter spectra from selected parent ions. Samples were introduced into the source by a direct chemical ionization method. Methane and ammonia were employed independently as the chemical ionization gases (0.4 - 0.7 torr) and argon was used as the collision gas. The collision energy was 30 eV.

Statistical Modeling and Analysis

The response surface design is a statistical approach used to predict growth and alkaloid production of callus and liquid cell suspensions over a wide range of hormonal environments (variable auxin and cytokinin combinations). The two factor experiments for solid and liquid media were designed to test the interaction of seven concentrations of auxin and cytokinin in a factorial fashion (7 x 7) without an excessive number of treatment groups. The three dimensional graph then generates predictive data for finite concentrations and combinations not tested (within the concentration limits). The response surface design also allows for predictive empirical modeling of growth and alklaoid production under specific hormonal conditions (205).

Statistical analysis to suggest differences in growth and alkaloid production between treatment groups was

obtained by the two most common methods in agricultural research. These are the least significant difference (LSD) test for planned pair comparison, and Duncan's multiple range test (DMRT) for unplanned pair comparison. The LSD is the simplest and the most commonly used procedure to compare treatment means with specific pairs of treatments. The DMRT procedure is used with a large number of treatment groups requiring the evalualtion of all possible pairs of treatment means. The comparison of means in both procedures were carried out at the 5 % level of significance (205,206).

TABLE 7. INITIATION OF PEYOTE CALLUS TISSUE ON VARIOUS MSK MEDIA

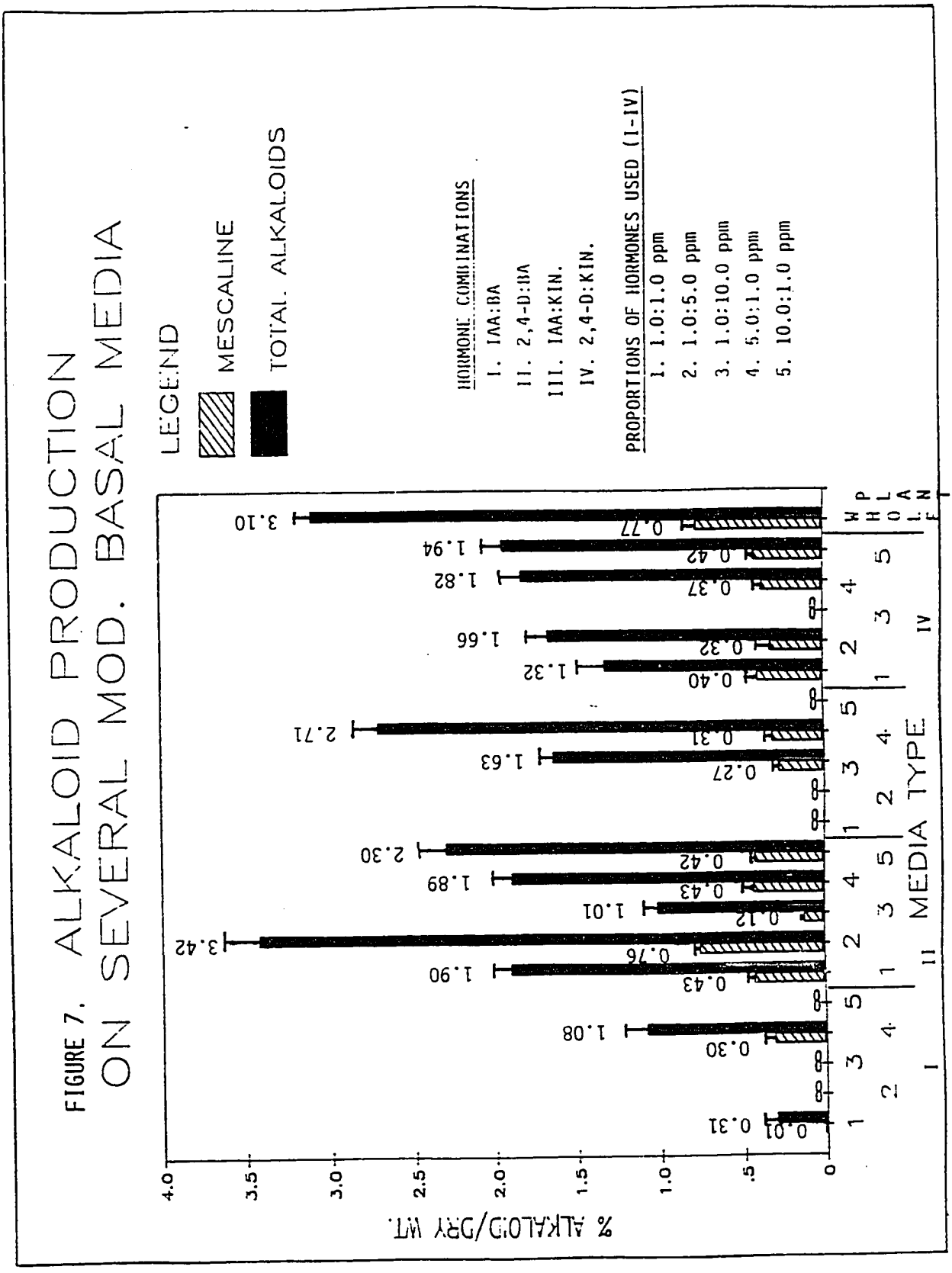
MSK MEDIUM INCLUDING:	INITIATION ABILITY
1 PPM 2,4-D + 0 % L.E.	-
+ 5 % L.E.	+
+ 10 % L.E.	+++
+ 15 % L.E.	+++
5 PPM 2,4-D + 0 % L.E.	-
+ 5 % L.E.	-
+ 10 % L.E.	-
+ 15 % L.E.	+

L.E. (LIQUID ENDOSPERM FROM GREEN COCONUTS);
(-) NO CALLUS DEVELOPMENT, (+) WEAK ABILITY TO INITIATE CALLUS TISSUE, (++) MODERATE ABILITY, (+++) STRONG ABILITY

TABLE 8. GROWTH OF PEYOTE CALLUS TISSUE ON SOLID MSK MEDIUM WITH AUXIN AND CYTOKININ MANIPULATION

GROWTH REGULATOR TREATMENTS	RELATIVE GROWTH	COMMENTS
I. IAA:BA		
1.	+	ONE RT-LIKE PROJECT.
2.	+	
3.	-	
4.	+++	ONE RT-LIKE PROJECT.
5.	-	
II. 2,4-D:BA		
1.	++++	LOOSE, FRIABLE
2.	+++++	LOOSE, FRIABLE
3.	++	
4.	+++	LOOSE, FRIABLE
5.	+++	
III. IAA:KIN		
1.	+++	MANY RT-LIKE PROJECT.
2.	-	
3.	-	
4.	+++	MANY RT-LIKE PROJECT.
5.	+++	MANY RT-LIKE PROJECT.
IV. 2,4-D:KIN		
1.	+++	LOOSE, FRIABLE
2.	+++	LOOSE, FRIABLE
3.	-	
4.	+++	
5.	+++	

TREATMENTS (PPM:PPM); 1. 1:1; 2. 1:5; 3. 1:10; 4. 5:1; 5. 10:1
RELATIVE GROWTH; (-) NO GROWTH; (+) POOR GROWTH; (++) MODERATE GROWTH; (+++) GOOD GROWTH; (++++/+++++) EXCELLENT GROWTH.
RT-LIKE PROJECT; ARE AERIAL, HAIRY TAP-ROOT PROJECTIONS.



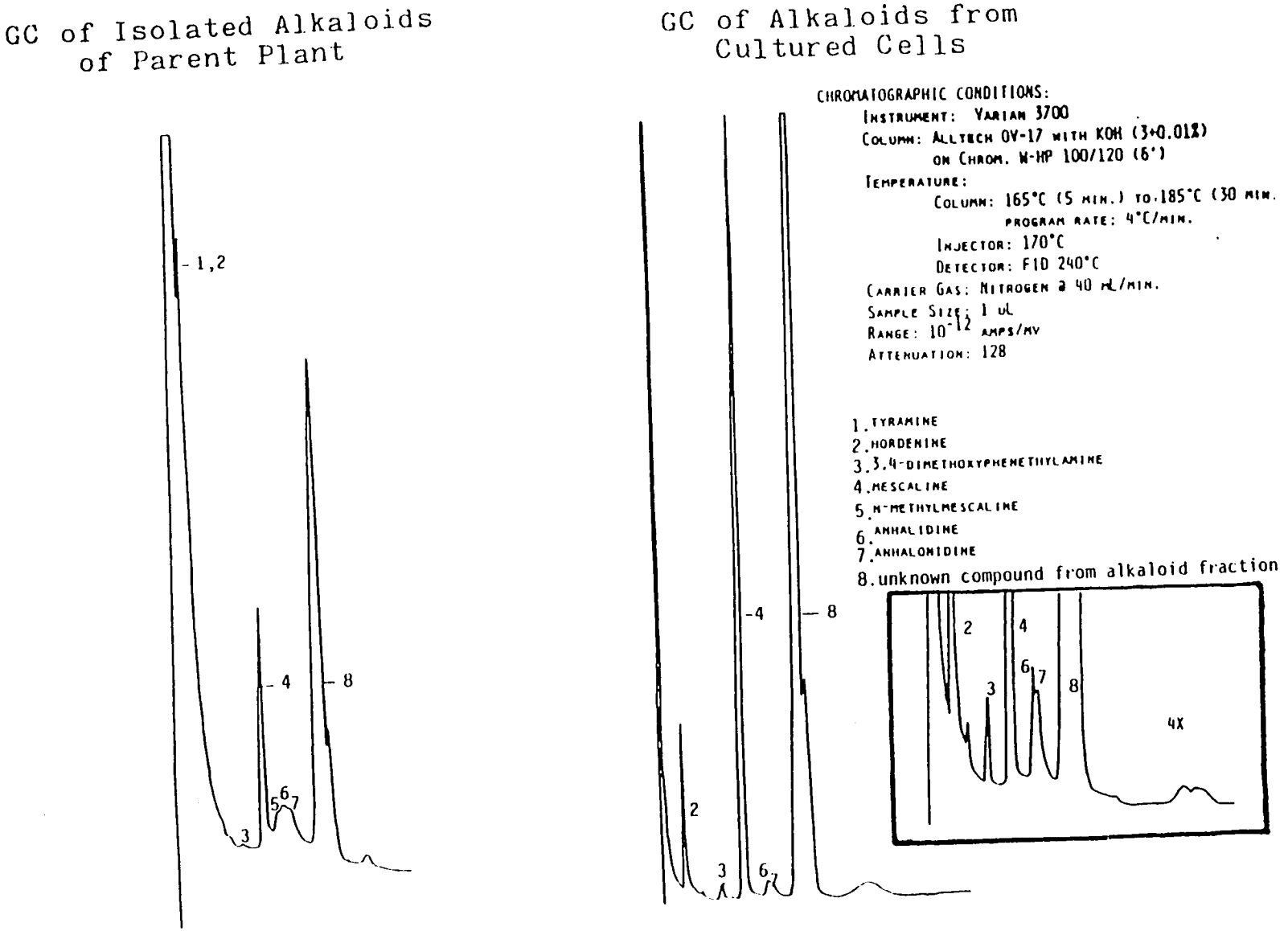


FIGURE 8. GC OF PARENT PLANT AND CALLUS CULTURE ALKALOIDS.

FIGURE 9. GC OF OLDER CALLUS TISSUES AFTER SEVERAL SUBCULTURES.

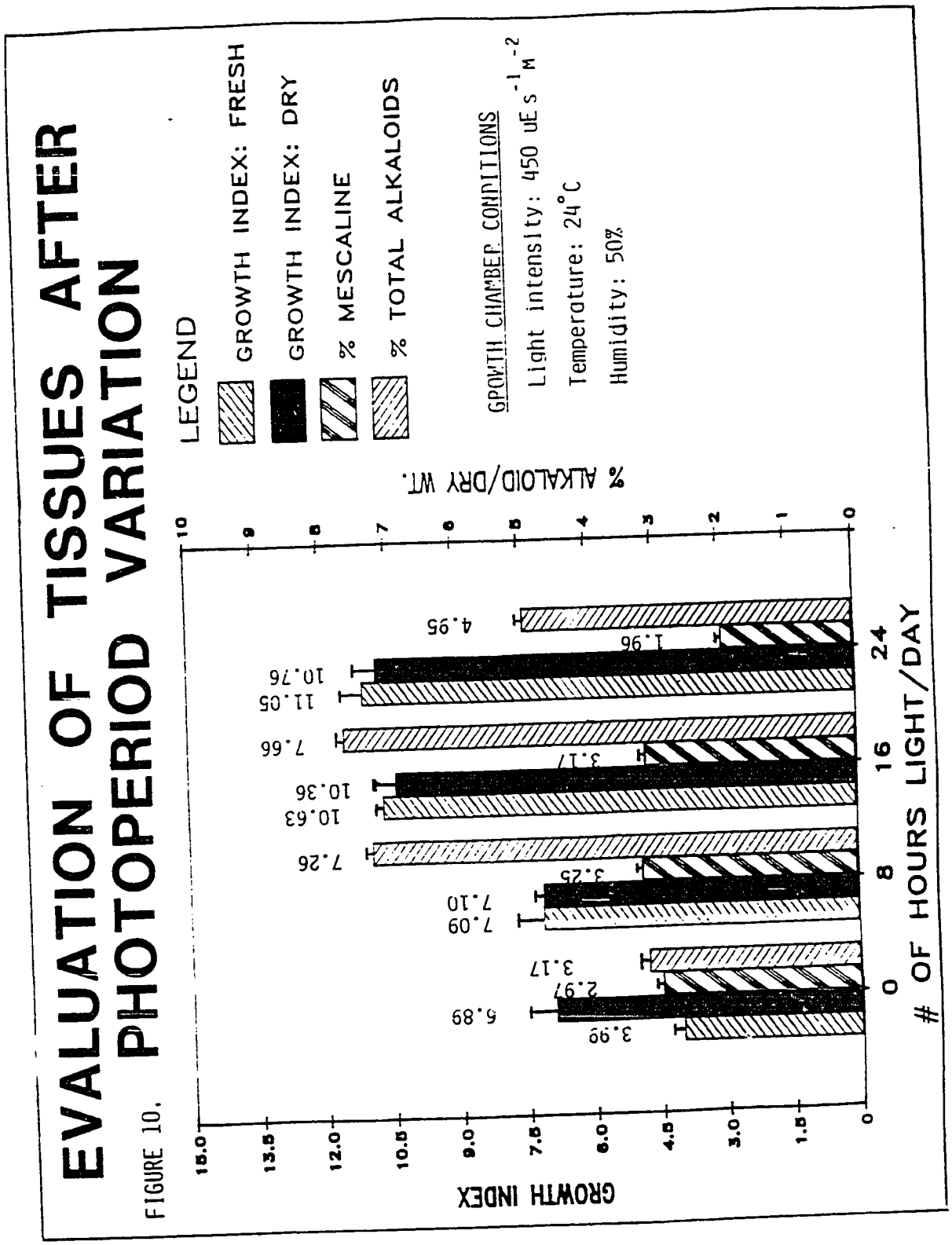


FIGURE 10.

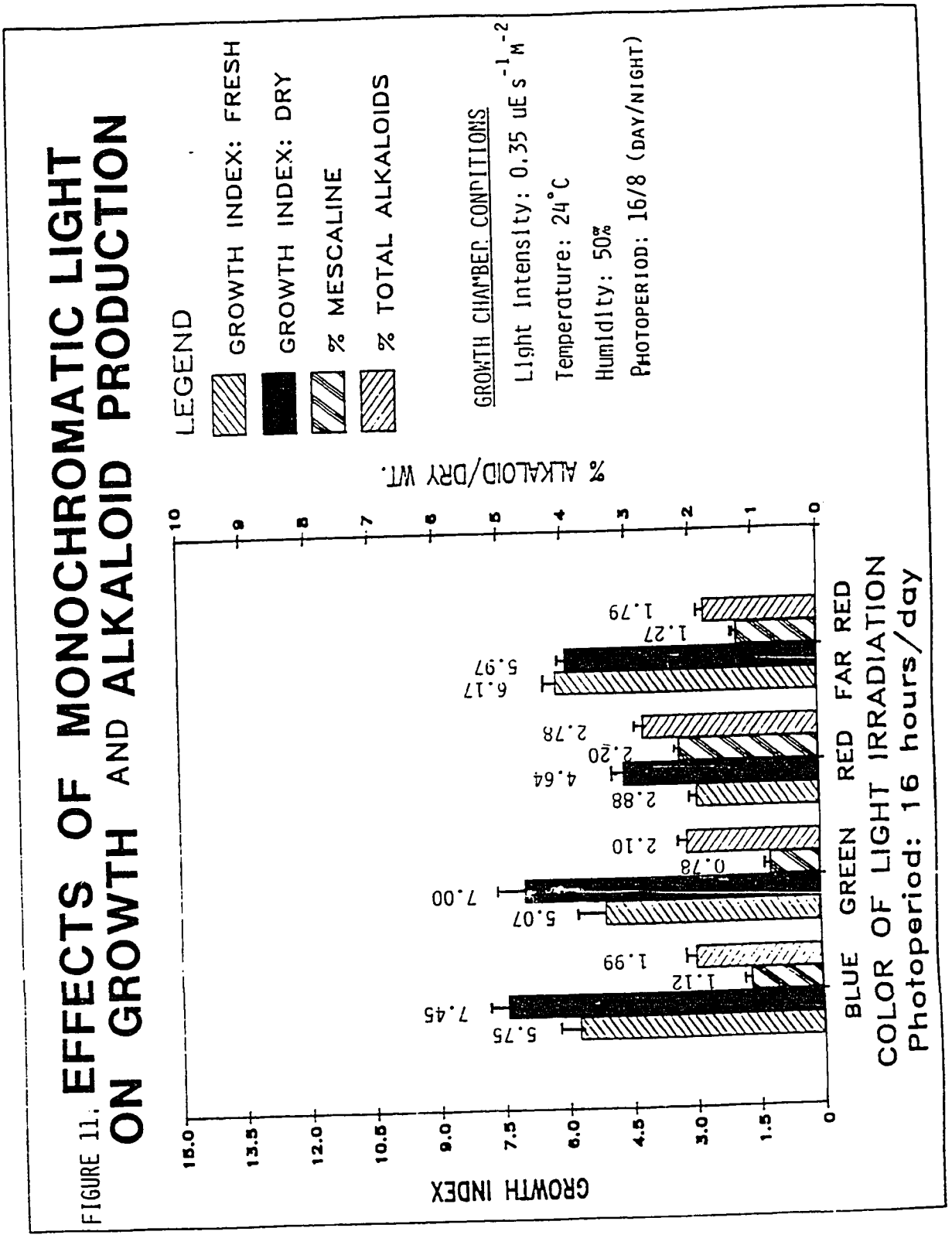
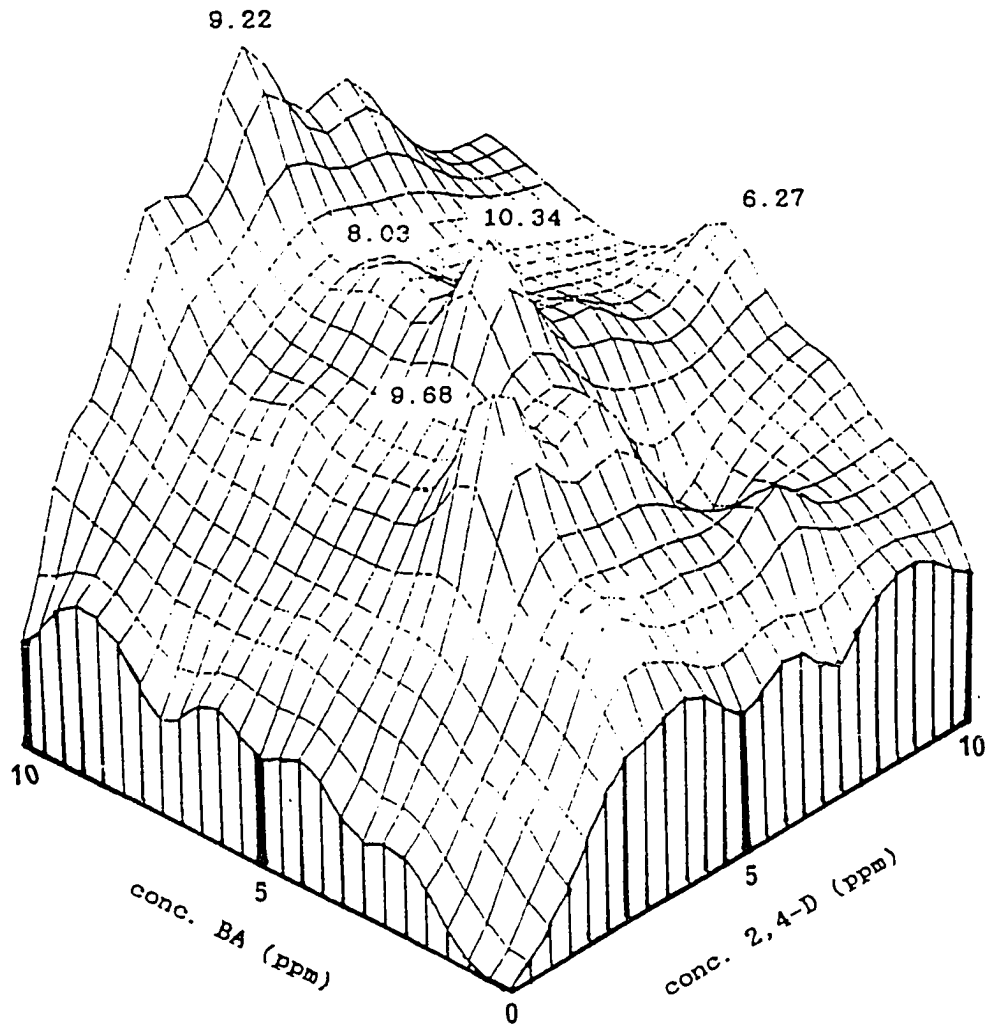


FIGURE 11.

