THE BIOGENESIS OF TROPIC ACID IN *DATURA STRAMONIUM*

by

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ABSTRACT

There have been two pathways postulated for the biogenesis of tropic acid in Datura stramonium. The first of these, involving the amino acid tryptophan, was proposed by Goodeve and Ramstad[13]. The second utilizes another amino acid, in this case phenylalanine, and was put forward by Leete[15] and independently by Underhill and Youngken Jr.[16].

Since there have been these two mechanisms postulated for the biogenesis of tropic acid in this plant, one must conclude that either the two amino acids act via a common pathway or that there are in fact two separate mechanisms for tropic acid biogenesis. The purpose of this investigation was to distinguish between these two possibilities.

Tryptophan-3-C^{14}, tryptophan-(2-indolyl)-C^{14}, indoleacetic acid-2-C^{14}, phenylalanine-3-C^{14}, and uniformly ring labelled phenylalanine-C^{14}, were fed to separate, one week old, sterile, root tissue cultures of Datura stramonium. The time allowed for the metabolism of the radio-active precursor varied from three to twenty-one days. The tissue was then extracted with ethanol and this extract subjected to two-dimensional paper chromatography. Autoradiography was employed to determine the location of labelled metabolites. These metabolites were then identified by direct comparison to authentic samples chromatographed in an identical fashion.

The results of this investigation show that all of the labelled precursors do give rise to labelled tropic acid. The pathway from phenylalanine appears to be the predominate mechanism for tropic acid formation in isolated Datura stramonium root tissue. The pathway from tryptophan, although apparently playing a minor role in tropic acid biosynthesis in this tissue, was shown to be a unique and independent system for the biogenesis of this aromatic acid.

It is concluded therefore that there are two separate mechanisms involved in the biogenesis of tropic acid in Datura stramonium root tissue.

We accept this abstract as conforming to the
required standard

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LITERATURE REVIEW

L-Hyoscyamine is the most commonly occurring alkaloid in plants of the family Solanaceae. This alkaloid is an ester consisting of a tropinol and a tropic acid portion.

The biogenesis of L-hyoscyamine is thought to occur principally in the roots of Datura stramonium and from here the alkaloid is transported via the transpiration stream, through the xylem to the aerial portions of the plant, especially the leaves, where it is finally stored. There is however, some evidence that L-hyoscyamine can be synthesized in the leaves, especially the young leaves, although in far smaller quantities than that synthesized by the roots.

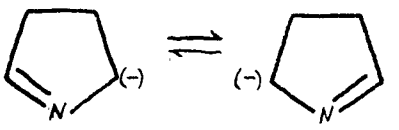
Elucidation of the pathway involved in the biogenesis of the tropinol portion of L-hyoscyamine began with the work of James[1] in 1949. James carried out experiments involving the feeding of several possible amino acid precursors to detached leaves of Belladonna. He found that M/100 L-arginine and M/100 ornithine supplied in addition to 1% sucrose caused increases in the amount of alkaloid produced per leaf both over the initial values at the time of picking and over the value for leaves supplied with 1% sucrose only. The increases were small but statistically significant. He further found that neither M/100 L-proline nor M/100 ammonium sulfate caused similar increases.

Glutamic acid, which is a precursor of both ornithine and proline, was investigated by French and Gibson[2] for its possible role in the biogenesis of hyoscine and hyoscyamine. Glutamic acid in concentrations of 20 and 60 ppm was fed to root tissue cultures of Datura tatula L. French and Gibson conclude that under the conditions and concentrations used in this investigation, L-glutamic acid produces a statistically significant decrease in alkaloid content, although growth rate was improved.

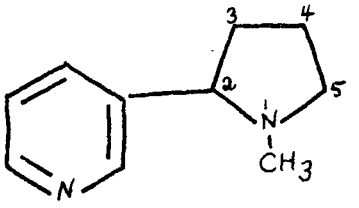
Gibson and Abbot[3] investigated proline as a possible precursor for tropinol in Datura tatula L. As in the case of James they conclude that proline is not indicated as a precursor of L-hyoscyamine, but merely enters the metabolic pool as part of synthesized proteins. When these proteins are degraded by the plant some of the activity may then reside in the alkaloid fraction. In a more recent paper, Gibson and Sullivan[4] found that proline is incorporated into tropinol, but that the degree of incorporation is too

small for proline to be an immediate precursor of tropinol.

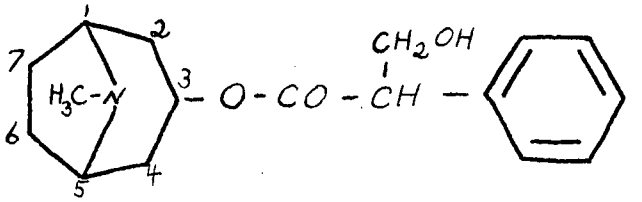
Until rather recently it was thought that a parellel existed between the biosynthesis of the pyrrolidine ring of nicotine and the tropinol portion of hyoscyamine. The postulated common intermediate was:



Investigators such as Dewey, Byerrum and Ball[5], and Leete[6], Leete and Liegliud[7], have shown that when whole tobacco plants (Nicotinia tabacum L) are grown on a medium containing ornithine-2-C^{14} they produce nicotine labelled in positions two and five of the pyrrolidine ring.



In similar experiments using Datura stramonium L, Leete, Marion and Spenser[8] have demonstrated that ornithine-2-C^{14} is incorporated into hyoscyamine biosynthetically, the C^{14} appearing on the bridgehead carbons. They did not ascertain whether the incorporation of the label was assymetric, and therefore the labelling may have occurred at position one, five, or at both positions randomly.



Later work by Botherner-By, Schutz, Dawson, Scot[9], and independently by Leete[10], indicates that ornithine-2-C^{14} stereospecifically labels one, but not both of the bridgehead carbons of hyoscyamine. There is no mixture of labelling in both the one and five positions as there had been in the two and five positions of nicotine. This of course indicated that a symmetrical intermediate such as the one postulated for the biogenesis of nicotine could not be involved in the biogenesis of hyoscyamine. In 1964 Leete[11] was able to conclude that ornithine-2-C^{14} labels only position one of the tropinol portion of hyoscyamine, and has therefore solved the biochemical stereospecificity of the incorporation of ornithine into tropinol.

Kaczhowski, Schutte, and Mothes[12] fed acetate-1-C^{14} and acetate-2-C^{14} to the excised roots of Datura metel. They found that these two compounds are especially incorporated into positions two, three, and four of the tropinol portion of hyoscyamine. Position three is only labelled if acetate-1-C^{14} is fed, whereas acetate-2-C^{14} is not incorporated into this position. The mechanism for this incorporation of acetate into tropinol is thought to involve the condensation of two acetate units to form acetoacetic acid. This molecule then becomes attached through its carbon atom number two to the intermediate formed from ornithine. Following this condensation the carboxyl group of the acetoacetic acid is eliminated, leaving carbons two, three, and four derived from acetoacetic acid.