FIGURE 12.
3-DIMENSIONAL DISPLAY OF CALLUS GROWTH INDEX ON SOLID MEDIA (FRESH WT.)

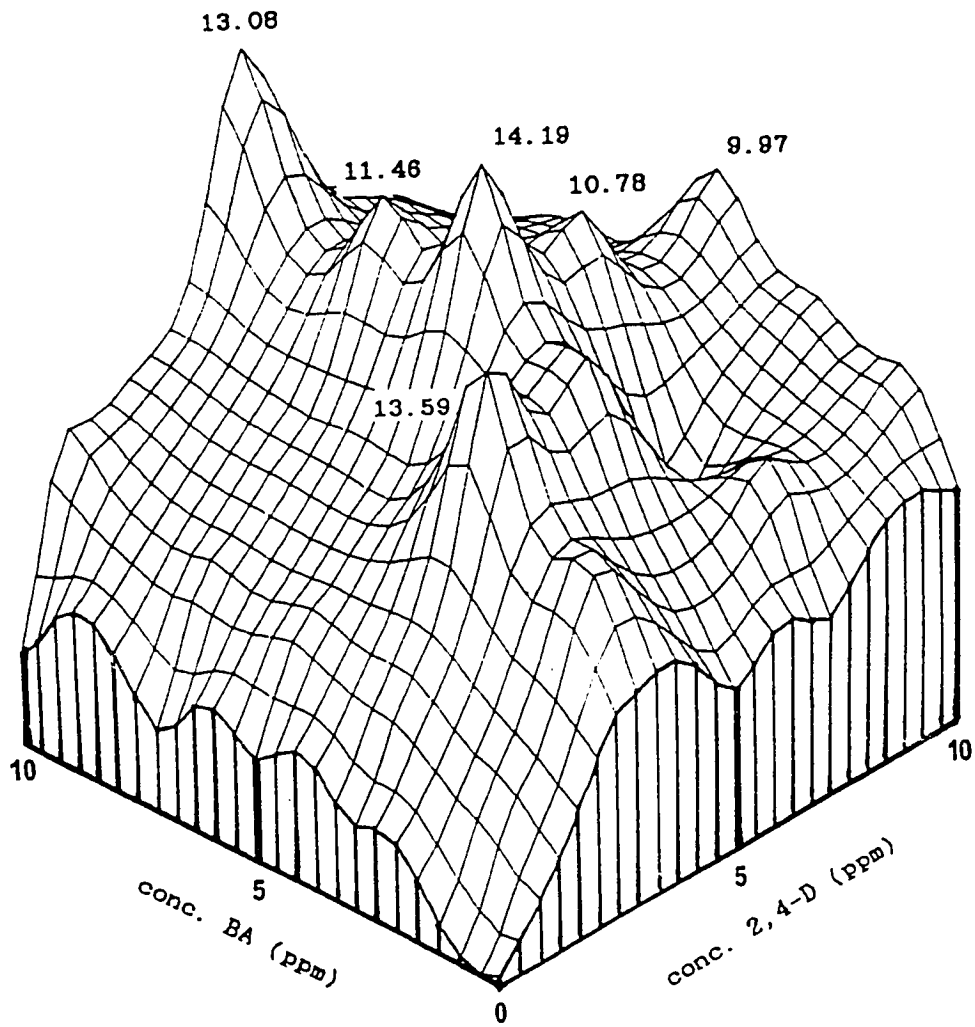


conc. of 2,4-D:BA (ppm)	mean G.I.	S.D.
3.0 : 3.0	9.68	3.64
5.0 : 5.0	10.34	3.01
5.0 : 7.0	8.03	3.65
5.0 : 10.0	9.22	1.85
10.0 : 5.0	6.27	1.37

2,4-D = 2,4-Dichlorophenoxyacetic acid

BA = Benzyl adenine

FIGURE 13.
3-DIMENSIONAL DISPLAY OF CALLUS GROWTH INDEX ON SOLID MEDIA (DRY WT.)



conc. of 2,4-D:BA (ppm)	mean G.I.	S.D.
3.0 : 3.0	13.59	1.99
5.0 : 5.0	14.19	1.45
5.0 : 7.0	11.46	2.67
5.0 : 10.0	13.08	2.18
7.0 : 5.0	10.78	2.46
10.0 : 5.0	9.97	2.08

2,4-D = 2,4-Dichlorophenoxyacetic acid

BA = Benzyl adenine

Table 9. ALKALOID ACCUMULATION OF PEYOTE CALLUS TISSUES ON SOLID MEDIA (DRY WT.)

conc. of 2,4-D:BA (ppm)	mean G.I.	S.D.	% Total alkaloid/wt.	Total alkaloids (mg)
3.0 : 3.0	13.59	1.99	1.98 ± 0.13	1.29
5.0 : 5.0	14.19	1.45	1.54 0.17	0.77
5.0 : 7.0	11.46	2.67	1.30 0.08	0.66
5.0 : 10.0	13.08	2.18	1.12 0.15	0.65
7.0 : 5.0	10.78	2.46	0.73 0.22	0.44
10.0 : 5.0	9.97	2.08	0.98 0.10	0.52

2,4-D = 2,4-Dichlorophenoxyacetic acid

BA = Benzyl adenine

G.I. = Growth Index = $\frac{\text{Final wt. - Initial wt.}}{\text{Initial wt.}}$

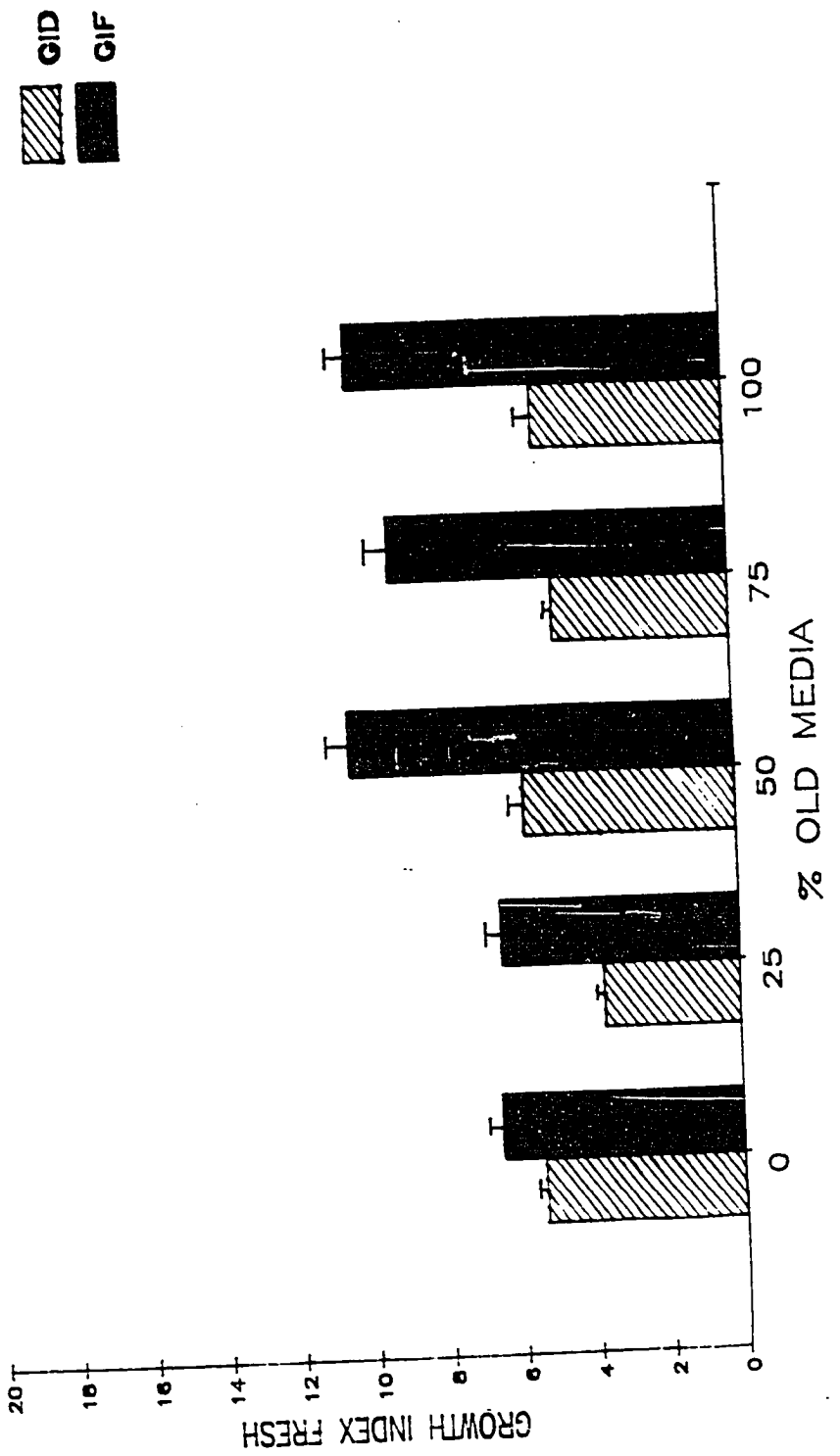
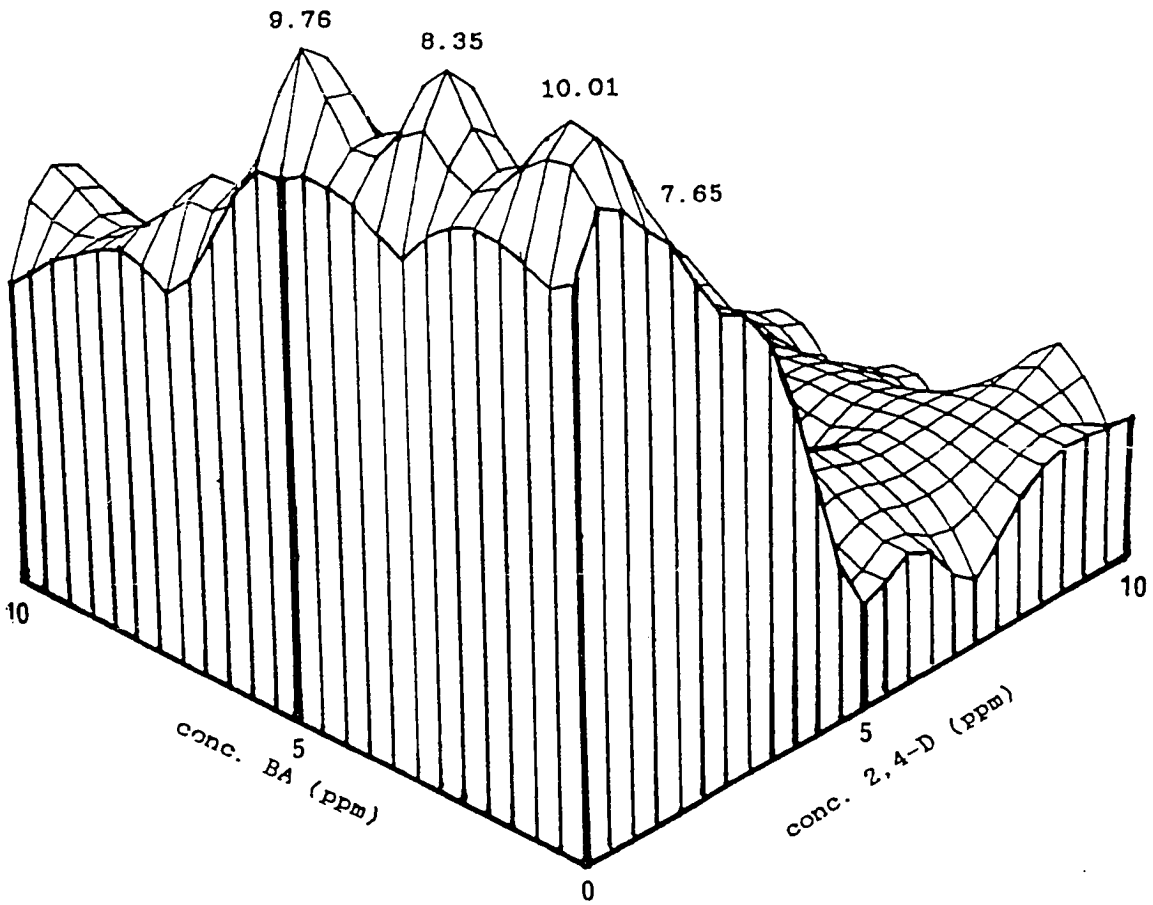


FIGURE 14.

CONDITIONED MEDIA
GROWTH INDEX
FRESH AND DRY WEIGHTS

FIGURE 15:
3-DIMENSIONAL DISPLAY OF LIQUID CELL SUSPENSION GROWTH INDEX (DRY WT.)

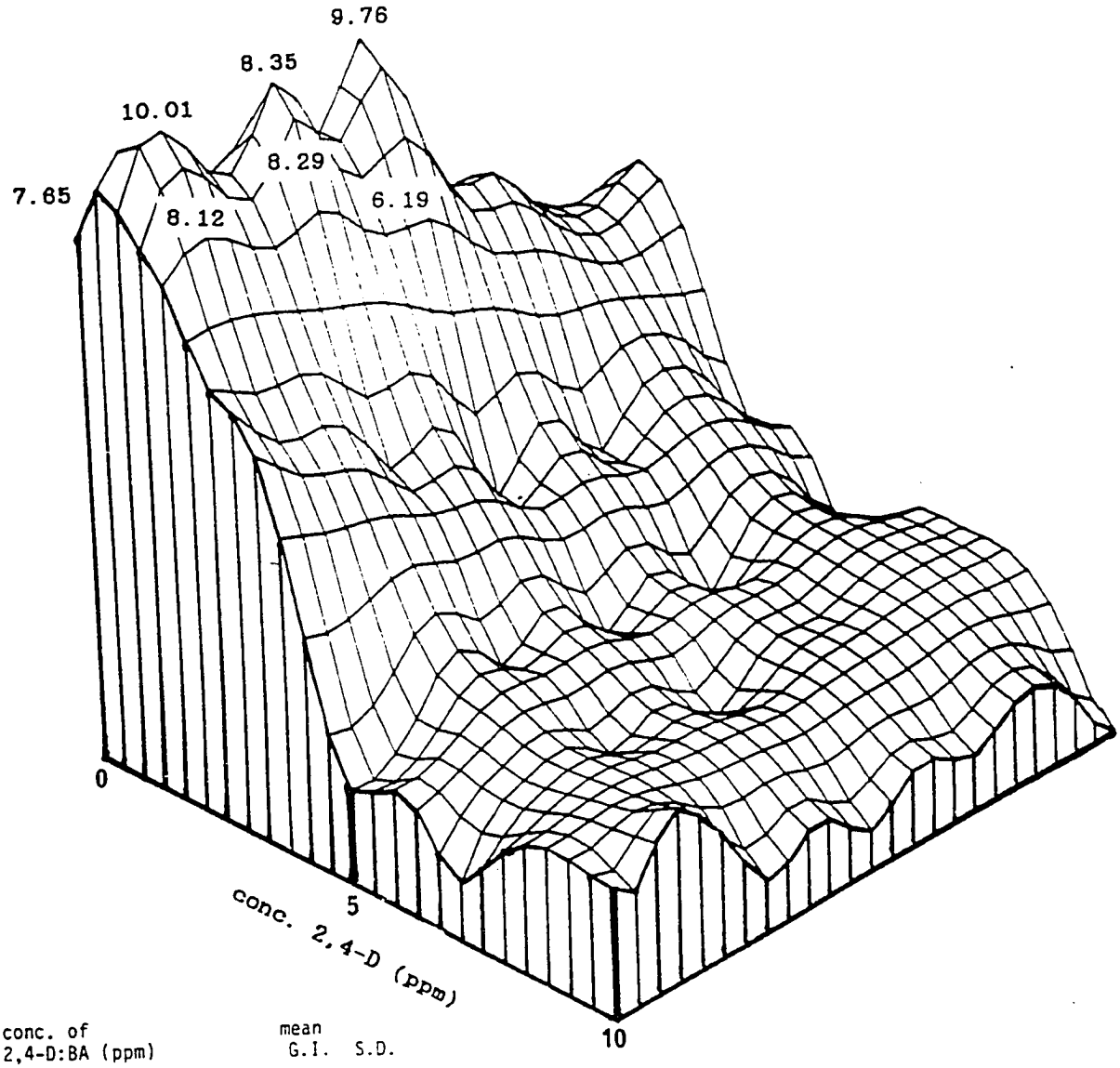


conc. of 2,4-D:BA (ppm)	G.I.
0.5 : 0.0	7.65
0.5 : 0.5	10.01
0.5 : 3.0	8.35
0.5 : 5.0	9.76

2,4-D = 2,4-Dichlorophenoxyacetic acid

BA = Benzyl adenine

FIGURE 16:
3-DIMENSIONAL DISPLAY OF LIQUID CELL SUSPENSION GROWTH INDEX (DRY WT.)
:AFTER 90 DEGREE CLOCKWISE ROTATION



conc. of 2,4-D:BA (ppm)	mean G.I.	S.D.
0.5 : 0.0	7.65	0.81
0.5 : 0.5	10.01	1.07
0.5 : 3.0	8.35	1.62
0.5 : 5.0	9.76	0.66
1.0 : 0.5	8.12	0.14
1.0 : 1.0	7.17	1.02
1.0 : 3.0	8.29	0.75

2,4-D = 2,4-Dichlorophenoxyacetic acid

BA = Benzyl adenine

Table 10. ALKALOID ACCUMULATION OF PEYOTE CELLS IN LIQUID SUSPENSION (DRY WT.)