There have been two pathways postulated for the biosynthesis of the tropic acid portion of L-hyoscyamine in Datura stramonium. The first of these mechanisms, that proposed by Goodeve and Ramstad[13], involves the amino acid tryptophan. Whole Datura stramonium plants grown in hydroponic culture were fed tryptophan-3-C^{14} and harvested at various time intervals. The radioactive metabolites of tryptophan-3-C^{14} were then identified chromatographically. From the results of this work Goodeve[14] was able to propose the pathway outlined in figure 1 for the biosynthesis of tropic acid from tryptophan. The elucidation of this scheme included the isolation of all of the proposed intermediates involved. The most interesting reaction in this metabolic sequence is of course the unique cleavage of the indole nucleus to give a free amino group rather than the more conventional and well studied reaction product, an aminoformyl group. The new amino acid, α-phenyl-β-amino propionic acid is closely related to phenylalanine, differing only in the positions of the amino and phenyl groups.

The second mechanism proposed for the biogenesis of tropic acid was put forward by Leete[15]. Phenylalanine-3-C^{14}, sodium formate-C^{14}, and formaldehyde-C^{14} were added to separate nutrient solutions and fed to two month old Datura stramonium plants. He found that phenylalanine-3-C^{14} was incorporated into carbon atom number two of the tropic acid portion of hyoscyamine while the sodium formate and formaldehyde gave most of their activity in the tropinol portion of the molecule. He concluded that phenylalanine is a direct precursor of tropic acid.

Underhill and Youngken Jr.[16] investigated further the role of phenylalanine in the biogenesis of tropic acid. They administered dl-phenylalanine-3-C^{14}, phenylacetic acid-1-C^{14}, sodium acetate-2-C^{14}, sodium propionate-2-C^{14}, sodium formate-C^{14}, dl-serine-2-C^{14}, and dl-zinc lactate-2-C^{14} to Datura stramonium L plants grown in hydroponic culture. Phenylalanine-3-C^{14}, phenylacetic acid-1-C^{14}, and sodium acetate-2-C^{14} were the most efficient precursors of hyoscyamine, followed by sodium propionate-2-C^{14}, sodium formate-C^{14}, and serine-3-C^{14} in that order. Zinc lactate-2-C^{14} was found to be a poor precursor for hyoscyamine. Administration of phenylalanine-3-C^{14} and phenylacetic acid-1-C^{14} produced labelling of only the tropic acid portion of the molecule, while sodium acetate-2-C^{14}, sodium propionate-2-C^{14}, and sodium formate-C^{14}, and serine-3-C^{14} labelled almost exclusively the tropinol portion of the molecule. The pathway postulated by Underhill and Youngken Jr. is outlined in figure 2. It should be noted however that the proposed intermediates have not been isolated and therefore only represent a logical sequence of events to explain the experimental findings.

Grass and Schutte[17] further substantiated the fact that phenylalanine was a good precursor for tropic acid in Datura metel by finding that phenylalanine-3-C^{14} would act as an intermediate in the biosynthesis of both tropic and benzoic acids in this species.

FIGURE 1.

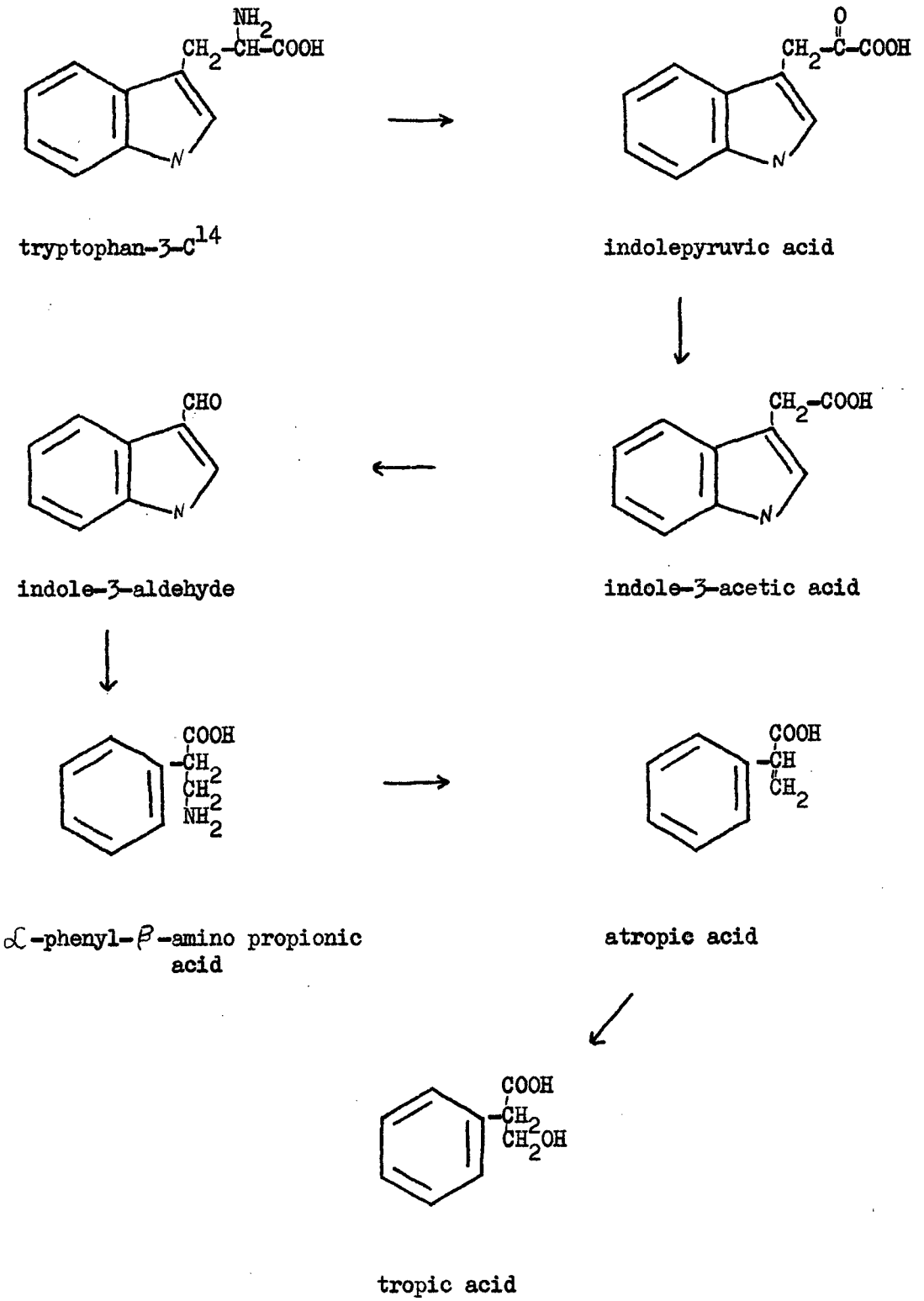
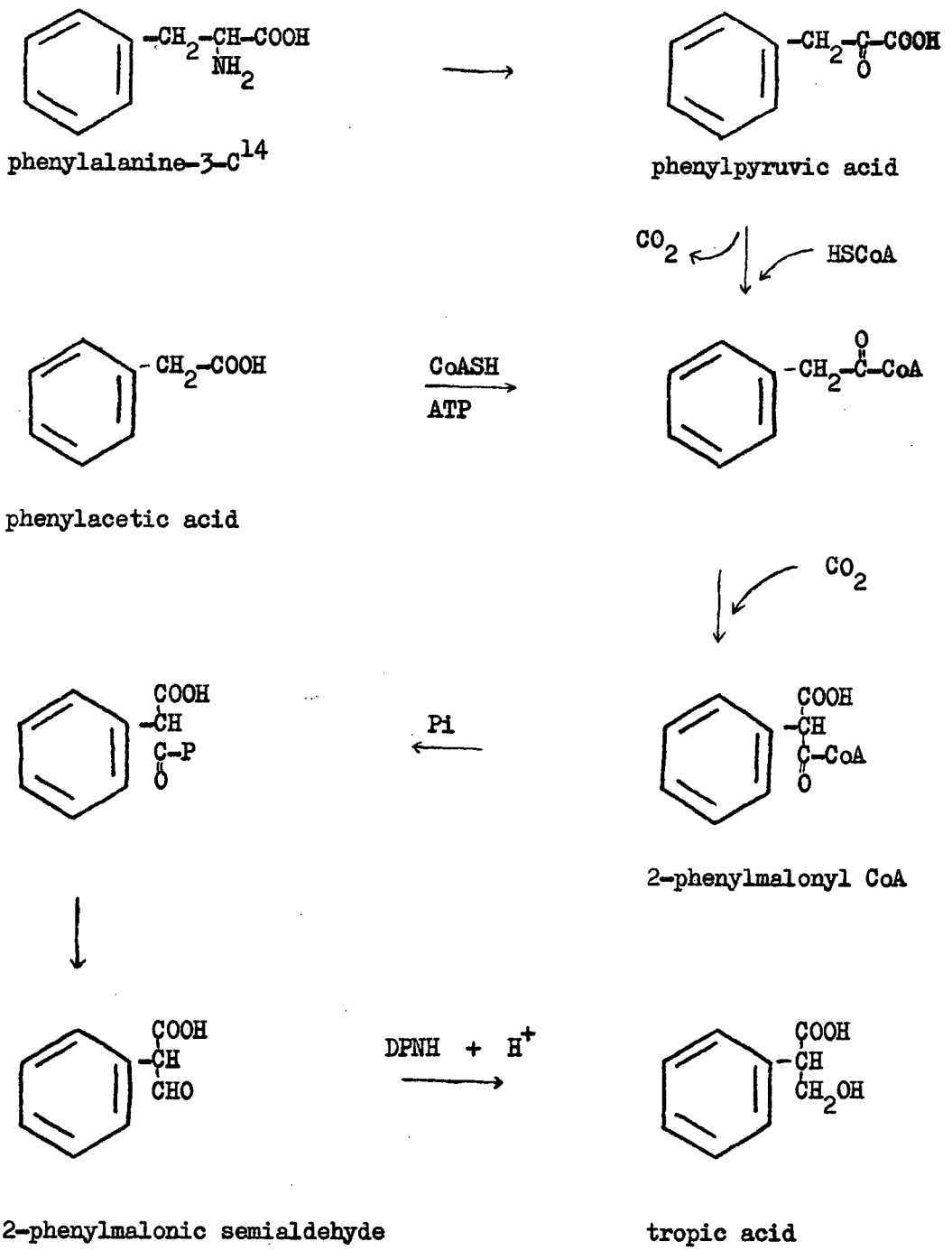

tryptophan-3-C14
indolepyruvic acid
indole-3-aldehyde
indole-3-acetic acid
α-phenyl-β-amino propionic acid
atropic acid
tropic acid

FIGURE 2.


-CH2-CH-COOH
NH2
phenylalanine-3-C14
-CH2-C-COOH
O
phenylpyruvic acid
CO2
HSCoA
-CH2-COOH
CoASH
ATP
O
-CH2-C-CoA
phenylacetic acid
CO2
COOH
-CH
C-P
O
Pi
COOH
-CH
C-CoA
O
2-phenylmalonyl CoA
COOH
-CH
CHO
DPNH + H+
COOH
-CH
CH2OH
2-phenylmalonic semialdehyde
tropic acid

INTRODUCTION to the PROBLEM

In view of the fact that there have been two mechanisms postulated for the biogenesis of tropic acid in Datura stramonium, and further, since these two schemes involve amino acids which have not been reported as being interconvertible, it was decided to compare the two biosynthetic schemes under identical conditions.

The two possibilities which were considered are:

1. That there are in fact two separate mechanisms involved in the formation of tropic acid in Datura stramonium.
2. That the amino acids involved are first interconverted or at least form a common intermediate which is in turn converted to tropic acid.

The purpose of this investigation was therefore to distinguish between these two possibilities.

METHODS and MATERIALS

Since the purpose of this investigation was to compare two biosynthetic pathways, it was necessary to maintain easily reproducible, constant, environmental conditions. Because of this need plant tissue culture was chosen as the technique to be used since it fulfilled these requirements and further offered a system which would be free from bacterial and fungal contamination.

The use and evaluation of plant tissue culture as an experimental method can be found in review articles prepared by Butcher and Street[18] and by Carew and Staba[19]. The actual methods employed in plant tissue culture are best dealt with by White[20] in his book The Cultivation of Animal and Plant Cells.

Excised root tissue cultures were used in this investigation since the biogenesis of L-hyoscyamine is thought to occur primarily in this region of the plant. White's liquid nutrient medium[20] was chosen to support the growth of the isolated root tissue since it satisfies the hormone requirements of isolated Datura stramonium roots as shown by Bonner[21]. These hormone requirements, as determined by Bonner, are nicotinic acid, thiamine, and pyridoxine.

White's liquid nutrient medium consists of three parts. They are: an inorganic salt solution, a hormone supplement, and a carbohydrate solution. Preparation of this medium was carried out as follows:

Using the best grades of analytical chemicals the following were weighed out:

$Ca(NO_3)_2$	20 gms.	$MnSO_4$	0.45 gms.
Na_2SO_4	20 gms.	$ZnSO_4$	0.15 gms.
KCl	8 gms.	H_3BO_3	0.15 gms.
NaH_2PO_4	1.65 gms.	KI	0.075 gms.

These compounds were then dissolved one at a time in sufficient distilled water to produce eight liters of solution. If the hydrates of any of the salts were used the proper compensations in weights were made. Thirty-six grams of magnesium sulfate were dissolved separately in two liters of distilled water. The two solutions were then mixed slowly and stored under refrigeration. This stock solution is ten times the concentration required in the complete nutrient solution.

A second stock solution was prepared by dissolving three hundred milligrams of thiamine, fifty milligrams of nicotinic acid, ten milligrams of pyridoxine, and three hundred milligrams of glycine in one hundred milliliters of distilled water. This solution was also stored under refrigeration and is one hundred times the concentration used in the complete nutrient solution.