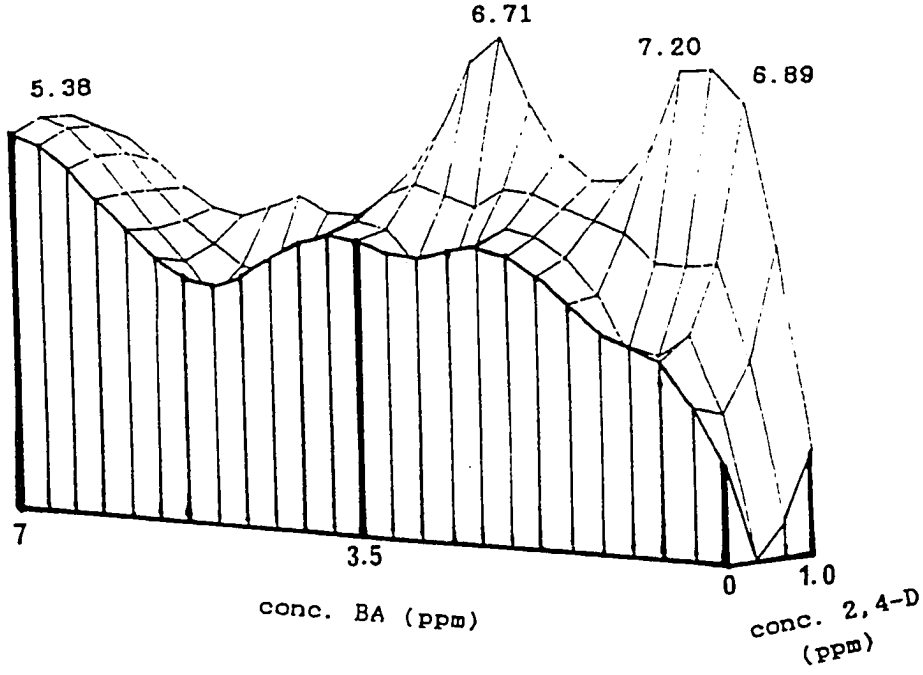
conc. of 2,4-D:BA (ppm)	mean G.I.	S.D.	% Total alkaloids/wt	Total alkaloids (mg)
0.5 : 0.0	7.65	0.81	0.35 ± 0.04	1.57
0.5 : 0.5	10.01	1.07	1.86 0.26	9.81
0.5 : 3.0	8.35	1.62	1.37 0.29	5.27
0.5 : 5.0	9.76	0.66	0.69 0.11	2.26
1.0 : 0.5	8.12	0.14	0.87 0.09	3.62
1.0 : 1.0	7.17	1.02	1.11 0.21	3.31
1.0 : 3.0	8.29	0.75	1.08 0.14	3.65

2,4-D = 2,4-Dichlorophenoxyacetic acid

BA = Benzyl adenine

G.I. = Growth Index = $\frac{\text{Final wt. - Initial wt.}}{\text{Initial wt.}}$

FIGURE 17.
3-DIMENSIONAL DISPLAY OF LIQUID CELL SUSPENSION GROWTH INDEX (FRESH WT.) PLATEAU REGION

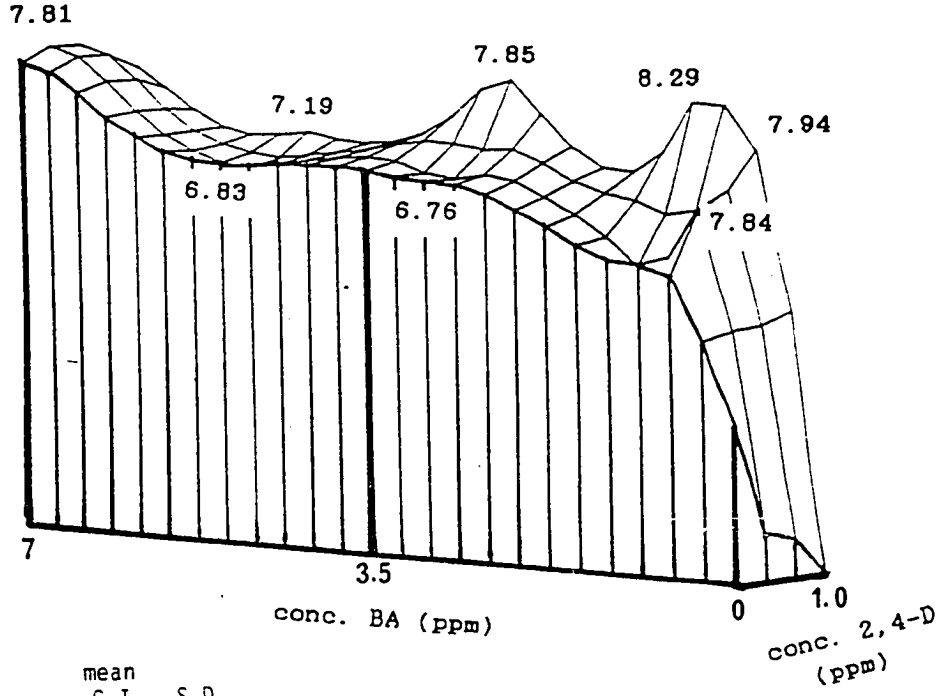


conc. of 2,4-D:BA (ppm)	mean G.I.	S.D.
0.5 : 7.0	5.38	1.27
1.0 : 0.5	6.89	0.84
1.0 : 1.0	7.20	1.10
1.0 : 3.0	6.71	1.05

2,4-D = 2,4-Dichlorophenoxyacetic acid

BA = Benzyl adenine

FIGURE 18.
3-DIMENSIONAL DISPLAY OF LIQUID CELL SUSPENSION GROWTH INDEX (DRY WT.) PLATEAU REGION



conc. of 2,4-D:BA (ppm)	mean G.I.	S.D.
0.5 : 0.5	7.84	0.96
0.5 : 3.0	6.76	1.15
0.5 : 5.0	6.83	1.31
0.5 : 7.0	7.83	2.26
1.0 : 0.5	7.94	1.80
1.0 : 1.0	8.29	2.23
1.0 : 3.0	7.85	2.07
1.0 : 5.0	7.19	1.08

2,4-D = 2,4-Dichlorophenoxyacetic acid

BA = Benzyl adenine

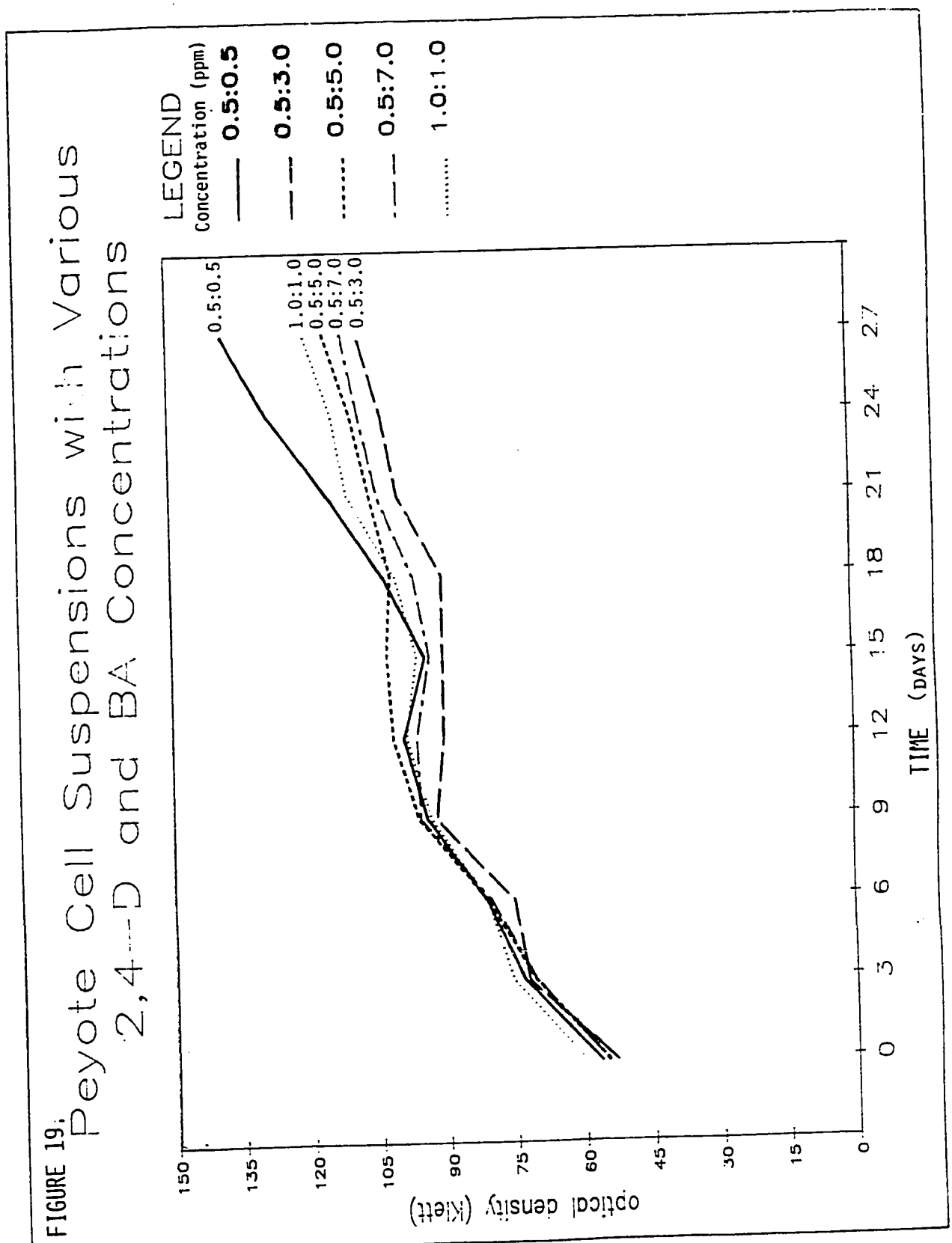
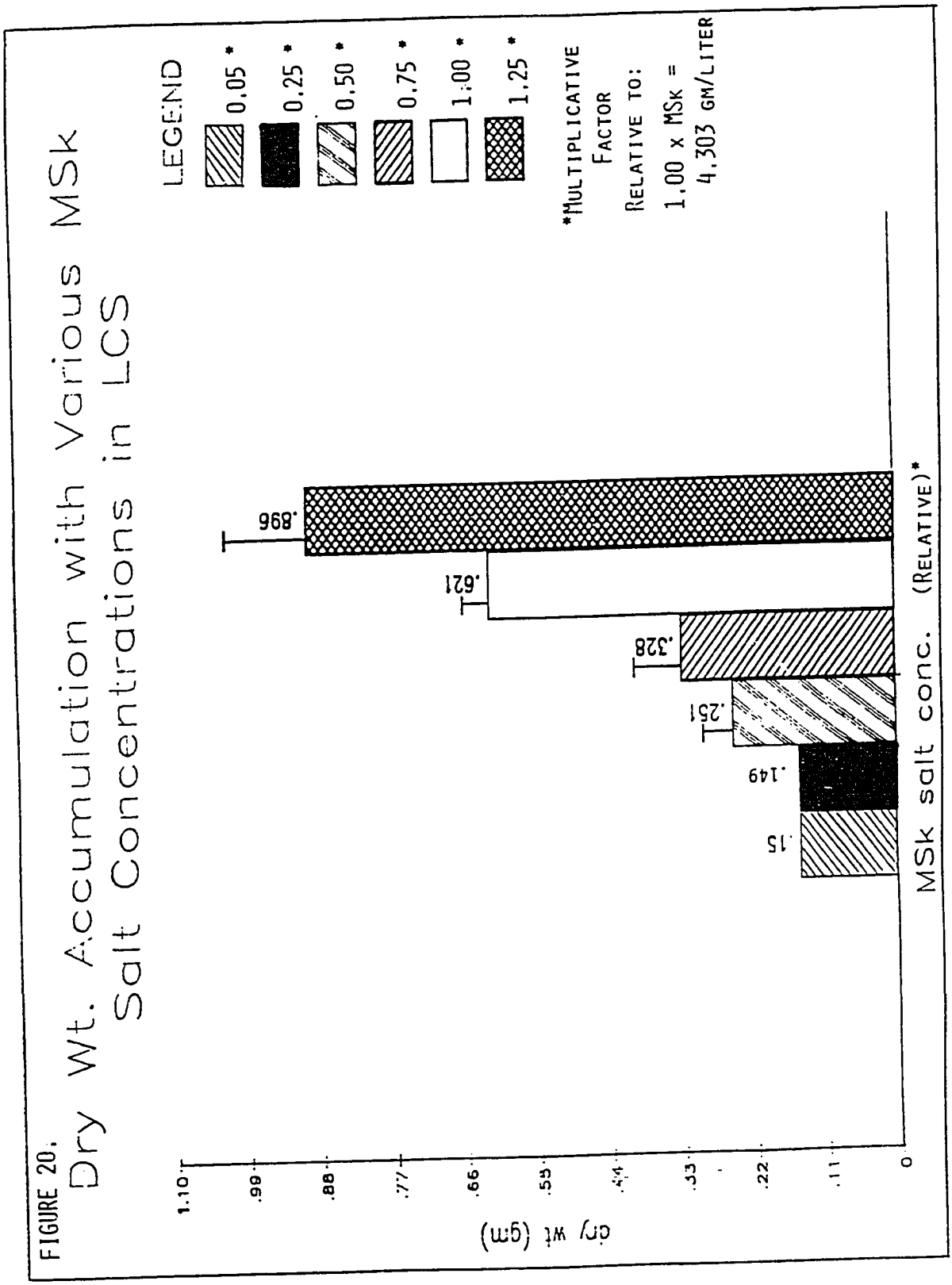


FIGURE 19.



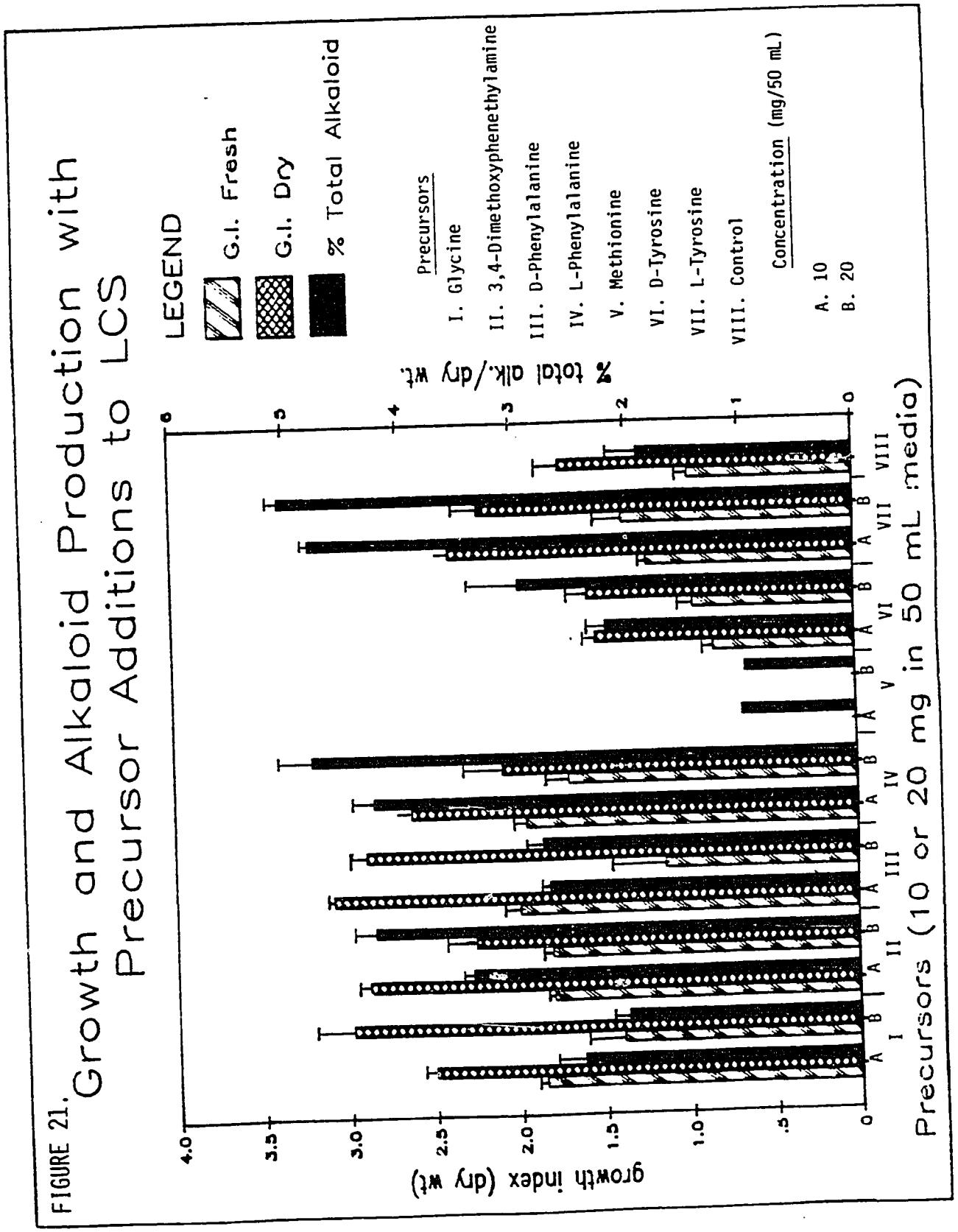


FIGURE 21.

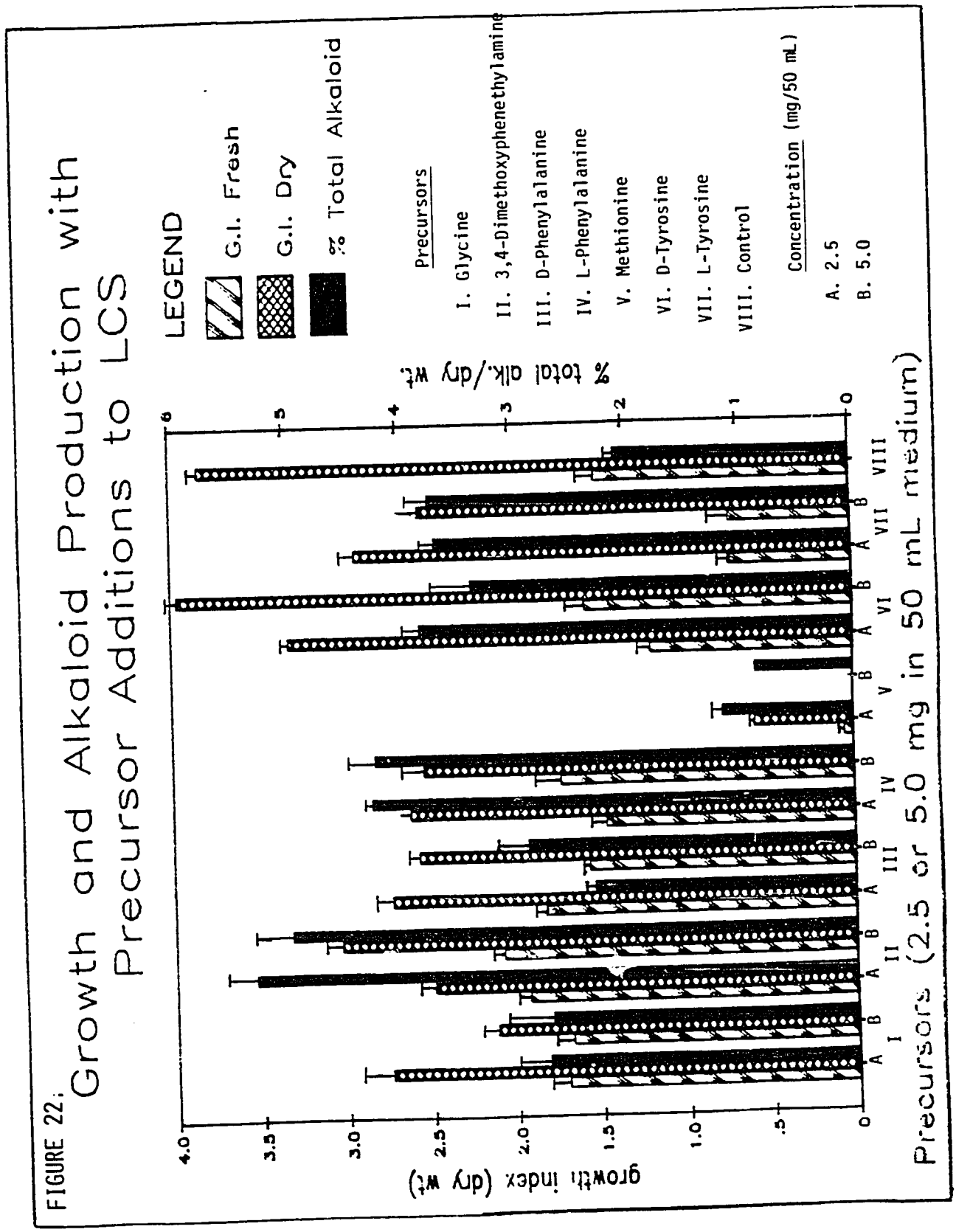


FIGURE 22.