In order to prepare two liters of the complete nutrient solution, forty grams of sucrose were dissolved in one liter of distilled water. Then, ten milligrams of ferric sulfate were dissolved in one hundred milliliters of distilled water, one-half was discarded, and the remainder was added to the sucrose solution. To these, two hundred milliliters of the stock salt solution and twenty milliliters of the hormone stock solution were added and the whole mixture was then made up to two liters with distilled water. This medium was then distributed into culture flasks, each flask containing four hundred milliliters of nutrient. The flasks consisted of one liter erlynmeyer flasks equipped with cotton plugs. When feeding experiments were being done, it was at this point that the radio-isotopes were added. The culture flasks with their nutrient solution were autoclaved for thirty minutes at one hundred and twenty degrees centigrade and fifteen pounds pressure.

White's liquid nutrient medium was notably deficient in copper and molybdenum. This was not of much importance in this investigation since there were no symptoms of the deficiency of either mineral and growth was satisfactory. It is probable that small amounts of both minerals are present as impurities in the other compounds used to prepare the stock salt solution.

The surface of unbroken capsules of Datura stramonium were sterilized by washing them with thirty per cent hydrogen peroxide solution for three to five minutes. The capsules were then broken open and the seeds removed aseptically. The seeds were then shaken vigorously in a stoppered erlynmeyer flask with a further portion of thirty per cent hydrogen peroxide for a period of ninety seconds. Following this sterilization period the seeds were placed in previously autoclaved petri dishes containing filter paper moistened with sterile distilled water. The petri dishes with the sterile seeds were then placed under an ultra-violet light in order to catalize the degradation of the remaining hydrogen peroxide. When all of the hydrogen peroxide had apparently degraded, the petri dishes were placed in an incubator at twenty-eight degrees centigrade and germination was allowed to con-

tinue for a period of seven to ten days. Following this period the largest and most rapidly growing roots were excised and placed in culture flasks containing White's liquid nutrient medium. The averate length for these freshly excised roots was about fwenty-five millimeters. Each one liter culture flask contained twenty excised roots and four hundred milliliters of White's liquid nutrient medium. These root tissue cultures were then placed in an incubator which maintained a constant temperature of twenty-eight degrees centigrade. Growth was allowed to continue for a period of seven days. On the seventh day the excised roots were aseptically transferred to fresh medium, but this medium also contained the radio-isotope which was being fed. Metabolism and growth were again allowed to continue under the conditions previously outlined. The metabolism time allowed before the cultures were sacrificed and extracted varied from feeding to feeding. Whenever this period extended beyond seven days the root tissue was placed in fresh nutrient solution containing more of the radio-isotope at the end of each seven day period.

Following the period allowed for metabolism the tissue was removed from the culture flask, blotted dry on filter paper, and placed in a glass mortar. A small amount of sand and ethanol were added and the tissue was ground until a thin paste was formed. This paste was then placed into a soxhlet extraction thimble and extracted in a micro-soxhlet apparatus with approximately twenty milliliters of ethanol for a period of six hours. The ethanolic extract thus formed was then evaporated to dryness by allowing it to stand under a stream of air at room temperature.

The dried extract was redissolved in a small quantity of ethanol and spotted on eight inch squares of Whatmann number one filter paper. This chromatography paper was equipped with corner-punched holes suitable for use on Smith's Universal Apparatus[22]. The spotted sample was then subjected to two-dimensional chromatography. The solvent system employed was isopropanol, ammonium hydroxide, and water (20:1:2) and n-butanol, acetic acid, and water (12:3:5). The first solvent system was run for fifteen hours. The paper was then allowed to dry in a current of air at room temperature for thirty minutes and then placed in the second solvent system and run until the edge of the chromatogram was reached (approximately six hours). The chromatogram was then removed from the chromatography chamber and again allowed to dry in a stream of air, this time for twenty-four hours.

This prolonged drying period is necessary since it is important that all traces of organic solvents are removed before the chromatogram is used in the preparation of auto-radiograms.

The location of radio-activity on the chromatograms was accomplished by preparing auto-radiograms. Auto-radiograms were prepared by placing the chromatogram between two sheets of Kodak No-screen Medical X-Ray Film. The film and chromatograms were then placed in an exposure holder for a suitable time period. Following this time period, one of the films was removed and developed.* The second film was usually left in contact with the radio-active chromatogram for twice the exposure period of the first film. The advantage of having two exposures for each chromatogram is that the first exposure is available for visualization after only a short waiting period while the visualization of radio-active compounds present in only small amounts can be done on the second film. Thus results are available quickly and accuracy is not sacrificed.

The compounds chosen for feeding purposes were:

1. tryptophan-3-C^{14}
2. indoleacetic acid-2-C^{14}
3. tryptophan-(2-indolyl)-C^{14}
4. phenylalanine-3-C^{14}
5. phenylalanine-C^{14} (uniformly ring labelled).

These compounds were administered at either the five or ten microcurie per week level.

Two additional solvent systems were employed to aid in the identification of radio-active spots which were eluted from the original chromatogram. Ethanol, ammonium hydroxide, and water (16:1:3) and n-propanol, eucalyptol, formic acid, and water (5:5:2 sufficient to saturate) were used in a two-dimensional system for the identification of neutral and acidic spots. Similarly n-butanol, acetic acid, and water (12:3:5) and n-butanol, 1N sodium acetate, and 1N hydrochloric acid (7:120:60) were used in the identification of basic spots. The relative acidities or basicities of the radio-active spots to be rechromatographed were judged by their position on the original chromatogram. Details on the preparation of any of the above

*Acknowledgement with thanks is extended to Miss M. Harrison, X-ray technician at the University of British Columbia Health Services, for technical assistance in developing the X-ray films.

solvent systems or their use can be found in Smith's book, Chromatographic and Electrophoretic Techniques, Volume I.[22]

Details of all of the root tissue culture feeding experiments can be found in table 1. The method employed in the production and maintenance of cultures, extraction of tissues, and the separation and identification of radio-active metabolites was in all cases identical with the methods previously outlined.

The administration of three of the radio-active materials was also accomplished by a vacuum infiltration method. In the first of these experiments, one seven day old culture of Datura stramonium root tissue was divided into four portions of three hundred milligrams each. These four portions were then treated in the following manner. Two of the portions were placed into separate beakers, each beaker containing ten microcuries of tryptophan-3-C^{14}. The remaining two portions were similarly placed into solutions of phenylalanine-3-C^{14}. The four beakers were then placed in a bell-jar. The air in the bell-jar was evacuated until bubbles of gas formed in abundance in the solutions contained within the four beakers. The tissue was left inside the bell-jar under this partial vacuum for a period of fifteen minutes. Thirty minutes after removal from the bell-jar, one beaker of tissue containing tryptophan-3-C^{14} and one beaker of tissue containing phenylalanine-3-C^{14} were emptied into separate glass mortars. These tissues were then sacrificed, extracted, and chromatographed as previously described. The remaining two portions of tissue were treated in a similar fashion after having been allowed to metabolize the radio-active material for one hundred and eighty minutes following removal from the bell-jar.