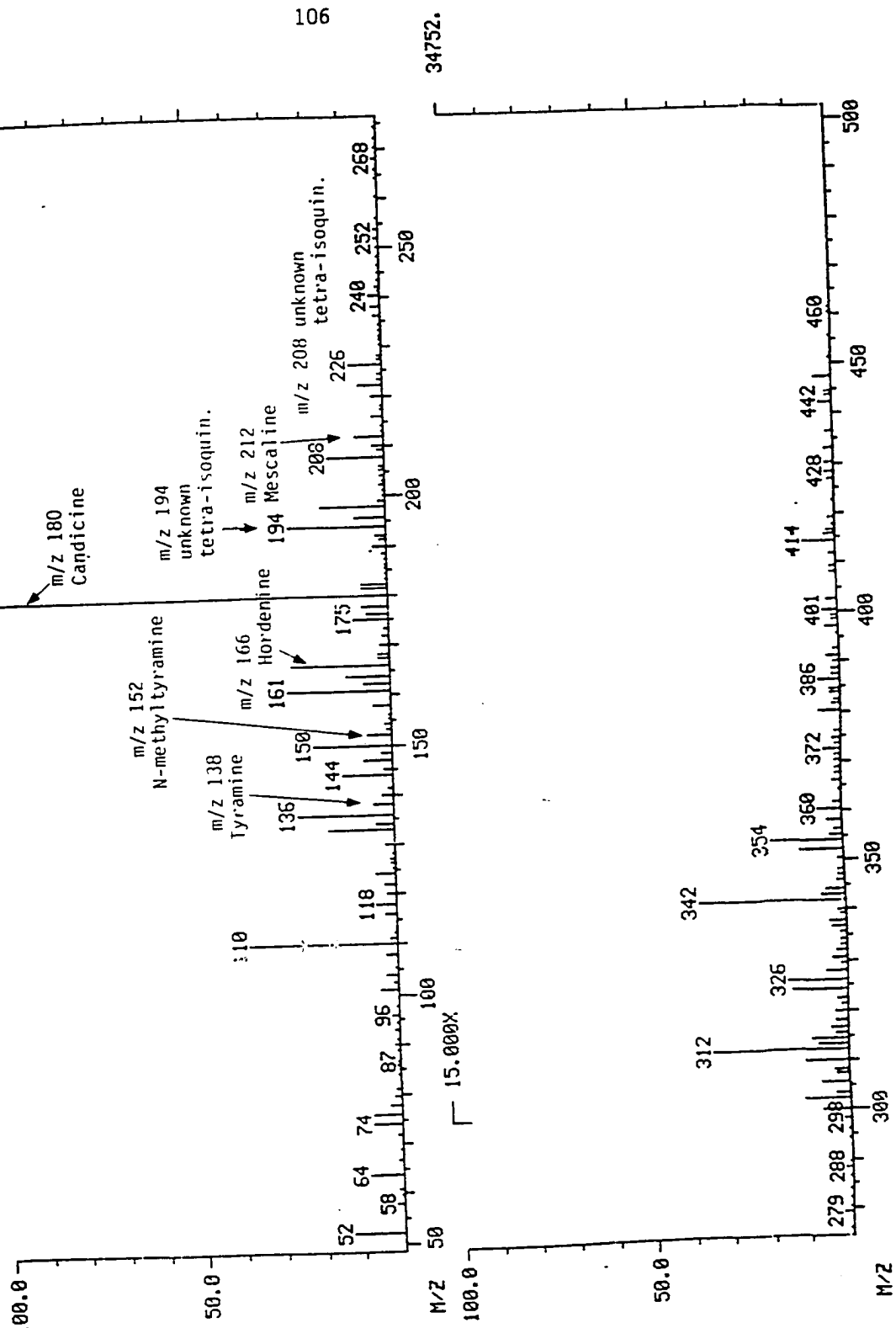


FIGURE 23.

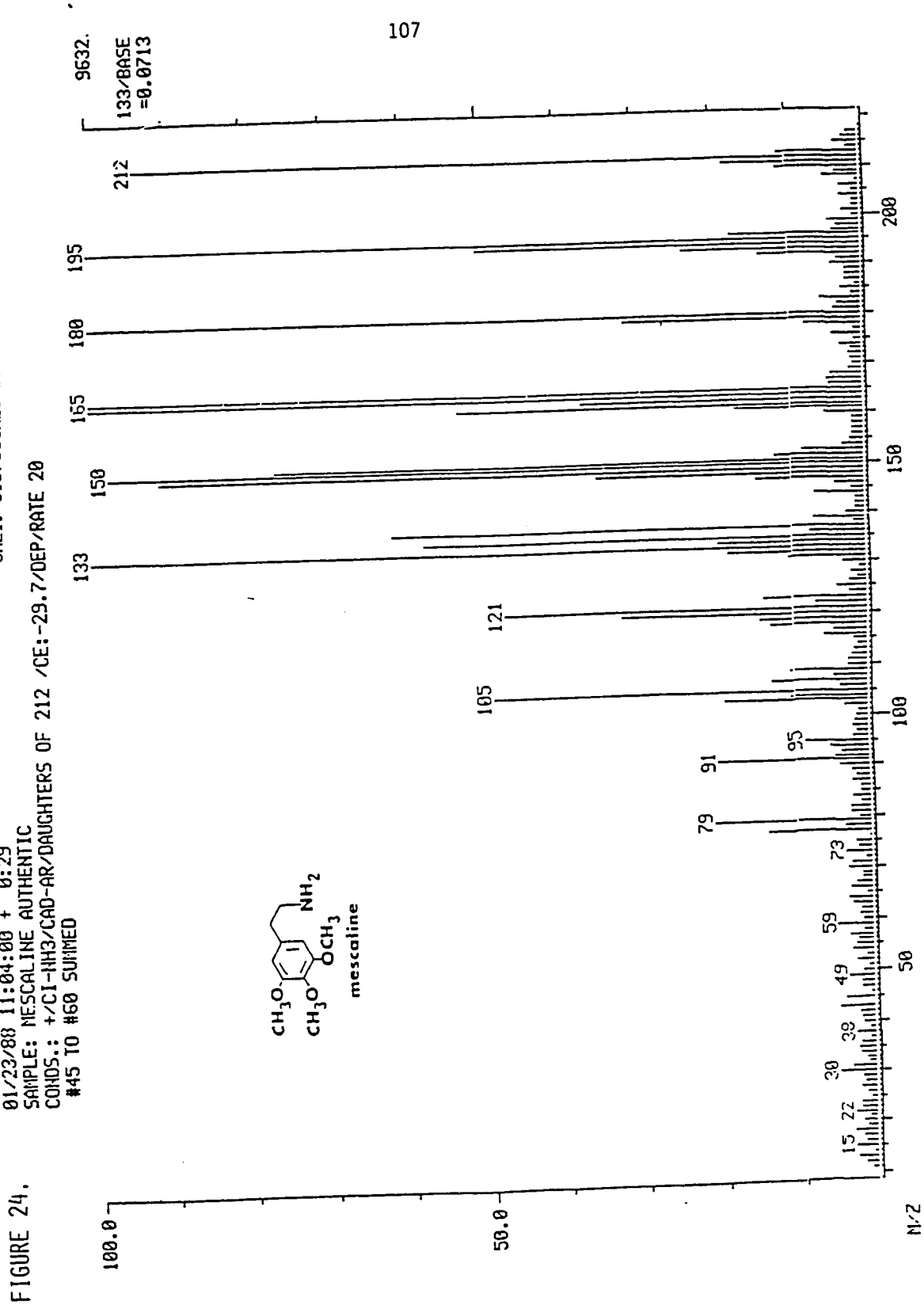


FIGURE 24.

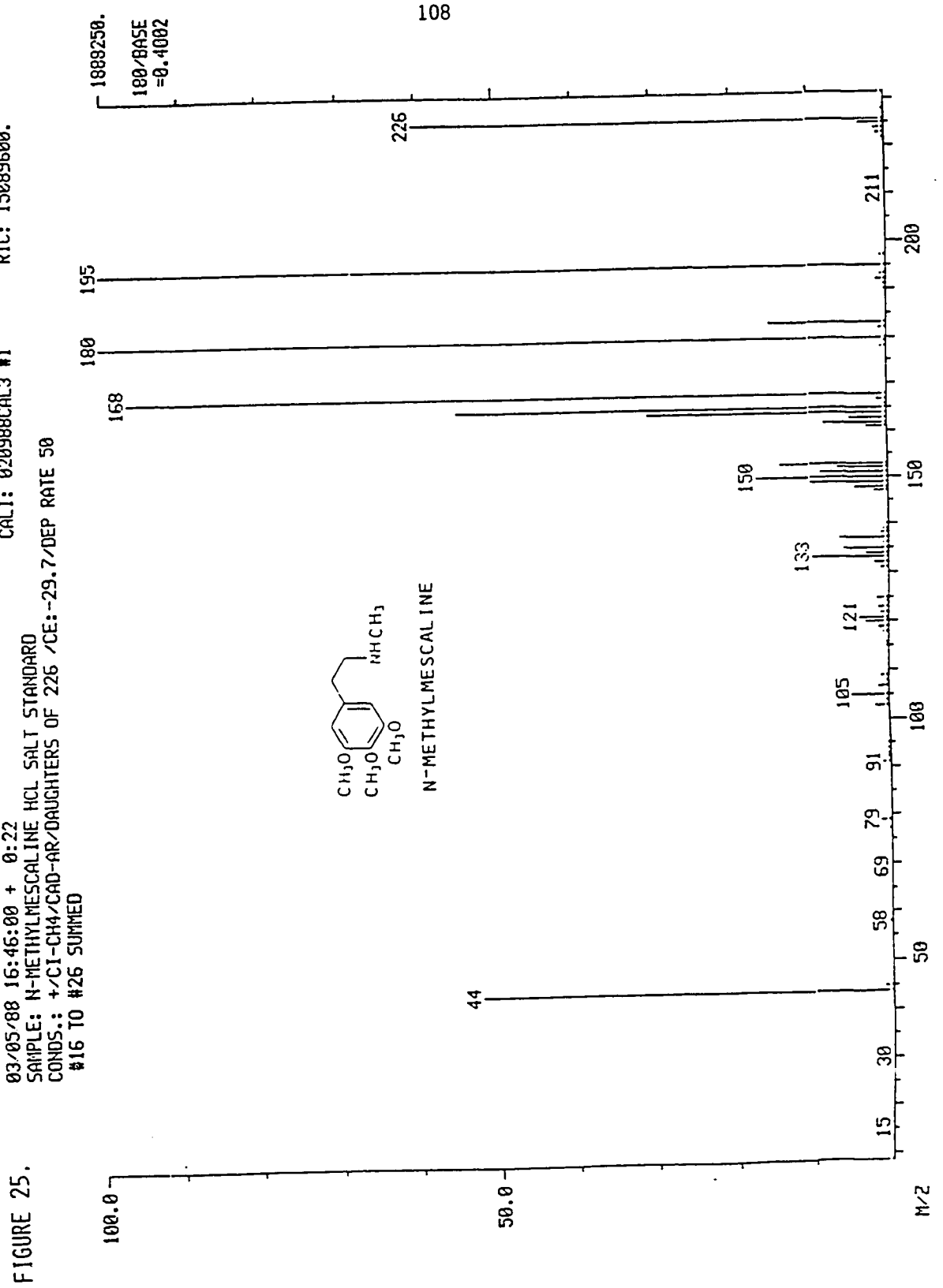
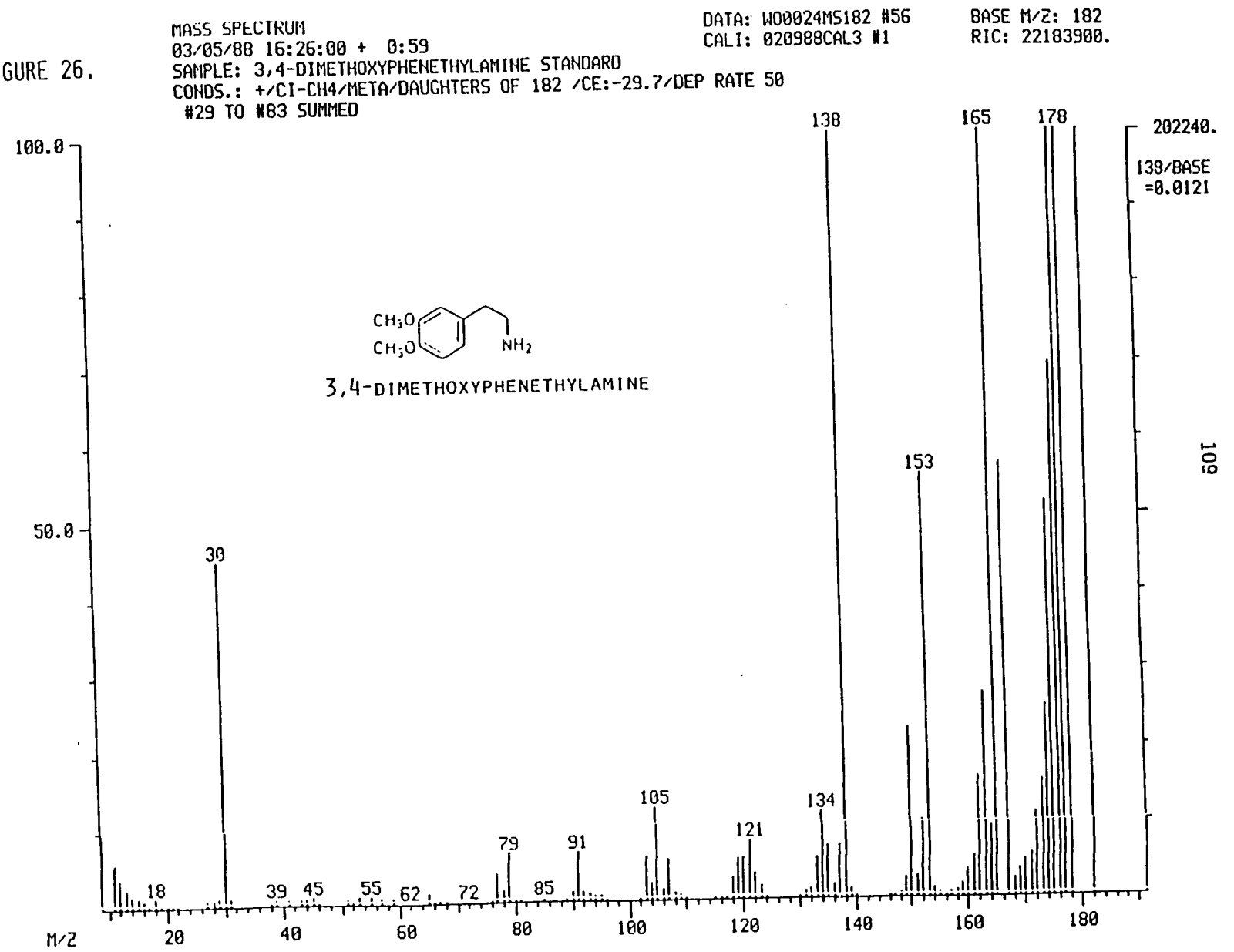


FIGURE 25.

FIGURE 26.



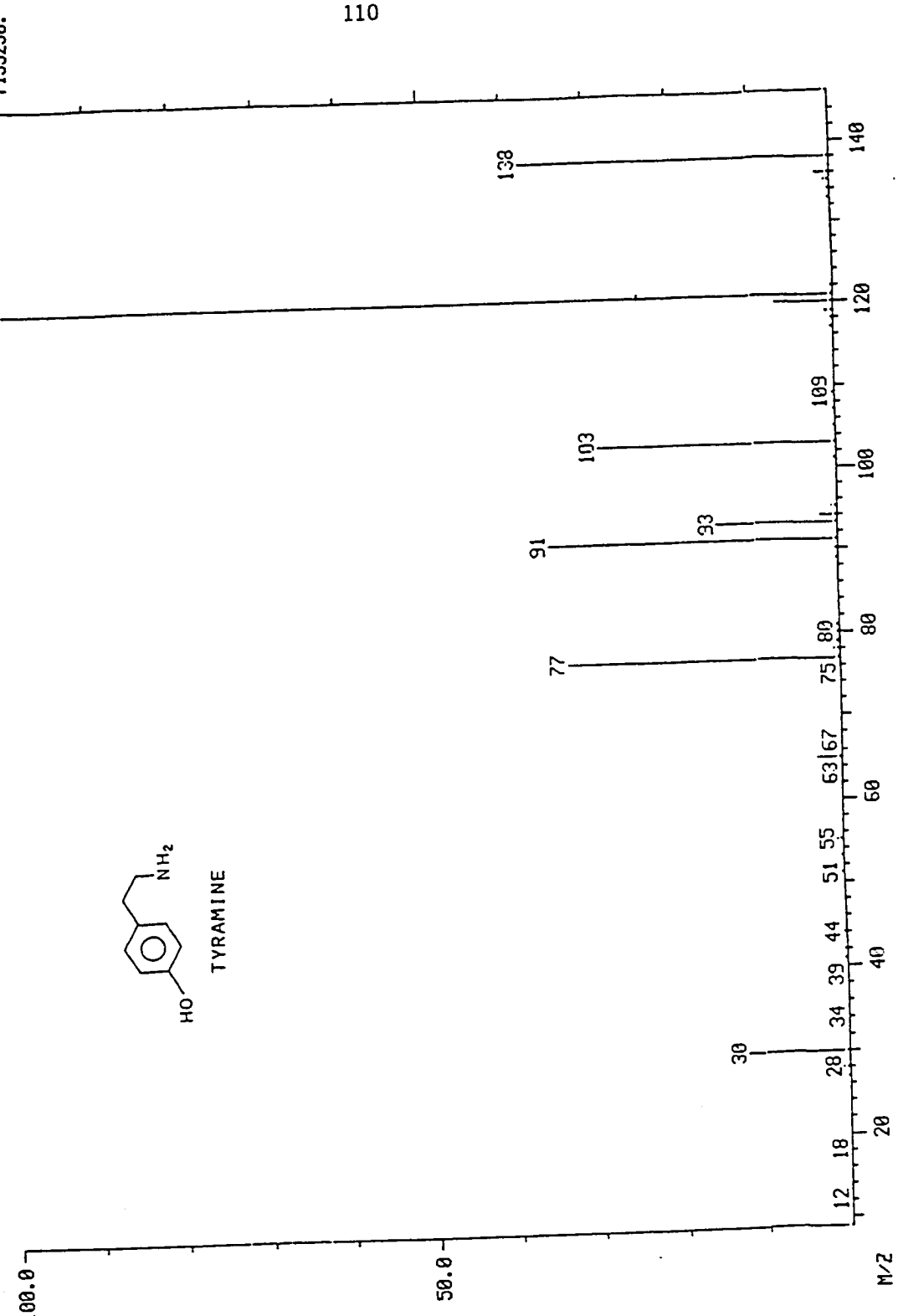


FIGURE 27.