The second experiment involving the use of the vacuum infiltration procedure was done in exactly the same manner. This time the material infiltrated was five microcuries of indoleacetic acid-2-C^{14}. The times allowed for metabolism in this instance were fifteen minutes, thirty minutes, sixty minutes, and one hundred and twenty minutes.

Data on the two vacuum infiltration experiments can be found in table 2.

TABLE 1.

DATA ON THE ROOT TISSUE CULTURE FEEDING EXPERIMENTS

ISOTOPE	LEVEL ADMINISTERED	METABOLISM TIME (days)	LEVEL OF ACTIVITY CHROMATOGRAPHED	EXPOSURE OF AUTO-RADIOGRAMS (days)	
PHENYLALANINE-3-C^{14}	10 uc	7	1700 cpm	10	17
	5 uc	3	500 cpm	30	90
	5 uc	6	500 cpm	30	90
	5 uc	9	500 cpm	30	90
	5 uc	12	500 cpm	30	90
TRYPTOPHAN-3-C^{14}	10 uc	7	1500 cpm	10	17
	5 uc	3	500 cpm	19	90
	5 uc	6	500 cpm	30	90
	5 uc	9	500 cpm	30	90
	5 uc	12	500 cpm	30	90
	5 uc	21	1500 cpm	30	90
INDOLEACETIC ACID-2-C^{14}	5 uc	3	500 cpm	30	90
	5 uc	6	500 cpm	30	90
	5 uc	9	500 cpm	30	90
	5 uc	12	500 cpm	30	90
PHENYLALANINE-C^{14}(ring Labelled)	5 uc	3	500 cpm	60	90
	5 uc	6	500 cpm	60	90
TRYPTOPHAN-(2-INDOLYL)-C^{14}	5 uc	21	1000 cpm	30	—

Note: The level of activity chromatographed was determined by means of a thin window Geiger Tube in direct contact with the chromatogram.

TABLE II.

DATA ON THE VACUUM INFILTRATION EXPERIMENTS

ISOTOPE	LEVEL ADMINISTERED	METABOLISM TIME (minutes)	LEVEL OF ACTIVITY CHROMATOGRAPHED	EXPOSURE OF AUTO-RADIOGRAMS (days)	
TRYPTOPHAN-3-C^{14}	10 uc	30	1300 cpm	7	18
	10 uc	180	1400 cpm	7	18
PHENYLALANINE-3-C^{14}	10 uc	30	1400 cpm	7	18
	10 uc	180	1500 cpm	7	18
INDOLEACETIC ACID-2-C^{14}	5 uc	15	6000 cpm	15	37
	5 uc	30	3000 cpm	15	37
	5 uc	60	4600 cpm	15	37
	5 uc	120	3000 cpm	15	37

Note: The level of activity chromatographed was determined by means of a thin window Geiger Tube in direct contact with the chromatogram.

The following materials were used as reference compounds to aid in the identification of spots which appeared on the auto-radiograms:

Organic acids:

γ-amino butyric
2-amino-3-phenyl butanoic
amygdalic
atrolactic
atropic
benzoic
caffeic
chlorogenic
cinnamic
ferulic
glutaric
glycolic
homogentisic
p-hydroxycinnamic
5-hydroxyindoleacetic
p-hydroxyphenylpyruvic
indole-3-acetic
indole-3-DL lactic
indole-3-propionic
kynurenic
malonic
oxalic
phenylacetic
α-phenyl-β-amino propionic
phenyllactic
phloretic
potassium fumarate
pyruvic
quinaldic
D-quinic
shikimic
sinapic
sodium lactate
succinic
tropic
vanillic

Amino acids:

β-alanine
L-alanine
L-arginine hydrochloride
aspartic acid
L-cystine
2,4-dihydroxyphenylalanine
L-glutamic acid
glycine
L-histidine
L-histidine hydrochloride
L-isoleucine
L-leucine
L-lysine hydrochloride
DL-methionine
DL-ornithine hydrochloride
phenylalanine
L-proline
DL-serine
DL-threonone
tryptophan
L-tyrosine
DL-valine

Miscellaneous compounds:

DL-alaninol hydrochloride
atropine sulfate
benzyaldehyde
benzyl alcohol
hydratropaldehyde
hyoscine hydrochloride
hyoscyamine
3-indol aldehyde
indole
indole acetonitrile
indolyl-3-acetamide
phenethyl alcohol
phenethylamine
phenylalamine
putrescine chloride
tryptamine hydrochloride

The above reference compounds were dissolved in ethanol to produce a solution of ten milligrams per milliliter concentration where possible. Compounds which had solubilities in ethanol lower that this value were dissolved in other suitable organic solvents. These reference solutions were then spotted on chromatography paper in the same manner as the plant extracts previously mentioned. The amount used for spotting the chromatograms was fifteen microliters. Visualization of the reference compounds on the chromatograms was accomplished using bromphenol blue reagent for the organic acids, ninhydrin-acetic acid reagent for amino acids and amines, sulphanilic acid reagent and iodine reagent for the miscellaneous compounds. All of these reagents were prepared according to Smith[22].

Atropic acid and α-phenyl-β-amino propionic acid were the only two reference compounds not obtained from chemical manufacturers. These two compounds were synthesized in the laboratory in the following manner:

Atrolactic acid was converted to atropic acid by distillation under reduced pressure according to the method of McKenzie and Wood[23]. At a pressure of ten to fifteen millimeters of mercury, five hundred milligrams of atrolactic acid were distilled. The vapour distilled at one hundred and eighty degrees centigrade and quickly condensed to form a white crystalline solid. This solid was dissolved in warm seventy per cent ethanol and hot water was added to this solution until it became turbid. Upon cooling crystals of atropic acid separated. These had a melting point of one hundred six to one hundred seven degrees centigrade which agrees with the value quoted in the literature[23]. The yield was approximately two hundred milligrams.

α-phenyl-β-amino propionic acid was synthesized by a method outlined by Mannich and Ganz[24]. Sixty grams of diethylphenylmalonate were hydrolyzed by heating at reflux temperature with two hundred milliliters of a fifteen per cent aqueous sodium hydroxide solution for two hours. The material was then placed in an ice bath until it began to freeze and cautiously neutralized with concentrated hydrochloric acid. The mixture was made slightly acidic and extracted three times with ether. The ether was allowed to evaporate spontaneously, leaving behind crystals of phenylmalonic acid. Twenty-seven grams of phenylmalonic acid were chilled in an ice bath and made faintly alkaline with a twenty-eight per cent solution of ammonium hydroxide. A further twenty-seven grams of phenylmalonic acid were then added and the mixture was stirred until it was homogenous. Thirty milliliters of a thirty-three per cent formaldehyde solution were next added to the mixture which was then stirred for fifteen minutes at zero degrees centigrade. The mixture was allowed to stand at room temperature. Decarboxylation began after thirty minutes and crystals began to separate after eighteen hours. The The reaction continued for seventy-two hours. Following this period the crystals of α-phenyl-β-amino propionic acid were filtered off and recrystalized in ninety-five per cent ethanol. The yield was approximately fifteen grams of recrystalized material. The observed melting point was two hundred and six to two hundred and ten degrees centigrade. The melting point range found in the literature was two hundred and two to two hundred and twelve degrees centigrade[24].