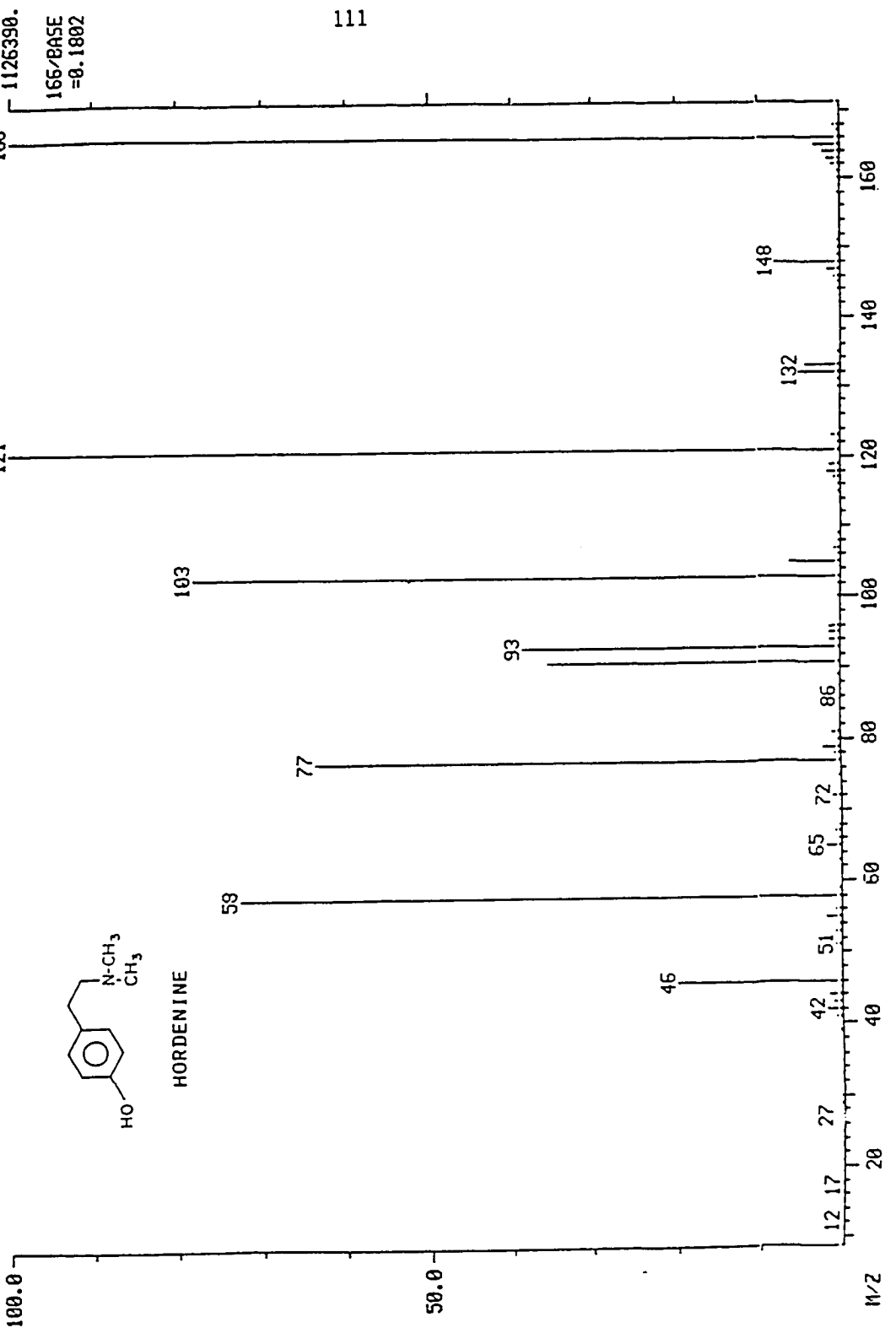


FIGURE 28.

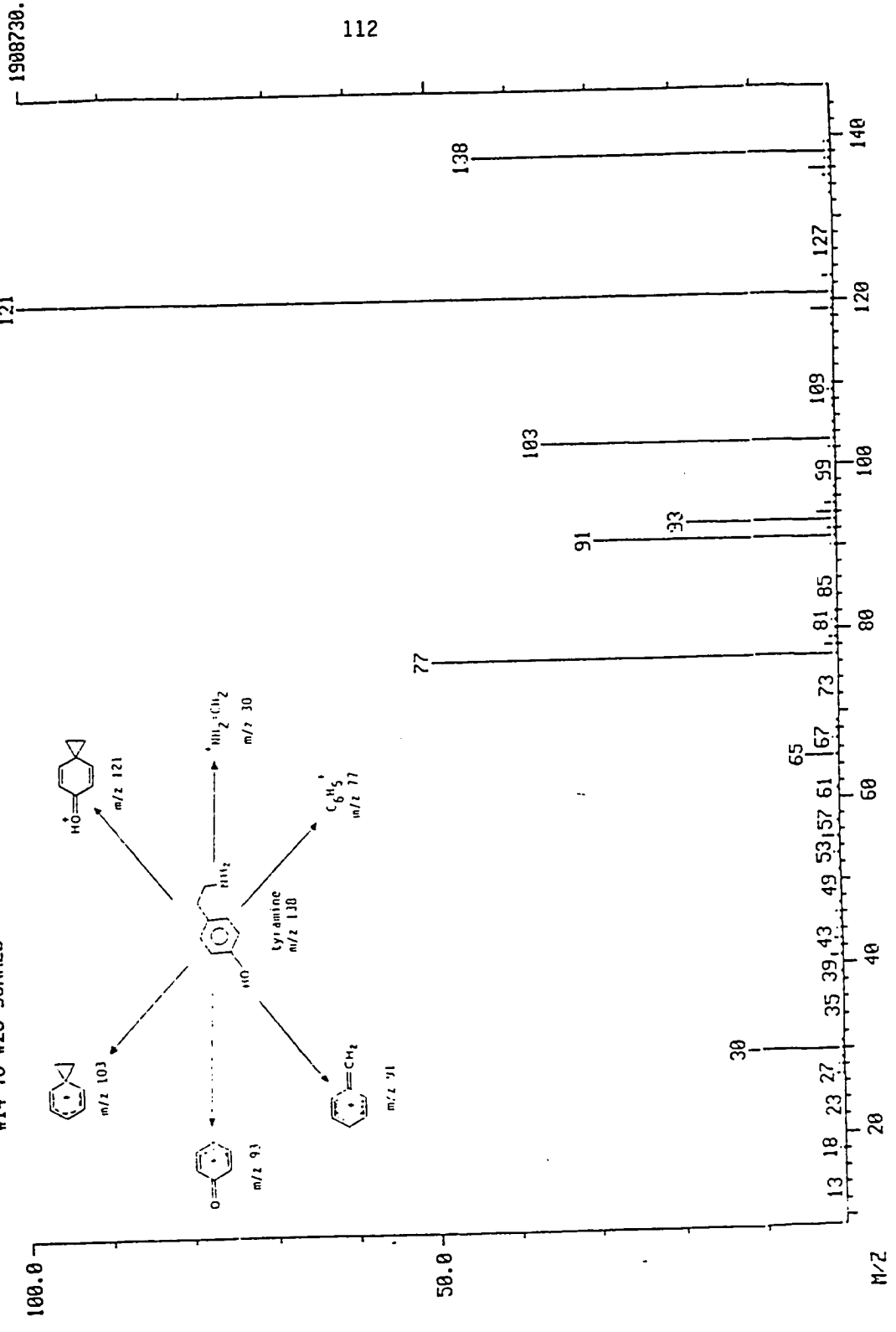


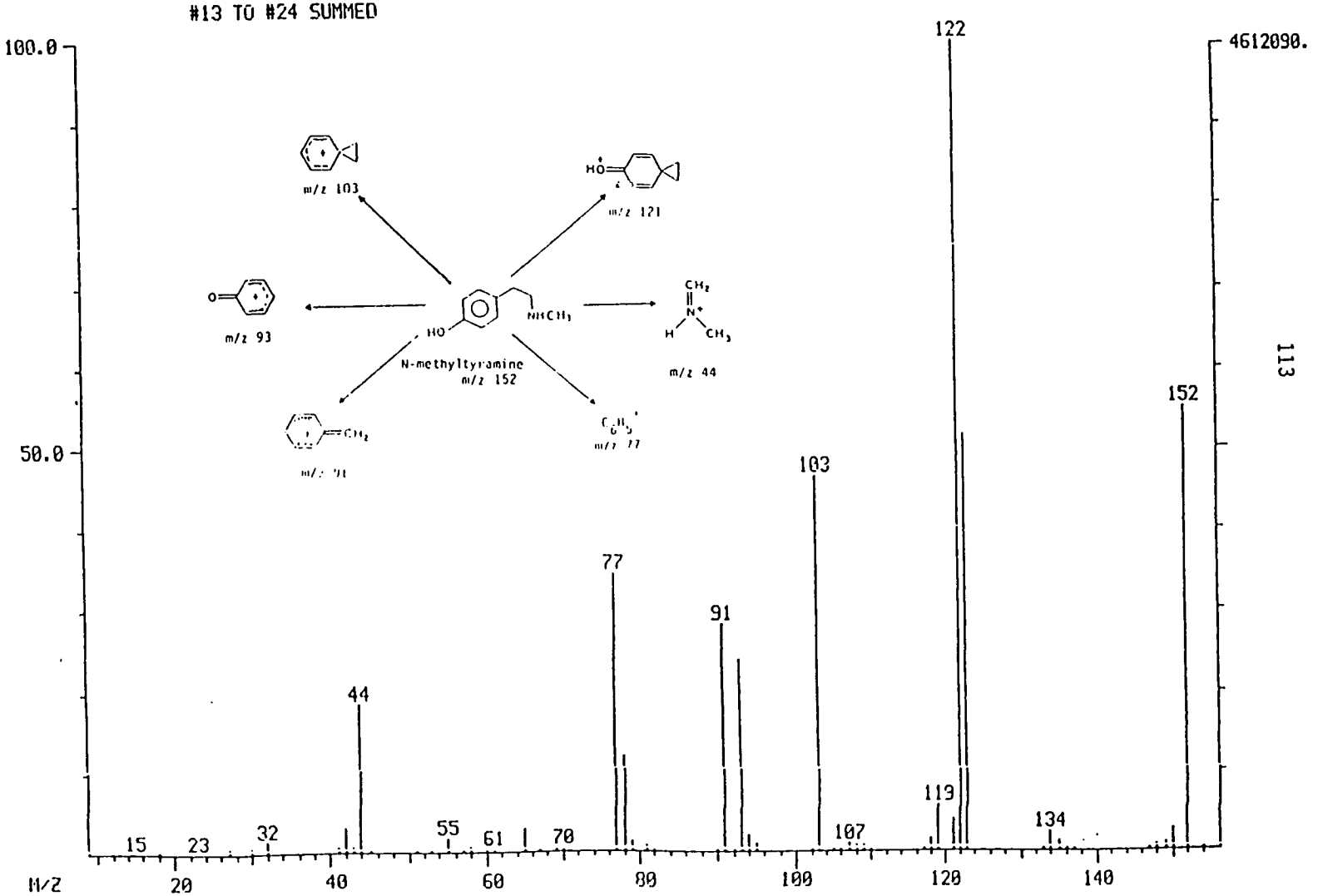
FIGURE 29.

FIGURE 30.

MID MASS SPECTRUM
02/06/88 14:06:00 + 0:06
SAMPLE: BILL OBERMYER FRACTION #2-3 TISSUE CULTURE
CONDS.: +/CI-CH4/N2/CAD-AR/DAUGHTERS OF 152 /CE:-30.0/DEP
GC TEMP: 20 DEG. C
#13 TO #24 SUMMED

DATA: W0012W0007 #18
CALI: 020288CAL3 #1

BASE M/Z: 122
RIC: 19136500.



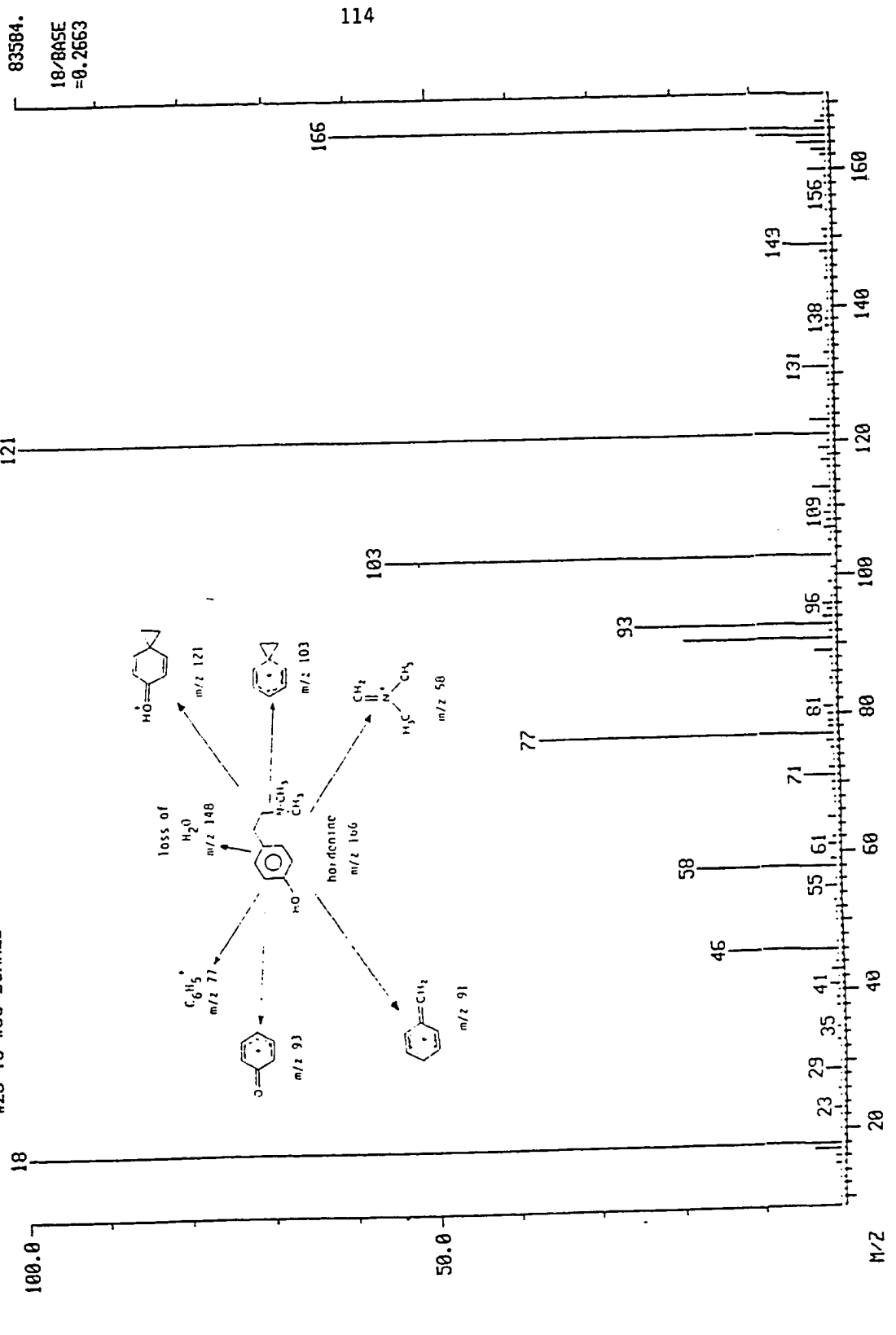


FIGURE 31.

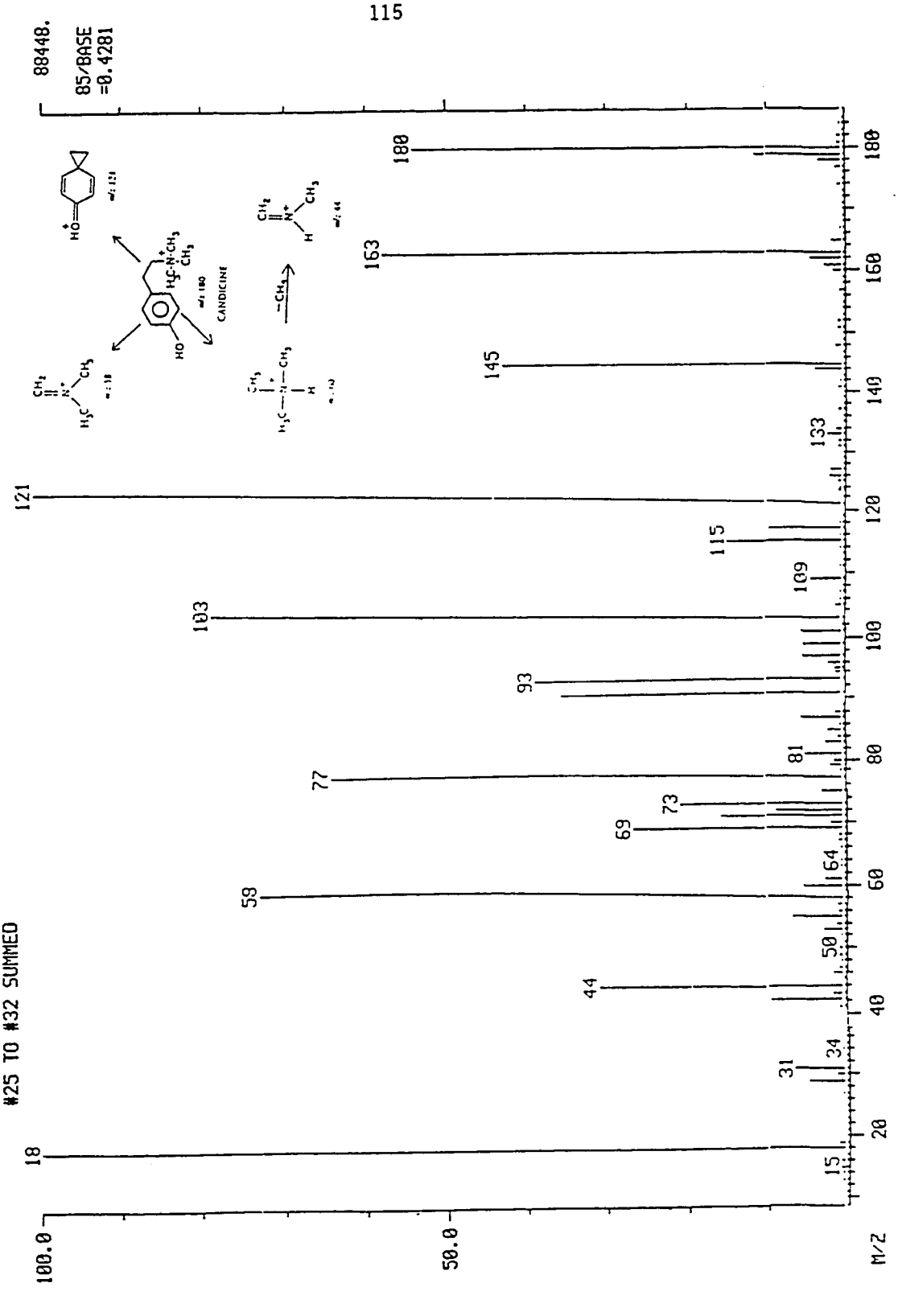


FIGURE 32.