Two methods of degradation were employed on the sample of radio-active tropic acid produced in the tryptophan-(2-indolyl)-C^{14} twenty-one day feeding experiment to determine the position of the C^{14} label. The sample of tropic acid was eluted from the chromatogram by soxhlet extraction with ether for six hours. The ether was then removed and two hundred milligrams of cold carrier tropic acid were added. Sufficient absolute alcohol was added to the sample of tropic acid to produce twenty milliliters of solution. This solution was then divided into two equal portions, and both were evaporated to dryness. Decarboxylation of one of the two samples of tropic acid was carried out according to the method outlined by Walling and Wolfstirn[27]. Ten milligrams of fine copper dust and one milliliter of quinoline were added to the sample of tropic acid. The mixture was then heated to two hundred and fifteen degrees centigrade for a period of one hour. The carbon dioxide

which was liberated was trapped in a tenth molar solution of hyamine 10-X manufactured by Rohm and Haas. The styrene produced by the decarboxylation of tropic acid was recovered by acidifying the contents of the reaction flask with dilute hydrochloric acid and extracting this solution with several portions of ether. The combined ether extract was washed several times, first with acidified water and finally with dilute sodium hydroxide. The ether was then evaporated to dryness, leaving behind a light brown residue of styrene. The styrene isolated was then weighed in order to determine the percentage yield of the reaction.

The second sample of tropic acid was oxidized to benzoic acid by the method described by Underhill and Youngken Jr.[16]. The tropic acid was refluxed with five hundred milligrams of potassium permanganate and one hundred milligrams of sodium hydroxide in ten milliliters of water for two hours. The hot solution was filtered, acidified with dilute hydrochloric acid, and extracted with several portions of ether. The ether was removed and the residue was subjected to sublimation which produced pure benzoic acid. The melting point of the benzoic acid produced was one hundred and twenty-two degrees centigrade. This agrees with that given by Underhill and Youngken Jr.[16]. The benzoic acid isolated was accurately weighed to determine the percentage yield of the reaction.

The results of these degradation studies and the percentage of C^{14} label at carbon atoms number one, three, and on the benzoic acid portion of the molecule of tropic acid are shown in table X.

Control procedures for this investigation included the chromatography of each radio-active compound to establish its purity, unless such information was already supplied by the manufacturer, as well as the chromatography of each radio-active compound after it had been autoclaved with a small amount of White's liquid nutrient medium for thirty minutes at one hundred and twenty-one degrees centigrade and fifteen pounds pressure. In all cases the radio-active compounds proved to be radio-chemically pure.

Extracts were made of the culture media by acidifying it slightly with hydrochloric acid and then extracting with ether. The ether solution was evaporated to dryness. The dry residue was dissolved in a small amount of ethanol and treated in the same manner as the extracts of the root tissue cultures. It was found that while small amounts of materials were secreted into the medium, these same materials were contained in the extract of the root tissue in greater abundance. Further investigation into the matter of excretion into the medium was therefore not made.

RESULTS

The results of the work done on both the plant tissue culture feeding experiments as well as that done on the vacuum infiltration experiments are summarized in tables III to VII. Any compounds which are listed under the list of reference compounds but not mentioned in these tables were not identified in any of the tissue extracts. Failure of these compounds to appear in the tissue extracts may be due to the fact that; they are not metabolites of the radio-isotope administered, the concentration of these compounds in the tissue is not sufficient for detection, or the solubility in ethanol under the conditions of extraction is not sufficiently high to allow adequate removal from the tissue.

Table VIII is a review of the solvent systems used in this series of experiments. Identification of all of the compounds listed in tables III to VII was made by chromatographing the plant extract and the corresponding reference compound in solvent system number one. Further substantiation of the identity of these compounds was made by eluting the radio-active spot with ethanol in a soxhlet apparatus and re-chromatographing this extract with the reference compound in either solvent system number two or three. The results of this work are shown in table IX.

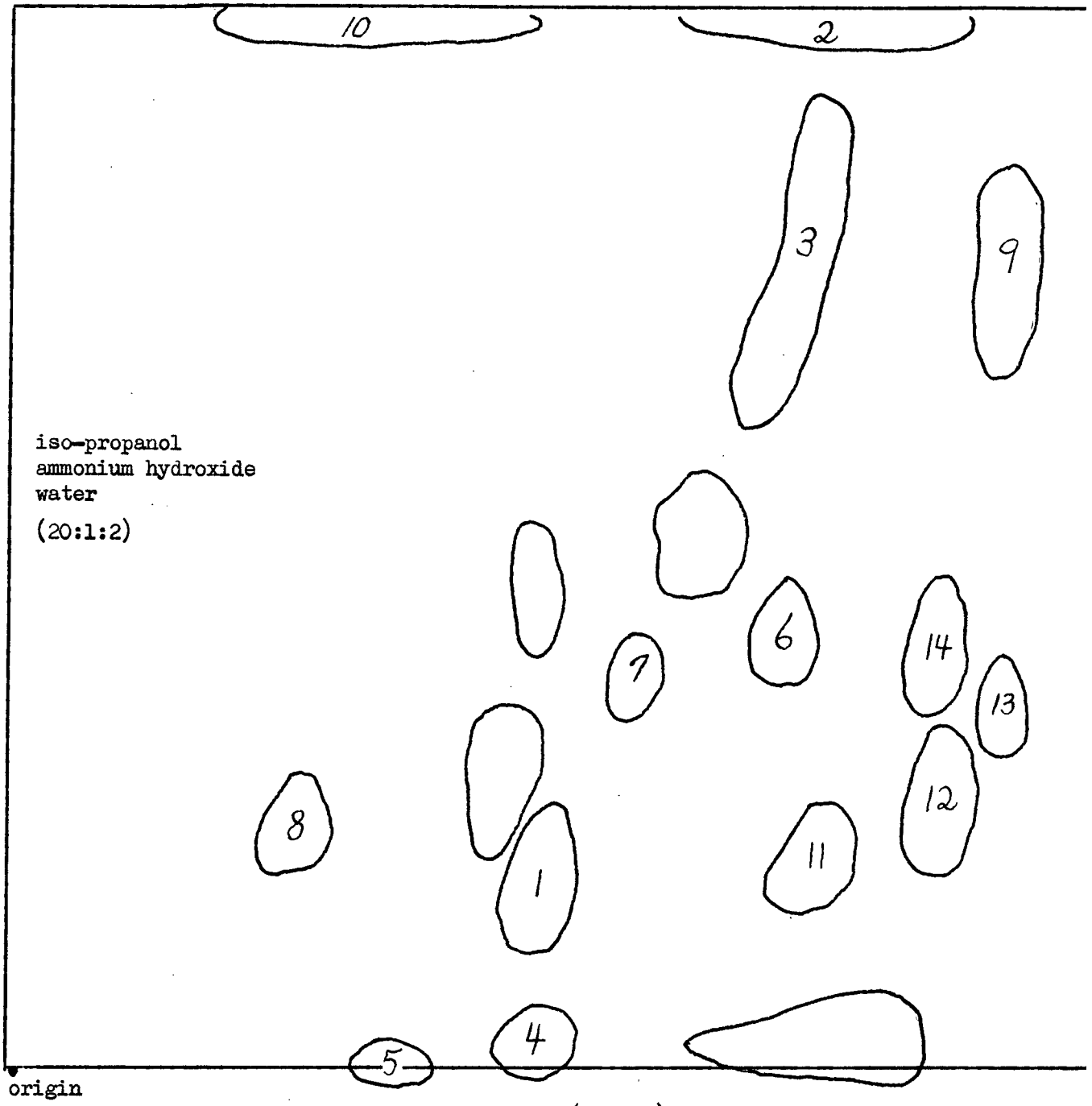
TABLE III.

RESULTS OF THE TRYPTOPHAN-3-C^{14} FEEDING AND INFILTRATION EXPERIMENTS

COMPOUND ANTICIPATED	LENGTH OF FEEDING (days)						METABOLISM TIME AFTER INFILTRATION (minutes)	
	3	6	7	9	12	21	30	180
tryptophan	+	+	+	+	+	+	+	+
tryptamine	-	+	+	+	+	+	-	+
indole-3-aldehyde	-	-	+	+	+	+	-	+
indole acetonitrile	-	-	-	-	-	-	-	-
kynurenine	+	+	+	+	+	+	+	+
alanine	-	+	+	+	+	+	+	+
kynurenic acid	-	-	+	+	+	+	-	+
quinaldic acid	-	-	+	+	+	+	-	+
skatole	-	-	-	-	-	-	-	+
α-phenyl-β-amino propionic acid	-	-	+	+	+	+	-	-
atropic acid	-	-	+	+	+	+	+	+
tropic acid	-	-	-	-	-	+	-	-
phenylalanine	-	-	-	-	-	-	-	-
tryptophanol	-	-	+	+	+	+	+	+
hyoscyamine	-	-	-	-	-	-	-	-
indole-3-DL lactic acid	+	+	+	+	+	+	+	+
indole-3-acetic acid	-	-	-	-	+	+	+	+
indole-3-propionic acid	-	-	-	-	-	+	-	+
5-hydroxyindole acetic acid	-	-	-	-	-	-	-	-
indole-3-acetamide	-	-	-	-	-	-	-	-
lactic acid	-	-	-	-	-	-	-	-

FIGURE 3.

DIAGRAM OF TYPICAL AUTORADIOGRAM FROM TRYPTOPHAN-3-C^{14} FEEDING



Note: see following page for identification of compounds.

FIGURE 3. (continued)

COMPOUND NUMBER	IDENTITY
1	tryptophan
2	tryptamine
3	indole-3-aldehyde
4	kynurenine
5	alanine
6	quinaldic acid
7	kynurenic acid
8	α-phenyl-β-amino propionic acid
9	atropic acid
10	tryptophanol
11	indole-3-lactic acid
12	indole-3-acetic acid
13	indole-3-propionic acid

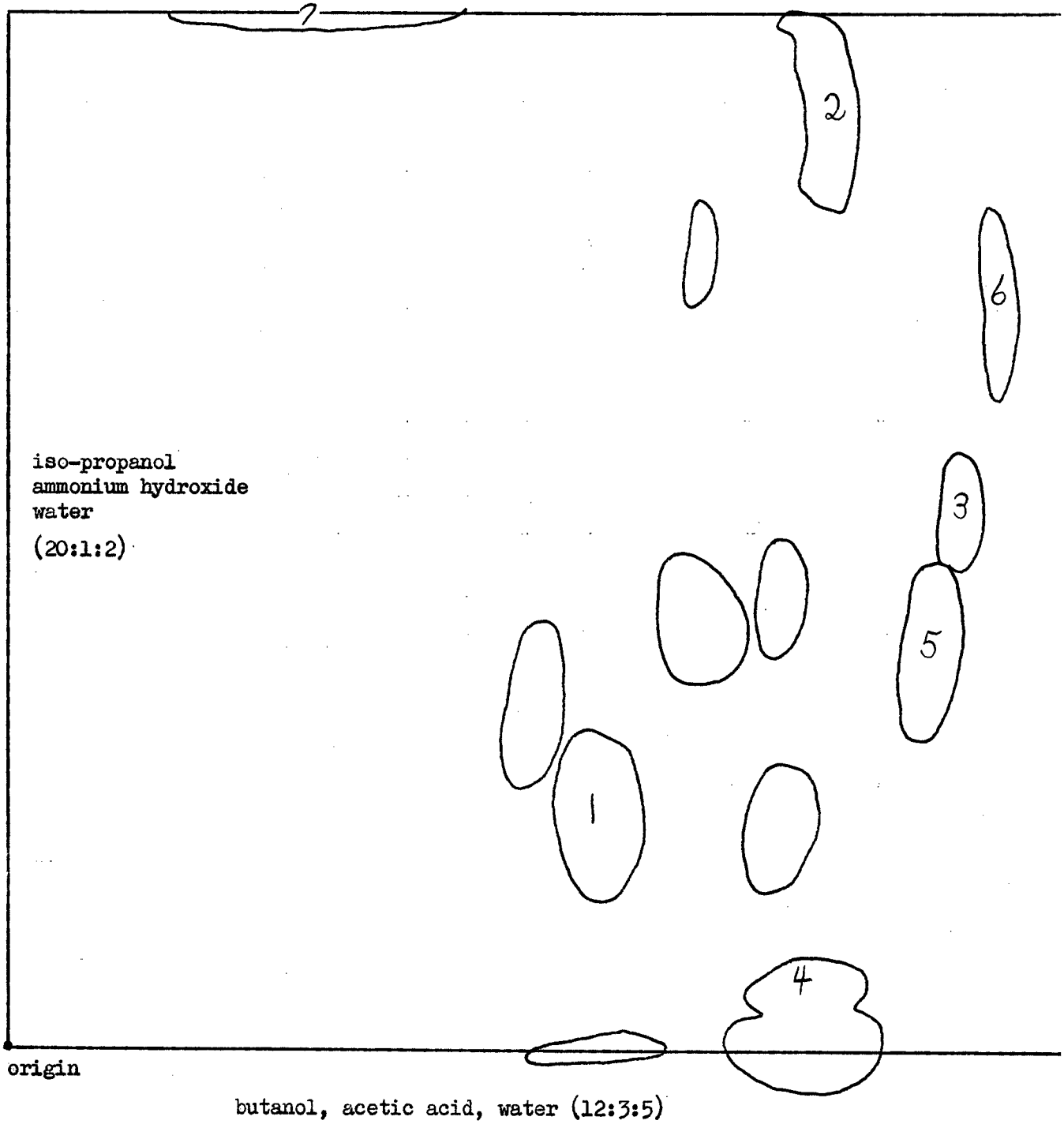
TABLE IV.