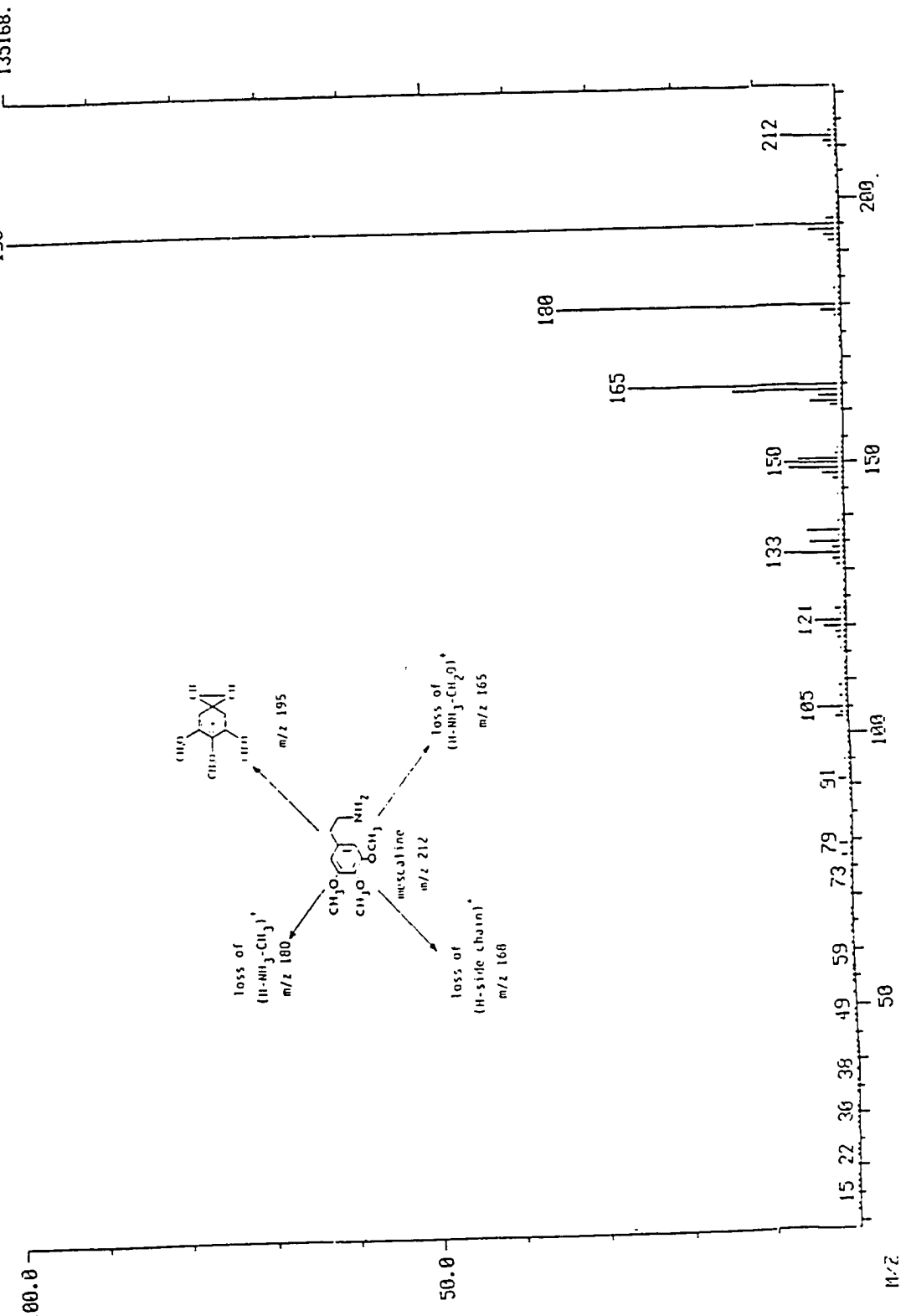


FIGURE 33.

FIGURE 34. BIOSYNTHESIS OF N-METHYLATED PHENETHYLAMINES IN PEYOTE

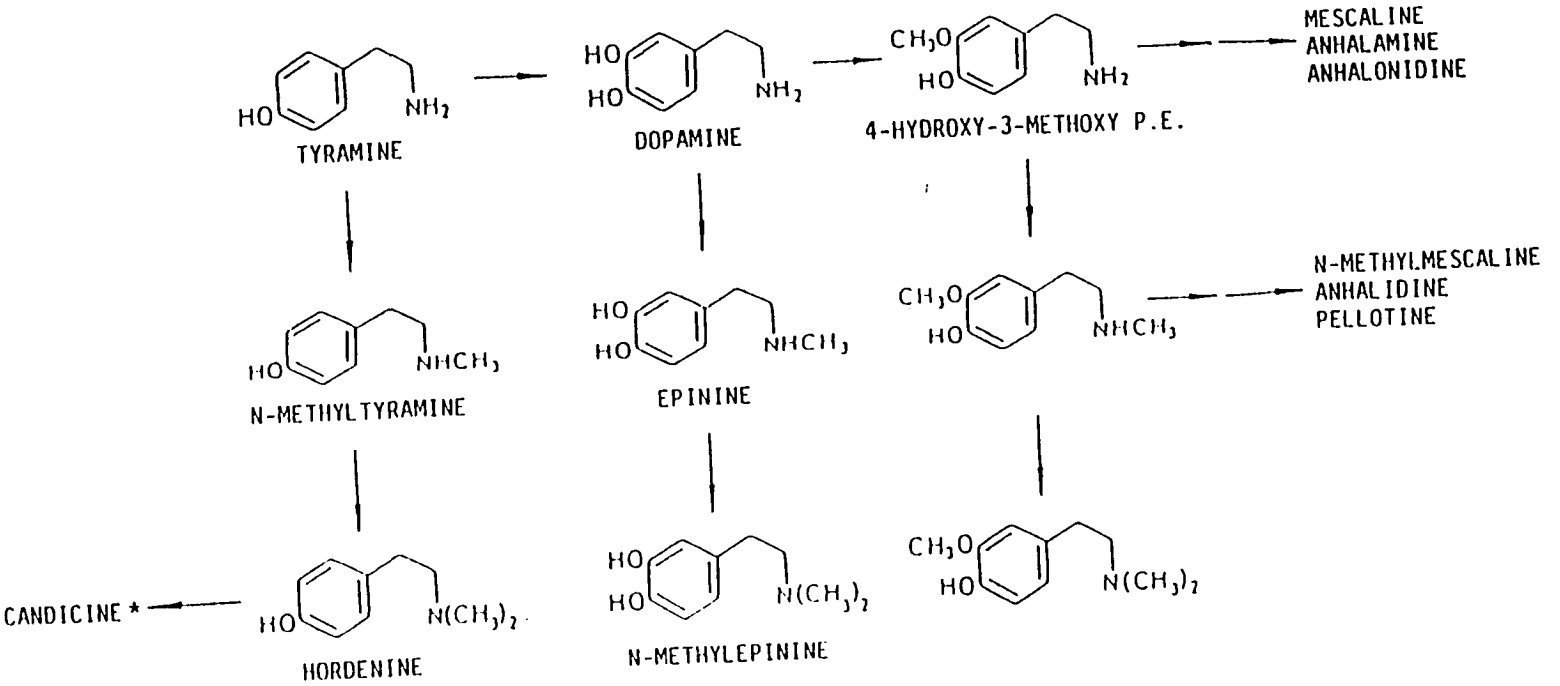


Figure 34. Biosynthesis of N-methylated phenethylamines in peyote*

* modified pathway proposed for candicine

Results and Discussion

Initiation of the Peyote Callus Tissue

The occurence of mescaline and related alkaloids has been well established in the Lophophora species and other cacti (59-122). However, the whole plants are not very suitable for laboratory experiments due to slow growth, limited manipulation of hormonal influences on the complex biosynthetic pathways,and problems such as translocation, permeability and segregation of metabolic pools. To date, there have only been few reports of successful in vitro callus culture of any cacti (207-211). The successful formation of calli is generally hindered by several factors, not least of which are choice of incorrect explant source (4,6,19,207,215) and the failure to formulate the correct initiation medium. The latter is directly attributable to the complex mixtures of inorganic salts, growth regulators, nitrogen and carbon sources which characterize plant tissue culture media (1-37,215).

Every plant must be subjected to specific experimention to determine the source of rapidly growing cells needed to establish a continuous callus culture. Attempts to form cultures of cacti from stems, roots,

stamens, pistils and ovules have shown a tendency to begin callus formation due to a wound response (207-211), but permanent cultures rarely could be established from them. Previous to these intial efforts to promote callus growth, the only other endeavor to obtain cultures from the peyote were from dormant seeds. These efforts did not result in formation of callus tissue (212). Inhibition of seed germination or callus production may be attributed to an ABA-like (abscisic acid) compound common to seeds of the Cactaceae (36,37,51,). From the results of tests of the various plant tissues, the only area of the whole plant that provided actively growing tissues to promote continuous callus growth was the stem sections of very young plants (1.0-1.5 cm dia.). The roots and root sections from these same plants did not continue to grow nor did they provide any callus growth. Although the roots could not be grown on this medium, the proper medium formulation may provide growth of these organs. This will provide avenues for further experiments to test for the separate biosynthetic capabilites. Furthermore, similar work would be required to for the culture and analysis of other plant parts (anthers, ovaries, etc.).

In early studies involving tissue culture of plants, many individual formulations for the inorganic salts, vitamins and other constituents were used for the basal medium (4,5,11). Eventually, a formulation was devised by

Murashige and Skoog (MSk) providing a salt base and vitamin mixture that could be used as a good foundation for most plant cultures (11). General modifications of this basic medium are made by additions of various types and amounts of growth regulators and coconut water to help stimulate callus initiation. Coconut water, the liquid endosperm of Cocos nucifera L., is considered an undefined source of free and combined amino acids, nonvolatile organic acids, sugars, nucleic acids and other growth stimulators (4-6,201,215,216).

A previously proven medium used to promote callus growth in other slow growing plants in our laboratory, contained the basic MSk mixture with 1.0-5.0 ppm of 2,4-D and 2-15 % of coconut water (218). After several experiments, it was found that combinations that initiated the peyote callus tissue were 1 ppm 2,4-D with 10 or 15 % liquid endosperm. No callus growth was initiated at higher concentrations of 2,4-D and the association of 5 ppm 2,4-D with the liquid endosperm appeared to be toxic to some stem and root sections (refer to Table 7, p. 86).

The premise for the site of actively growing cells in cacti and the general medium formulation used to initiate callus tissues of the peyote were later verified by a much earlier, infrequently cited paper. The researchers showed initiation of callus cultures from another mescaline producing cactus, Trichocereus spp. (207), but could not

show evidence of alkaloid production. This South American columnar cactus, produced a continuous source of callus tissue only from stem sections on a solid medium containing an inorganic salt mixture formulated by Hildebrant (207), autoclaved coconut water, and 2,4-D. Although Steinhart did not use the advanced MSk salt mixture in their experiments (MSk contains more trace elements along with different types and proportions of nitrogen sources)(4-8,11,12,215). The optimum levels used to produce enough callus tissue for subculturing was 2.5 ppm 2,4-D with 10 % coconut water. These results compare favorably to the medium formulation and tissue sections needed to stimulate callus cultures in the peyote. Unfortunately, we could find no other work published which continued studies of the growth and alkaloid biosynthesis in Trichocereus spp. callus tissues.

Growth Response of Callus Tissues to Individual Growth Regulators

While coconut water contains a variety of factors which can act as growth stimulators, a more consistent, defined medium is needed for reproducible results (4-8,11,12,215). Besides the basic nutritional constituents of coconut water, the complex mixture of substances that promote cell division can vary with the age and geographical source of the liquid endosperm (L.E.)(215). The typical

means of creating a defined medium experimentally starts with simplified additions of individual auxins or cytokinins, then progresses to complex combinations of these two types of growth regulators.

The generic term "auxin", originated by Kogl and Haagen-Smit in 1931, refers to substances that are capable of promoting plant growth similar to that of native growth hormones (4-6). Today, a generally accepted definition is that an auxin is an "organic substance which at low concentrations (<0.001 M) promotes growth (cell enlargement) along the longitudinal axis, when applied to shoots of plants freed as far as practical from their own inherent growth-promoting substances, and inhibits the elongation of roots."(36,37). Some authors restrict the term to substances active in the Avena Coleptile Curvature Test (213,214). There are a wide variety of substances that are classified as auxins (for example see 214), but relatively few are commonly used for PTC purposes. The compounds most frequently employed are IBA, IAA, NAA and 2,4-D. Indole-3-butyric acid (IBA) has stimulated callus formation in cereals (6,11,36,37) and is a particularly effective rooting agent (6,11,29,36,37). In most cases, a naturally occuring auxin (such as IAA) must be used in relatively higher concentrations (1-20 ppm), because it may be readily degraded by light and enzymatic oxidation (6,11,29,36,37). Synthetic chemicals such as NAA and 2,4-D are not subject to

the same enzymatic oxidation and may be added in much lower concentrations (0.1-6.0 ppm)(36,37). The auxin most widely used to promote callus proliferation is 2,4-D and it is often used without any exogenous cytokinin in concentrations less than 2.0 ppm to avoid toxic effects (11,29,36,37). At high concentrations, 2,4-D is a powerful herbicide and has been shown to suppress organogenesis and secondary metabolite production (3-13,18,21,23,26-29,36,37).

Cytokinins can exhibit a variety of responses similar to other plant growth substances, but are required by definition to promote cell division in plant tissues under certain bioassay conditions (36,37,213,214). These compounds are mainly N^6-substituted aminopurine derivatives obtained from both natural and synthetic sources (36,213,214). The cytokinins most widely used in culture media are kinetin, benzyladenine, and zeatin. Zeatin is naturally occurring and can elicit responses at extremely low concentrations (0.00001 ppm). Most other cytokinins are less effective than zeatin and are used in concentrations of 0.1 - 10 ppm. At very low concentrations, kinetin favors root development, but, at higher concentrations, it can suppress root initiation and promote callus initiation (3-13,18,21,23,26-29,36,37).

During this experiment (Expt 2), various concentrations of IAA, 2,4-D, BA or KIN were used, but only 2,4-D exhibited any positive growth response at the levels

tested. The best growth was achieved at 1 ppm 2,4-D, but it was always less than the growth obtained by medium supplemented with coconut water. Increased toxicity of the 2,4-D was noted by a decreased rate of growth in cultures beyond concentrations of 1 ppm. At 20 ppm of 2,4-D, all callus cultures appeared to be dead after several days of exposure. Both the cytokinins tested produced little or no extended growth on an individual basis. This also suggests that the cultures were not producing any endogenous hormones and need an external application to initiate or maintain callus growth. Since the individual regulators did little to promote cell division and accelerate callus growth in these tissues, an auxin must be applied exogenously in conjunction with cytokinins to create the proper defined growth regulator combination.

Growth and Alkaloid Accumulation in Response to Combinations of Growth Regulators

Previous experimental attempts to enhance the growth of the peyote cultures on a defined medium with individual growth regulators (Expt. 2) suggested that a combination of auxin and cytokinin was needed for proper growth. Another indication of this auxin/cytokinin synergism was observed in growth stimulation from the undefined initiation medium containing low amounts of 2,4-D and relatively high

concentration of cytokinin-like and other growth stimulators present in the liquid endosperm.

Generally, plant growth regulators can affect not only growth, but can influence secondary metabolite production as well (3-13,18,21,23,26-29,36,37). In most biosynthesis directed tissue culture experiments, economically valuable biochemicals are mainly formed in differentiated tissues or accumulated in specialized tissues or organs (3-13,18,22-31,36,37), while the majority of undifferentiated callus tissues produce only low concentrations of secondary metabolites (3-13,18,22-31). Growth, differentiation, genetic expression, and organogenesis within the cells and tissues of plants are regulated by the relative levels of auxin and cytokinin. Explants generally produce undifferentiated callus when supplied exogenously with auxin and cytokinin of approximately the same molar ratio. When the auxin level is lowered, relative to the cytokinin concentration, shoots are induced or create a hormonal environment similar to that in shoots. If the auxin concentration is higher than that of the cytokinin, a hormonal environment for roots is created and roots may be formed (3-13,22-31,36,37). Ideally, chemical production could be increased by rapidly growing single cells and small aggregates (with limited differentiation) rather than resorting to complete morphological specialization.

The growth regulator treatments in this experiment were various combinations of IAA or 2,4-D with BA or KIN at proportions that would simulate the hormonal conditions for organogenesis and tissue growth (Table 8, p. 87). Combinations of IAA with either type of cytokinin (BA or KIN) at 1 ppm:1 ppm, 5 ppm:1 ppm, and 10 ppm:1 ppm produced RT-like projections (nongeotropic, aerial roots with numerous root hairs). Although not unexpected in conjunction with higher concentrations of auxin, the formation of these organs at equal proportions of auxin to cytokinin was a surprise. A higher growth index and a larger number of these RT-like projections were noted when the cytokinin source was KIN. Little or no growth was observed when IAA was combined with BA or KIN at proportions that would stimulate shoot expression or shoot formation in the callus tissues.

The combinations of 2,4-D with various concentrations of cytokinin produced different effects in tissue growth and organogenesis than that noted with IAA. No macroscopic indications of organ formation (RT-like projections or shoots) were noted in any combinations of cytokinin with this auxin. This conforms with previous reports of the suppression of organogenesis by 2,4-D. Loose, friable tissues with good to excellent growth were noted with 2,4-D combinations of either cytokinins at 1 ppm: 1 ppm and 1 ppm: 5 ppm, but best growth was observed with BA at 5 ppm. Increasing the concentration of either cytokinin to 10 ppm

produced much lower growth rates. Increasing the auxin concentration to 5 and 10 ppm produced relatively moderate to good growth rates and the callus tissues appeared to be more densely packed, making them more difficult to subculture.

The patterns of alkaloid formation and accumulation in the callus tissues relative to the parent plant were variable throughout the various treatment groups. Generally, any growth regulator combination that provided a high growth rate in the callus tissues, also showed an increased concentration of total alkaloids compared to cultures which showed much lower growth indices. The best overall growth rates correlated well with the highest accumulations of total alkaloids with the treatment group using 2,4-D:BA (Figure 7, p. 88). The percentage of mescaline of the total alkaloids accumulated in the cultures with 2,4-D and either cytokinin was similar to the parent plant (25 % of total alkaloids), but relative concentrations differed in most cases. The overall distribution and concentration of alkaloids was most similar to that of the parent plant when medium containing 1 ppm 2,4-D and 5 ppm BA (Figure 8,9, p. 89,90). This ratio generally would be expected to favor shoot expression. Other treatment groups did not produce many of the secondary or tertiary amines characteristic of the parent plant, but no conclusion could be made with the lack of alkaloid production by combinations of the other

growth regulators.

In these studies, the callus tissues that produced an alkaloid distribution pattern similar to the whole plant were ultimately selected as the most "desirable". Besides the quantitative effects of treatments on alkaloid accumulations, observations on distribution and proportion were noted. These results suggest that manipulation of the medium can readily alter the alkaloid content, both in quality and quantity, to suit the needs of a particular industrial or experimental application.

Although little work has been done to locate the site of biosynthesis of alkaloids in cacti, the results of this work suggests that their alkaloid formation may be in the stem section. This compares favorably to the findings in the cactus, Trichocereus spp. as the site of alkaloid formation in the chlorophyllyous epidermal layer and the cortical parenchyma of the stem sections. The formation of *N*-methylated compounds (*N*-methyltyramine (II), hordenine (III), candicine (IV), and epinine (VI)) in our callus tissue culture experiments, regulated by shoot proportions of hormones, disagrees with the previous findings by Todd (Table 4, p. 33) (74). Todd's work inferrs that the enzymes needed for *N*-methylation only occur in the roots, followed by translocation of the products to the stem. The results of this experiment indicate that neither the roots nor the hormone combinations creating root expression are needed to

accumulate *N*-methylated compounds in the callus tissues.

Effects of Light Intensity and Quality on Callus Development

The characteristics of light radiation (intensity, spectral quality, and photoperiod) that influence normal plant development are the same as those which affect plant tissues in culture. Under normal tisssue culture conditions, callus cultures have been shown to demonstrate some photosynthetic activity (10-12,35,213,224,225), develop chloroplasts (22,224,225) and evolve oxygen similar to whole plants (6,7,16,22,224,225). However, the presence of an exogenous sugar in the medium has been reported to inhibit chlorophyll synthesis and photosynetic carbon fixation (10-12,224,225). Currently, no successful photoautotrophic cell cultures of the gymnosperms, monocotyledons, C_4-plants, CAM (crassulacean acid metabolism), or C_3 plants have exhibited their complete pattern of photosynthesis (22,213,214). Therefore, the changes observed are better described as photomorphogenic rather than photosynthetic effects of light. The term photomorphogenesis encompasses all regulatory effects of light on the development of plants or tissues, independent of the photosynthetic process (22,213,214).

In the photosynthetic process large amounts of

chlorophyll and accessory pigments (carotenes) are used in plants to convert light energy to chemical bond energy (213,214). In contrast, photomorphogenic responses use low amounts of sensory pigments, such as phytochrome and "cryptochrome" to take advantage of the available light energy (16,22,214). A variety of photoresponses are directly related to the phytochrome system in whole plants (213,214) and a similar function would be expected in cultured plant tissues. Some of the phytochrome photoreponses that would be applicable to PTC are promotion of shoot growth (221,222,224,225), increases in number of stomata (221,222) and chlorophyll content (221-225), accumulations of anthocyanin and other flavonoid pigments (221-225), as well as increased concentrations of alkaloids and phenolic compounds (223,224). The phytochrome system is active, more or less, over the whole range of visible and near-infrared radiation (213). Other photoreceptors in photomorphogenesis are referred to as "cyrptochromes" and which are described (213,214) as hypothetical photoregulatory pigments with the ability to sense light in the blue-ultraviolet spectral range. Although these sensory pigments are unidentified and their specific function unknown, they help explain photomorphogenetic blue-light responses that are not related to the phytochrome system (phototropic response of shoots and coleoptiles) (213,214,221,222).

Light and growth regulator interactions have been

reported to produce variable effects on culture growth (10-12,22,36,37,213,214,224,225), pigment formation (22,224,225), organogenesis (10-12,22,36,37), and secondary metabolite production (10-12,35-37,224,225). The responses of plant cultures to light are so diverse that very few specific correlations can be made for general tissue culture applications. This diversity creates the need for empirical, fundamental light experiments for characterization of the individual culture responses. Some of the response variations obtained may be explained by the spectral quality of a given light source, which will greatly affect photomorphogenesis or photosynthesis in the cultures. In general, tissue cultures require red or blue light for morphogenic responses, with blue light specifically promoting chlorophyll differentiation (22,224,225).

The evaluation of the cultured tissues after photoperiod variations (0/24, 8/16, 16/8, or 24/0 day/night regimens) provided data about growth rates, alkaloid accumulation and relative pigment production (Figure 10, p. 91). The culture showed increased fresh and dry growth rates which were observed until a plateau (no further growth) was reached at the 16/8 and 24/0 photoperiods. There was no statistical difference in either of the growth indices for these photoperiods. The cultures receiving no light (control) were the only treatment group to exhibit a noted difference between the fresh and dry growth indices.

Exposure to 8 hours of light produced similar dry growth indices to the control, but showed a higher fresh growth index. Exposure to 16 and 24 hours of light produced fresh growth indices slightly higher than the respective dry growth index. The increased percentage in the fresh to dry growth indices in the irradiated cultures may suggest that light induces cellular maturation and differentiation relative to the control. The effect of photoperiod on growth rates shows 16 vs. 24 hour light exposures to be similar. Hence, based on cost analysis for lighting, the 16 hour irradiation period is more economical.

Evidence of increases in relative cellular differentiation (relative increase of fresh to dry G.I.) is supported by the increased accumulation of total alkaloids per dry weight in cultures with light exposure. The percentage of total alkaloids was increased approximately two fold in the cultures exposed to 8 or 16 hours of light compared to the control. A lesser total alkaloid accumulation was noted in the cultures with continuous light exposure. This may imply a redirection of the photoreponse to other cellular processes, such as production of anthocyanin or flavonoid pigments to protect against the excessive light exposure. The pattern of alkaloids produced by the light exposed cultures was similar to the parent plant, while the control showed a decreased alkaloid diversity and a lower concentration of total alkaloids.

Mescaline was the chief alkaloid accumulated by the control and the percentage of mescaline per dry weight resembled that of the moderate light exposed cultures. Moderate light exposure did not cause any significant increase in mescaline production, but did increase the diversity of alkaloids produced. The cultures exposed to continuous light grew at a similar rate and relative cellular differentiation as the 16 hour cultures. This suggests that a period of darkness is needed to support an enhanced alkaloid accumulation as indicated by the 16 hour cultures. Similar results in other plant tissue cultures have shown lower accumulations of dopa with continuous light exposure, but which were increased with periods of darkness (22). Although mescaline is inexpensive to synthesize, the control group indicates that environmental factors can induce cultures to accumulate specified secondary metabolites of economical importance.

The extraction and spectral technique used to quantitatively measure photosynthetic and accessory pigments in whole plants (330,331), did not provide to be effective for plant tissue cultures. With sensitivity limitations, the relatively low amounts of the photosynthetic pigments (chloropyll a and *b*) in the cultures increased under the photoperiods tested, until a plateau was reached after irradiation of 16 hours of light. Red pigmentation was noted in cultures exposed to 16 hours or continuous light, but no other pigments (protochlorophyll, anthocyanins, or

carotenoids) were detected by spectrophotometic methods. The increased production of red pigmentation in these cultures may suggest that accumulation of anthocyanin or other flavonoid pigments were induced for light protection (22,213,214,224,225).

The effects of white light and photoperiod adjustments on the peyote cultures can be summarized as a positive influence on tissue growth, an increased relative proportion of cellular maturation, and tissue or cell differentiation (vacuole and chloroplast), and can be related to alkaloid diversification. The overall enhancement of these characteristics was best seen in the cultures exposed to a 16/8 (day/night) regimen. Future experiments with light could provide truly photoautotrophic cultures with rapid growth and increased metabolite production.

Spectral quality can also produce various effects on callus growth and primary or secondary metabolite production. In contrast to the intact plant, chloroplast differentiation is strictly blue-light dependent in cultured cells and not stimulated by both the red and blue spectral regions (22,213,214,225). Various degrees of cellular differentiation and growth have also been demonstrated to be stimulated or inhibited by specific wavelengths of light (3-13,18,22,26-28,213,214). Since the effects of spectral quality on cultures are so diverse, no specific conclusions can be drawn. In general, blue and red light elicit growth

and morphogenic responses, thus these regions of light may be effective for increased secondary metabolite production (10-12,224,225).

The irradiation of the peyote cultures with monochromatic light produced both relative stimulatory and inhibitory effects on growth and alkaloid production (Figure 11, p. 92). The results of this experiment cannot be directly compared to the white light experiments due to the decreased light intensities caused by the filters. The 16/8 (day/night regimen) photoperiod was carried over from the positive results elicited by the white light experiments. The growth indices of the cultures irradiated with blue and green were similar, while red light caused a relative reduction in growth. Reduction in the relative growth in the red light may be attributed to low phototransformation of P_r to P_{fr} chromophores in the normal phytochrome system and leading to direct decreases in protein synthesis needed for growth. The far-red region of light provided an indication of greater cellular maturation and differentiation as shown by the increased percentage of fresh growth compared to similar cultures in the white light experiment. They did not exhibit an increase in total alkaloid production. Increased growth with far-red light suggests that absorption by the P_{fr} chromophore is active in the photomorphgenic system in these cultures. The red and far-red treated cultures produced the highest percentage of mescaline of the total

alkaloids accumulated and decreased the relative concentrations of other alkaloids produced. This suggests a phytochrome-mediated reaction may be influencing the specific production of mescaline over other alkaloids. The green light group accumulated the least amount of mescaline, but produced a similar percentage of total alkaloids as the blue, or far-red groups, thus demonstrating the lack of any significant photosynthetic influence in the cultured cells. The "blue light" cultures showed a "normal" alkaloid diversity by producing a composition pattern similar to that observed in the whole plant. The light from each spectral region had some effect on growth or alkaloid accumulation indicating a complex interaction of light quality combined with photoperiod, but more specifically suggests that some independence between cellular growth and secondary metabolite production exists. If studied closer the effect of monochromatic light may help unravel regulatory systems controlling alkaloid biosynthesis and other processes involved in cell growth and differentiation.

Enhancement of Growth and Alkaloid Production of Callus Tissues by Auxin and Cytokinin Manipulation on Solid Media

Results from the "root" and "shoot" additions of growth regulators suggested that combinations of 2,4-D and BA produced the best growth response and alkaloid

accumulations in the peyote cultures. The previous growth regulator experiment (Expt. 3) did not systematically cover every possible combination of these two growth regulators within their physiological range for obvious reasons. A factorial experimental design was used which provided a finite set of concentrations and combinations of 2,4-D and BA to generate a response surface model (203,204). This type of experimental design reduces the number of experiments needed to predict the best concentrations and proportions within the range tested.

The 7 x 7 factorial design chosen for treatment with 2,4-D and BA provided increased and refined proportions of growth regulator combinations than were employed previously. The experiments with different combinations of the two growth regulators resulted in data which were best represented using a three-dimensional approach. The three-dimensional graph of the dry growth indices (Figure 13, p. 94), describes the growth response of the tissue after computer interpolation of the raw data. While visually useful to describe growth trends and predict other potential regulator combinations, it should be used as a general guide. The appearance of peak heights seen here may be misleading because of the illusion created by the rotation and tilt of the graph. The "smoothing" factor (peak sharpening) can aid or create problems for interpretation of the data, but generally personal preference dictates this

factor.

The highest growth index was suggested to be at 5 ppm 2,4-D and 5 ppm BA of the concentrations tested and appeared to be the focal point for three ridges of relatively high growth. The growth means and alkaloid percentages of the six major peaks (see Fig. 13, p. 94) were statistically different then the other growth regulator combinations. Two statistically similar growth mean groups are formed from these six peaks. The first group contains the combinations (3:3; 5:5; 5:10) and the second group (5:7; 7:5; 10:5). These ridges also produced the highest percentages of alkaloids (per dry weight) and final concentrations of total alkaloid accumulations relative to the other growth regulator combinations (Table 9, p. 95). The total alkaloid percentages were all statistically different. The front ridge follows a 1:1 ratio of growth regulators, while the other sets of peaks conform to 5 ppm of 2,4-D or BA and do not follow any general ratio. The ridge with 5 ppm BA and increasing 2,4-D exhibits decreasing growth and alkaloid accumulation. The relative decrease of the fresh to dry growth indices was correlated with a decrease of cellular differentiation and maturation relative to mitotic division. Improved growth rates with increased relative proportions of fresh to dry G.I., and alkaloid production were noted with equal concentrations of 2,4-D and BA or by increasing the proportion of BA to 2,4-D. These results were similar to the

trends demonstrated in Expt. 3 with root and shoot proportions of 2,4-D and BA, but the concentrations differed. Although these results are similar to previous experiments, the existance of a bias cell population may have been produced on a medium composition on which they were environmentally conditioned. Population dynamics may change and better growth may be obtained with other growth regulator combinations, if several subcultures are made which are then acclimated to the new environmental conditions.

Measurements of Growth in Liquid Cell Suspensions (LCS)

Plant cells grown in the liquid medium can supply further information about specific cellular growth characteristics (11-13,233-244), tissue and cell differentiation (21,27,35,233-244), exogenous accumulation of alkaloids (238,239,242,243) and ability to increase incorporation of added precursors (239-244). As in the solid media experiments, a high number of growth regulator treatment groups were tested to enhance growth and alkaloid production in the liquid cell suspensions. Therefore, methods to monitor growth needed to be simple and obtained with the equipment on hand. The growth characteristics of plant cells have been monitored in several ways, each with

their own inherent errors. In the literature, cell counts were the best indication of growth, but this is tedious and single cell cultures are necessary for accuracy (11-13,21,215). Conductivity of the cultures in the medium also provides accurate information on growth rates (215), but the initial cost is expensive. The other methods available to supply growth data are settled cell volume (SCV) (11-13,215), fresh and dry matter increase (11-13,35,215), and measurement of optical density (11-13,35). These methods were tested to provide a reproducible and nondestructive method to measure cell growth in LCS of peyote.

The direct method of measuring the cell accumulation by weight was similar to the growth determination used in the solid cultures. Because of the decreased amounts of cells, the main limitations of this method for measuring growth in LCS are based upon the sensitivity of the balance. In the literature, the direct weighing method forms the basis for fresh and dry weight accumulations measured by other growth determination methods (11-13,35,215), such as settled cell volume and optical density. Correlations for the relative degree of cellular maturation and differentiation were obtained by proportioning the fresh to dry G.I. (11-13,21,27,35,215,233-244). The direct method produced the best results for growth determination in conjunction with the "clump addition method" of cells The

latter method was used later to determine alkaloid accumulations and precursor incorporation.

Settled or packed settled cell volume are techniques that have been used to measure cell growth in various liquid cultures (11-13,215). These procedures measure the total amount of cell mass in a given volume of the culture by passive settling of the cells within a specific time (settled cell volume; SCV) or by compressing the cells to a constant volume with centrifugation (packed cell volume; PCV) in graduated conical tubes. The PCV method has less variations than the SCV procedure, but both have limitations of accuracy (11-13,215). The main sources of error in the determination of cell volume are 1) poor sampling of the original culture, 2) low cell numbers in culture, 3) calibration of the conical tube, 4) inadequate settling time or centrifugation, and 5) osmotic changes producing swelling of the cells and inflated values. Other considerations are the high time consumption for increased sample numbers, the removal and destruction of cells which will change the population dynamics of the culture and an increased possibility of contamination when samples are removed. Despite these potential errors the readings from the PCV were relatively consistent for cell growth and their relationship for fresh and dry weight accumulations. Predictions of the mean fresh and dry weights per milliliter were consistent within the repetitions, but produced high

deviations from the actual fresh and dry weights (19.91 and 20.20% respectively). The inaccurate predictions of fresh and dry weights by this method were probably due to limitations from the calibrations on the conical tube. The results do show good reproduceability, but inadequate calibration along with high time consumption per sample posed the biggest problems with this method for the determintion of cell growth.

The most sensitive method of growth determination was obtained from measurements of optical density of the cultures (11-13,21,27,35,215,233-244) composed of single cells and small aggregates with the Nephelo flasks. This procedure was an extremely easy, rapid method providing better calibration and the most reproducible results when compared to the other methods tested. The deviation of predicted to actual mean fresh and dry weights per Klett unit were 14.43% and 14.99% respectively. Since samples are not removed for measurement, this method did not disrupt the population dynamics or cause contamination, often associated with the other methods. Limitations occurred if the liquid became discolored during the growth period, but this increased background could be easily corrected by subtraction of a blank from that culture. This method was used when high sensitivity was needed to differentiate growth rates with growth regulator combinations that were statistically similar based upon the weight accumulations in

clump cultures.

The most reliable methods suggested by the literature for determining the growth of LCS cultures were obtained from the direct weighing and by measurment of optical density. In our experiments each method had a particular application based on the type of cultures and the sensitivity needed. The weighing method worked well in determining the final growth effects of growth regulators, once the overall growth curve was established with larger amounts of cells or tissue. Data from the optical density measurements became more useful when the "weighted" growth indices from various growth regulator combinations were statistically similar. The increased sensitivity of this method, along with a more uniform sizing of the culture provided better resolution of growth induced by the growth regulator combinations.

Establishment of Batch Cultures on Platform Shakers

Suspension cultures are normally initiated by transferring pieces of undifferentiated callus (2.0-3.0 g for 100 mL) to a liquid medium which is agitated during incubation (11-13,215,233-244). A more difficult process is to initiate cell suspensions by inoculating the liquid medium with an explant and waiting for the callus cells to

break off the innoculum. For subculture, the free cells and small cell aggregates are collected by selective filtering of large clumps or by pipetting. They are then transferred to a new medium. The culture medium used for the suspension culture is usually based upon that which maintains good callus growth. To achieve a high growth rate and a good cell suspension in liquid medium it is generally necessary to modify the growth regulators due to the increased cell contact (21,27,35,233-244).

Cultures in a fixed volume of medium (batch) increase in biomass by cell division and cell growth until a factor in the culture environment (nutrient or oxygen availability) becomes limiting (11-13,21,27,35,215,233-244). The cells pass in sucession through a lag phase, a short period of exponential growth, and a linear increase in the cell population. The cells then enter the stationary phase in which the cell dry weight decreases and the stability of the culture declines. The viability of the cells in the stationary phase depends on the type of limiting growth factor involved (Figure 35, p. 147).

Other considerations for establishment of LCS are the speed of the shaker (to limit cell shearing), volume of the medium (to allow proper gas phase composition), and addition of the critical cell density or "conditioned" medium (to limit death or the lag phase). Improper adjustments of any of these factors will cause most cultures to fail to grow on

subculture (11-13,21,27,35,215,233-244).

Initial attempts to establish a LCS of the peyote began by providing the medium composition which would support proper cellular growth. As suggested by the literature (11-13), the initial medium formulation was based upon the composition that maintained good callus growth on solid medium. Death in a large majority of cells occurred during the first several attempts using hormonal combinations (3 ppm:3 ppm or 5 ppm:5ppm 2,4-D:BA) that provided the best callus growth. Following the trend in the solid media of a hormonal ratio of 1:1, lower concentrations were attempted to initiate the peyote LCS. Death of the cells continued until concentrations of 0.5 ppm:0.5 ppm and 1 ppm:1 ppm 2,4-D:BA were used. These concentrations maintained good growth characteristics. Concurrent experiments with additions of conditioned media (Expt. 7) and a systematic approach to growth regulator combinations (Expt. 8) were also performed to provide the environmental conditions for proper maintenance of the peyote LCS.

Effect of "Conditioned" Medium Additions to LCS

Many researchers have noted that a volume of "conditioned" medium (from previously growing cell cultures) or a high number of cells in the initial inoculum must be

added to the LCS to inhibit retardation of the exponential growth phase or prevent death of the cells (11-13,21,27,35,215,233-244). During the beginning of these studies, retardation of growth was noted, necessitating another experiment to provide the appropriate level of "conditioned" medium to promote normal cellular growth characteristics and also insure that no growth inhibitors were being formed.

The "conditioned" medium was procured from previously subcultured single cells and small aggregates obtained by clump tissue additions to the liquid medium. The liquid medium was Millipore filtered to remove any residual cell fragments before the aliquots of "new" medium were added. Additions of at least 50 % (v/v) of conditioned medium produced normal growth curves and the highest growth indices, while lower additions showed retardation in growth (Figure 14, p. 96). No growth reduction was observed in the cultures grown in 100 % old medium. This suggests that no growth inhibitors are present and the medium does not lack (after 2 months) any essential nutritional component that limits culture growth. The treatment group consisting of 50 % additions of "conditioned" medium provided good growth and was statistically similar to the 75 % or 100 % treatment groups. Also the choice of using the 50 % "conditioned" medium in the following LCS experiments would permit replacement of any limiting essential nutrients, if any does

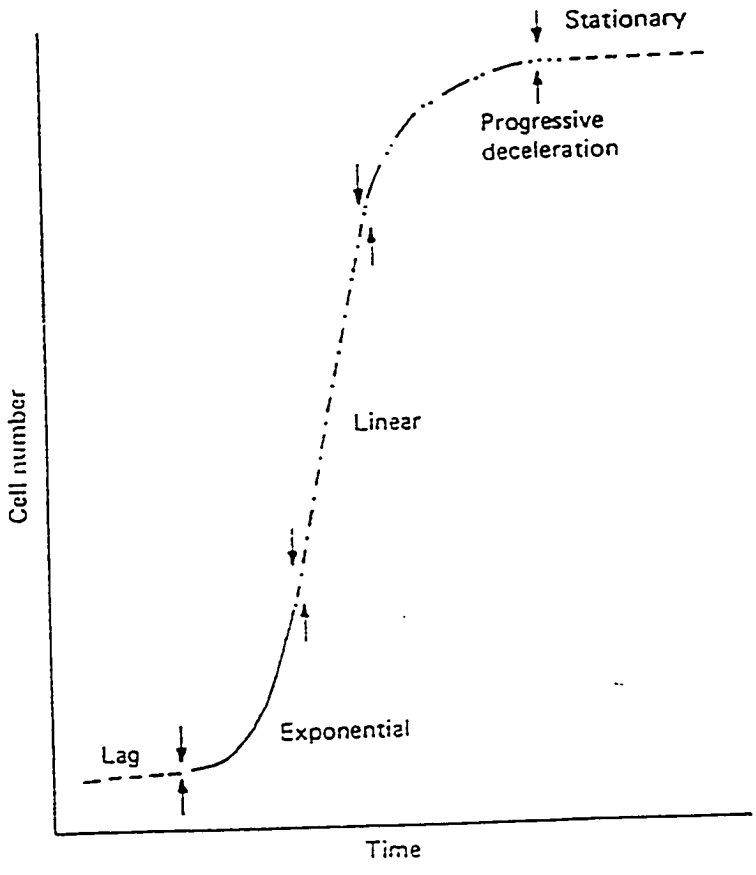


Figure 35. Growth curve of a cell suspension grown under batch conditions relating total cell number per unit volume to time. (215)

exist.

Unfortunately, it is not well known what additional components or factors are needed to formulate "conditioned" medium. It may be pH, metabolite or hormone excretion, or conversion of specific medium components to a usable form (21,27,233-244), but results from these experiments are consistent with the literature. This work suggests the addition of "conditioned" medium helps in the establishment and maintenance of "normal" cell growth characteristics of peyote in liquid cell suspensions.

Enhancement of Growth and Alkaloid Production of Suspended Cells by Auxin and Cytokinin Manipulation in Liquid Medium

Because of the increased cellular contact with the components of the medium, a new set of experiments was needed to determine the best proportions of 2,4-D and BA required to achieve good growth and alkaloid accumulations in both LCS and clump additions of peyote cells. Initial growth regulator experiments with LCS were started without "conditioned" medium, but low growth or death of the cells necessitated the running of concurrent experiments. The addition of "conditioned" medium and decreased growth regulator combinations were needed to establish good cellular growth characteristics of the peyote cells in liquid suspension.

The results obtained during the study of growth and alkaloid production of the LCS (Figures 15,16, p. 97,98) show that drastic differences are observed with growth regulator combinations in the liquid medium. Although the effective growth regulator concentrations are different, a general correlation exists between growth and the accumulation of alkaloids in the liquid and solid media. From the response surface graph, high growth indices are observed from low or "zero" levels of growth regulator additions. This is not seen with the growth on solid medium, but can be directly attributed to the low amounts of growth regulators and other factors associated with the additions of "conditioned" medium. The effectiveness of lower regulator concentrations can be associated with the increased celluiar contact of the liquid medium and the removal of the diffusion factor of the nutrients or regulators through the agar. On the solid medium, these compounds are actively transported from the cells in contact with the medium to the meristematic regions. The main growth trend appears to follow a ridge along 0.5 ppm 2,4-D with other peaks of less magnitude along 1.0 ppm 2,4-D (Figure 16, p. 98). Increased concentrations of growth regulators demonstrated toxicity by rapid decreases in growth or death of cultures. The best growth appears with 0.5 ppm 2,4-D and 0.5 ppm or 5.0 ppm BA. The production of alkaloids was greatest with the combination of 0.5 ppm 2,4-D with 0.5 ppm

or 3.0 ppm BA and 1.0 ppm 2,4-D with 1.0 ppm BA. The combination of 0.5 ppm 2,4-D with 0.5 ppm BA produced the highest total percentage of alkaloids and was statistically different than the growth regulator combinations. The general ratio of 1:1 (2,4-D:BA) provided enhanced growth combined with an increased alkaloid production. This concurs with general trends seen in the callus tissue grown on solid medium.

High standard deviations among the groups with enhanced growth did not provide statistical confidence that any one growth regulator combination induced better growth than any other (Table 10, p. 99). The increased standard deviations in growth are common when working with clump-type cultures due to the variability in the number of meristematic regions that may be involved in any one clump of tissue (11-13,233-244). Another set of growth regulator experiments was conducted with emphasis placed on the regulator combinations that exhibited enhanced growth and alkaloid accumulation.

The second experiment involving the growth regulator combinations used both the direct weight and measurements of optical density to measure growth rates in this plateau region (Figures 17,18, p. 100,101). The growth means obtained from the clump tissue additions did not exhibit any statistical difference, but better resolution of the growth differences were monitored by the optical density

measurements of the single cells and small aggregates. The increased resolution was obtained by the addition of improved cell populations (random distribution of meristematic cells and differentiated cells), along with the increased sensitivity of measurements by optical density. The optical density data (Figure 19, p. 102) shows good growth characteristics at 0.5 ppm:0.5 ppm and 1 ppm:1 ppm 2,4-D:BA. This suggests that the best growth regulator combinations during the time period and concentrations tested were in the general ratio of 1:1 (2,4-D:BA) and were similar to those obtained on the solid medium. The combination of 2,4-D and BA that gave enhanced growth appeared to be with the concentrations of both at 0.5 ppm and was statistically different (95 % confidence) then the other regulator combinations tested.

Effect of Varied Concentrations of MSk Salt Base in the Liquid Medium

Many investigators have shown that there are limiting components in most media that are often found in low concentrations and which are rapidly consumed preventing further growth of the culture (11-13,233-244). These were not evident in the peyote cultures. Another limitation of growth can be caused by osmotic stress from the inorganic salts found in the normal salt base. If the osmotic strength

is too high, the cells will be water stressed and will inhibit normal cellular growth characteristics. For this reason an experiment was designed to find the correct concentration of salt base, of a fixed composition, needed to insure proper growth without imposing extreme osmotic effects.

The results of this experiment (Figure 20, p. 103) shows minimum growth with low concentrations (0.05 and 0.25 MSk) of salt base. Further additions produced a linear increase in cell growth that directly correlates with the salt base additions. This would suggest that one or more components in the salt base aid in cell growth. Indications from the conditioned medium experiment (100 % old medium) are that no limiting essential nutrients appear to exist in this salt base formulation, since no significant decrease in cell growth was observed after two growth cycles. Therefore any components that promote growth are probably not in low enough concentrations to be depleted during a single growth period.

Some negative growth effects were noted in the cells, due to osmotic strength increases in the medium. Microscopic evaluation of the cells showed a relative decrease in cell size and turgor with increased osmotic stress. These changes did not inhibit growth or cause death of the cells. Some browning of the cells near the end of the growth period was observed with the high salt base additions (1.25 MSk).

Generally, cell and tissue browning is thought to be due to increased production of polyphenolic compounds and could be a the result of osmotic stress in the cultures (11-13,15,35,163-165). This class of compounds is also seen to be formed in whole cacti in response to tissue wounding (158-160).

Further experiments are needed to establish the specific salt base constituent(s) that promote enhanced growth of the peyote cells in liquid suspension, without causing extreme osmotic stress conditions.

Precursor Additions to LCS

The biosynthetic pathways leading to the phenethylamines and tetrahydroisoquinoline alkaloids in the whole peyote cacti have been fairly well characterized by previous investigators (59-74,144-154) in whole plants using radiolabeled precursors (Figure 2, p. 36). However, there are still a few minor conflicts in interpretation due to the complex nature of biosynthetic studies in whole plants. The plant tissue cultures in liquid suspension allow a more simplified and direct approach to study biosynthetic pathways then when using the whole plant. Precursor additions to plant LCS would regulate secondary metabolic pathways and be similar to the successful bioreactor systems

established from fungal and microbial sources. Although enrichment of the medium with precursors has produced some positive results, the lack of incorporation may be due to low uptake, conjugation, diversion into alternative pathways, or the lack of isomerases or other enzymes between the precursor and the product.

Because of the large number of treatment groups and repetitions for each, two large experimental sets were carried out to test various concentrations (Expt. 1, 10.0 and 20.0 mg per 50 mL (Figure 21, p. 104) and Expt. 2, 2.5 and 5.0 mg per 50 mL (Figure 22, p. 105)) of glycine, 3,4-dimethoxyphenethylamine, methionine, and D- or L-isomers of phenylalanine or tyrosine. A control group, without any precursors, was used to estalish any background variation in the growth characteristics of the LCS and clump-type cultures. Some variation in growth of the cultures was observed between the two experimental sets. This may be due to difference in age of the cultures, prolonged time periods in the stationary phase before transfer, or inequality in cellular maturation and differentiation. Trends in each experimental group were compared to the relative growth and alkaloid accumulations incurred by the control.

Glycine was used to establish base line increases in cell growth and alkaloid production from a nonspecific amino acid source (nitrogen source). The control-normalized

results of glycine additions showed a slight increase in fresh or dry matter accumulations and alkaloid production for each experimental set. These increases were consistent in each set and did not relate directly to concentration variations. Glycine, therefore, may be metabolized or directly used for protein synthesis and provide an indirect promotion of cell growth. Previous data suggested increases in alkaloid accumulation was associated with general cell growth. This could account for the slight increase of alkaloids in the glycine groups compared to those observed in the control group.

Additions of 3,4-dimethoxyphenethylamine to peyote cells provided a mescaline precursor that is in the mainstream of the alkaloid biosynthetic pathway. Conflicting data by other researchers show either high or very little incorporation of this compound into the phenethylamines (42,43,59-74). Other investigators report 3,4-dimethoxy-phenethylamine as a deadend product in the mescaline producing cactus, Trichocereus, and suggest a similar pathway is involved in Lophophora (42,43,59-74,144-154). Analysis of the liquid medium showed that neither an appreciable amount of the precursor material nor any alkaloids existed in the medium. The cells showed increases of total alkaloid content compared to the control approximately 1.8-2.5 times. Only trace amounts of the alkaloids were identified as 3,4-dimethoxyphenethylamine

with general increases in all other phenethylamines. This data disputes the idea that this compound is a final biosynthetic product in Lophophora and suggests that the alkaloid pathways are slightly different then found in Trichocereus. Incorporation of this precursor also may be a feedback effect caused by the reversal of the pathway because of high concentrations. Another unidentified compound was detected in higher than normal concentrations in these cells. Based on the biosynthetic pathways and predictive models for a homologous GC series, the suspected compound is probably a hydroxylated derivative of the precursor, but this structure could not be confirmed. The control-normalized growth indices were observed to have a proportionaly higher fresh to dry index, perhaps indicating a relative increase in cell differentiation in all additions of 3,4-dimethoxyphenethylamine.

Phenylalanine is another amino acid that has an unclear role as a precursor in the peyote alkaloid pathway. Low levels of incorporation to mescaline was observed in many cases when D,L mixtures of radiolabelled phenylalanine were injected into the whole plant (42,43,121-154). In general, only L isomers of most amino acids are usually biochemically active (unless a racemase enzyme is present) and thus would immediately eliminate half of the radiolabelled compound from incorporation (141). In our studies, the D isomer produced low level stimulation of

growth and alkaloid accumulation similar to that of glycine, indicating its use as a general nitrogen source. The L isomer showed similar growth patterns as the D isomer, but provided a much higher concentration of total alkaloids. The control-normalized data from the L isomer showed twice the alkaloid production with the lower concentrations of L-phenylalanine and an increase of 2.3-2.5 times with the higher levels of precursor addition. This data suggests that L-phenylalanine is an effective precursor in the alkaloid biosynthetic pathway found in cultured peyote cells.

Methionine or S-adenosyl-L-methionine has been postulated by many investigators as the source of *O*-methyl groups for catecholic and monophenolic phenethylamines (42,43,130-154). In our experiments all methionine concentrations tested caused cell death within several days and could not be evaluated for incorporation as an alkaloid precursor. The death of the cells in culture suggests that direct injection of radioactive methionine in previous experiments stimulated a wound response. This may cause increased accumulations of polyphenolic alkaloids (due to stress) by indirect incorporation the methyl and amine groups of methionine.

Tyrosine has been adequately demonstrated to be incorporated into many of the phenethylamine biosynthetic pathways in a variety of organisms, including fungi, cereal plants, as well as cacti. Although not as confusing as

phenylalanine incorporation, previous radiolabelled experiments also involved injections of D,L mixtures into whole plants (139-141). In our studies D-tyrosine produced a low level stimulation of alkaloid production similar to that of a nonspecific nitrogen source (glycine), but only equaled the control in growth characteristics. The L isomer produced the same low growth and alkaloid stimulations in low concentrations, but showed better growth and 2.5-2.7 times the alkaloid accumulation than the control.

The data from this experiment indicates that a nonspecific nitrogen source can cause a low stimulation of growth and, so, indirectly effect alkaloid accumulation. Better precursors for alkaloid production in bioreactor systems with peyote cells are from the L forms of phenylalanine and tyrosine or 3,4-dimethoxyphenethylamines. The production of abberant alkaloids in these systems may also be a future possibility when the details of enzyme specificities are learned and structurally similar compounds can be introduced to produce "unnatural" compounds.

Confirmation of Phenethylamine Alkaloids by Mass Spectrometry

The identification of the major phenethylamine alkaloids in the peyote tissue cultures has been tentatively established in the foregoing experiments by TLC, GC and HPLC

methods. Although these techniques are specific for quantitation and provide good resolution of the individual alkaloid constituents, positive confirmation of the constituents by MS or MS/MS (tandem mass spectrometry was necessary.

The use of MS/MS for characterization of secondary metabolites in plants and other complex mixtures is becoming more common (177-187). Several advantages are offered by the MS/MS over traditional methods of alkaloid studies, which typically involve lengthy chromatographic isolation procedures and laborious large-scale isolation. The ability to measure small quantities of crude samples directly with little or no sample preparation is of primary importance. This permits rapid characterization of plant samples of limited availability and also avoids possible formation of extraction artifacts. Current applications of this technique are in detection of new compounds, conduction of chemotaxonomic surveys, finding suspected biosynthetic intermediates, and mapping the distribution of alkaloids in plant tissues (177-187).

Several major alkaloid phenethylamine components of the cultured peyote cells were identified by MS/MS (Figures 22-33, p. 106-116). The phenethylamines under MS conditions provide characteristic peaks in their spectra unique to this class of compounds. The characteristic peaks are found at m/z 77, 91 and 103 for general phenethylamines. Mono or

dihydroxylation of the ring includes other major peaks at m/z 93 and 121 in the spectrum. *N*-substitutions of the amine show peaks at m/z 30 for a primary (no extra alkyl group), m/z 44 for a secondary, m/z 58 for a tertiary, and m/z 60, 58 and 44 for the quaternary compounds.

Besides the normal phenethylamine compounds expected in the whole plant (tyramine, *N*-methyltyramine, hordenine, and mescaline) a new constituent was found and confirmed. Candicine, which follows the *N*-methylated homologous phenethylamine biosynthetic series, has not been previously confirmed by MS to be found in peyote (Figure 32, p. 115)(62,183). With previous detection, purification and isolation quaternary salts were difficult with classical chromatographic methods, because of the complex polarity of plant extracts. Candicine is the most common quaterary cactus alkaloid and has been confirmed in many other alkaloidiferoius cacti (183). Based on a knowledge of the chemotaxonomic leads and biosynthetic pathways in other mescaline producing cacti, the presence of candicine was highly suspected in these peyote tissues. With confirmation of the rest of the *N*-methylated compounds in the peyote, the normal biosynthetic progression from hordenine to candicine has been proposed to exist in these cultured cells (Figure 34, p. 117).

Summary

Fundamental experiments, to grow and enhance alkaloid accumulations in tissue cultures of the peyote cactus, Lophophora williamsii (Lemaire) Coulter, were successful. The first major obstacle, as in any PTC project, was the proper selection of tissues from the parent plant to initiate callus growth. Another contingent factor to promote callus initiation was the selection of the correct medium formulation containing the proper nutrients and growth regulators. Callus formation from the stem and root sections from the whole plant was attempted. Only the stem sections were able to initiate a friable callus tissue on a medium containing 10% L.E. and 1 ppm 2,4-D. The initiation of the peyote callus tissues, had not been previously reported in the literature. The successful formation of callus tissues from the peyote combined the selection of meristematic tissues along with the proper combination of medium components to promote and maintain callus growth.

Further enhancement of peyote culture growth and alkaloid production was achieved by growth regulator combinations in both the solid and liquid media. The best growth and alkaloid accumulations were achieved with

combinations of 2,4-D and BA in a 1:1 ratio, but concentrations differed for the solid and liquid media. The inequality of hormone concentrations are due to different diffusion rates of the medium components in the two physical states. The regulator manipulations showed direct influences on growth, cellular differentiation and suggested potential control of the concentration and distribution pattern of the alkaloid production. Although the "ideal" alkaloid distribution pattern was one similar to the whole plant, some regulator environments did not produce the full complement of alkaloids. Regulator combinations shown to produce higher concentrations of specific components can be of economic importance. The production of specific metabolites may increase yield of minor constituents and may be useful to solve biosynthetic problems on a molecular level.

Variation of light intensity and quality provided other interesting results on growth and secondary metabolite production. While the role of light is not well understood in tissue cultures, the highest callus growth and alkaloid accumulation was achieved in white light and with a photoperiod of a 16/8 day/night regimen. As in the growth regulator experiments, several monochromatic light conditions provided redistribution of the alkaloid pattern and may help provide answers to photomorphogenic responses in whole plants.

Based on the comparative growth and alkaloid accumulations achieved on solid and liquid media, the overall data suggests that the medium in the solid state is superior. In theory, the liquid medium should have relatively higher growth and alkaloid production, because of increased cellular contact with nutrients. Decreased growth in the liquid medium may be caused by low oxygen levels and improper balance of nutrient concentrations. Increased growth on the solid medium may also be attributed to stimulation by the agar, acting as a carbon source or as a source of other stimulating substances.

Precursor additions to the peyote LCS enhanced growth and influenced alkaloid production by direct and indirect processes. Previous work with radioactive D,L mixtures of amino acid precursors to the phenthylamine pathway demonstrated limited incorporation into the alkaloids. Our data suggests that only the L isomer is active as a precursor for the phenethylamine alkaloids. The D isomers provide an indirect alkaloid production by increased cellular growth, possibly acting as a nitrogen source. The precursor, 3,4-dimethoxyphenethylamine, provided direct incorporation to other alkaloids and suggested that the phenethylamine biosynthetic pathway is different from that found in Trichocereus spp..

While the individual experiments produced good results, they have only touched the tip of the iceberg and

indicate that further work can be performed to enhance growth and secondary metabolite characteristics in the peyote cultures. The statistical approach, to test combinations of growth regulators, established trends for regulator influences on callus growth and alkaloid production. This work provided response surface data suggesting other combinations that may influence growth and alkaloid accumulation. This statistical approach reduced the necessary experiments to test the large number of treatment combinations. Otherwise, the treatment groups would be carried out in the traditional and laborious, "trial and error" or "brute" force experimental methods. Future considerations to enhance growth and alkaloid accumulations in the peyote tissues would combine all of the individual experimental relationships into one large empirical formula.

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Biographical Sketch

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