RESULTS OF THE PHENYLALANINE-3-C^{14} FEEDING AND INFILTRATION EXPERIMENTS

COMPOUND ANTICIPATED	LENGTH OF FEEDING (days)					METABOLISM TIME AFTER INFILTRATION (minutes)	
	3	6	7	9	12	30	180
phenylalanine	+	+	+	+	+	+	+
phenethylamine	-	+	+	+	+	-	+
β-phenyllactic acid	-	-	-	-	-	-	-
benzoic acid	-	+	+	+	+	+	+
tyrosine	-	-	-	-	-	-	-
p-hydroxyphenyl-pyruvic acid	-	-	-	-	-	-	-
fumaric acid	+	+	+	+	+	+	+
tropic acid	+	+	+	+	+	+	+
atropic acid	-	-	-	-	-	-	-
phenylacetic acid	-	-	+	+	+	+	+
mandelic acid	-	-	-	-	-	-	-
succinic acid	-	-	-	-	-	-	-
α-phenyl-β-amino propionic acid	-	-	-	-	-	-	-
tryptophan	-	-	-	-	-	-	-
hyoscyamine	-	-	-	+	+	±	+
phenethyl alcohol	-	-	-	-	-	-	-
benzyl alcohol	-	-	-	-	-	-	-
benzaldehyde	-	-	-	-	-	-	-
lactic acid	-	-	-	-	-	-	-

FIGURE 4.

DIAGRAM OF TYPICAL AUTORADIOGRAM FROM PHENYLALANINE-3-C^{14} FEEDING



Note: see following page for identification of compounds

FIGURE 4. (continued)

COMPOUND NUMBER	IDENTITY
1	phenylalanine
2	phenethylamine
3	benzoic acid
4	fumaric acid
5	tropic acid
6	phenylacetic acid
7	hyoscyamine

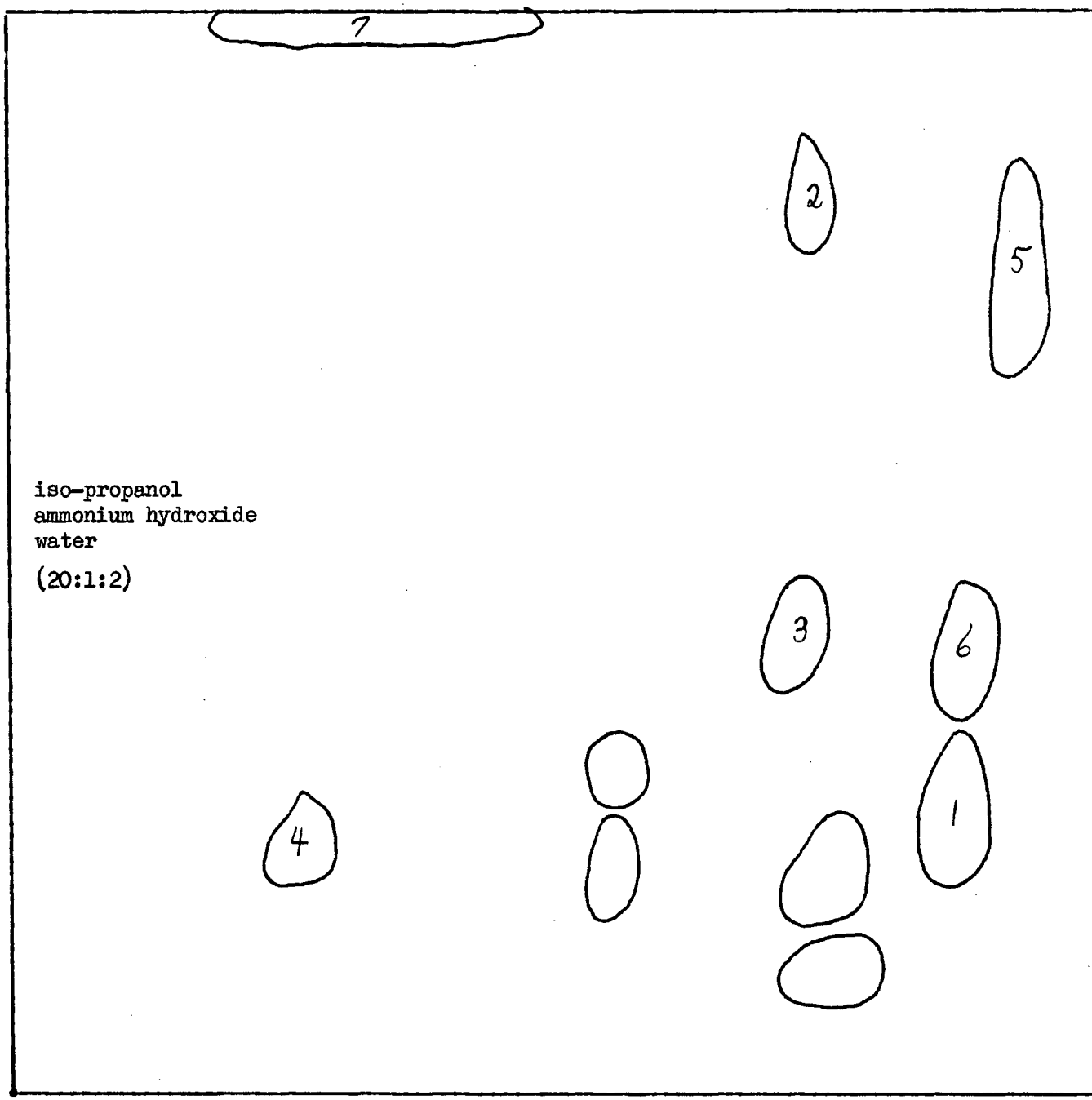
TABLE V.

RESULTS OF THE INDOLE ACETIC ACID-2-C^{14} FEEDING AND INFILTRATION EXPERIMENTS

COMPOUND ANTICIPATED	LENGTH OF FEEDING (days)				METABOLISM TIME AFTER INFILTRATION (minutes)			
	3	6	9	12	15	30	60	120
indole-3-acetic acid	+	+	+	+	+	+	+	+
indole-3-aldehyde	+	+	+	+	+	+	+	+
indoleacetonitrile	-	-	-	-	-	-	-	-
kynurenine	-	-	-	-	-	-	-	-
alanine	-	-	-	-	-	-	-	-
kynurenic acid	-	-	-	-	-	-	-	-
quinaldic acid	-	-	-	-	-	-	-	-
skatole	-	-	-	-	-	-	-	-
α-phenyl-β-amino propionic acid	-	-	-	-	-	-	-	-
atropic acid	-	+	+	+	+	+	+	+
tropic acid	-	-	+	+	+	+	+	+
hyoscyamine	-	-	-	+	+	+	+	+
5-hydroxyindole-acetic acid	-	-	-	-	-	-	-	-
indole-3-acetamide	-	-	-	-	-	-	-	-

FIGURE 5.

DIAGRAM OF TYPICAL AUTORADIOGRAM FROM INDOLEACETIC ACID-2-C^{14} FEEDING



Note: see following page for identification of compounds.

FIGURE 5.(continued)

COMPOUND NUMBER	IDENTITY
1	indoleacetic acid
2	indole-3-aldehyde
3	quinaldic acid
4	α-phenyl-β-amino propionic acid
5	atropic acid
6	tropic acid
7	hyoscyamine

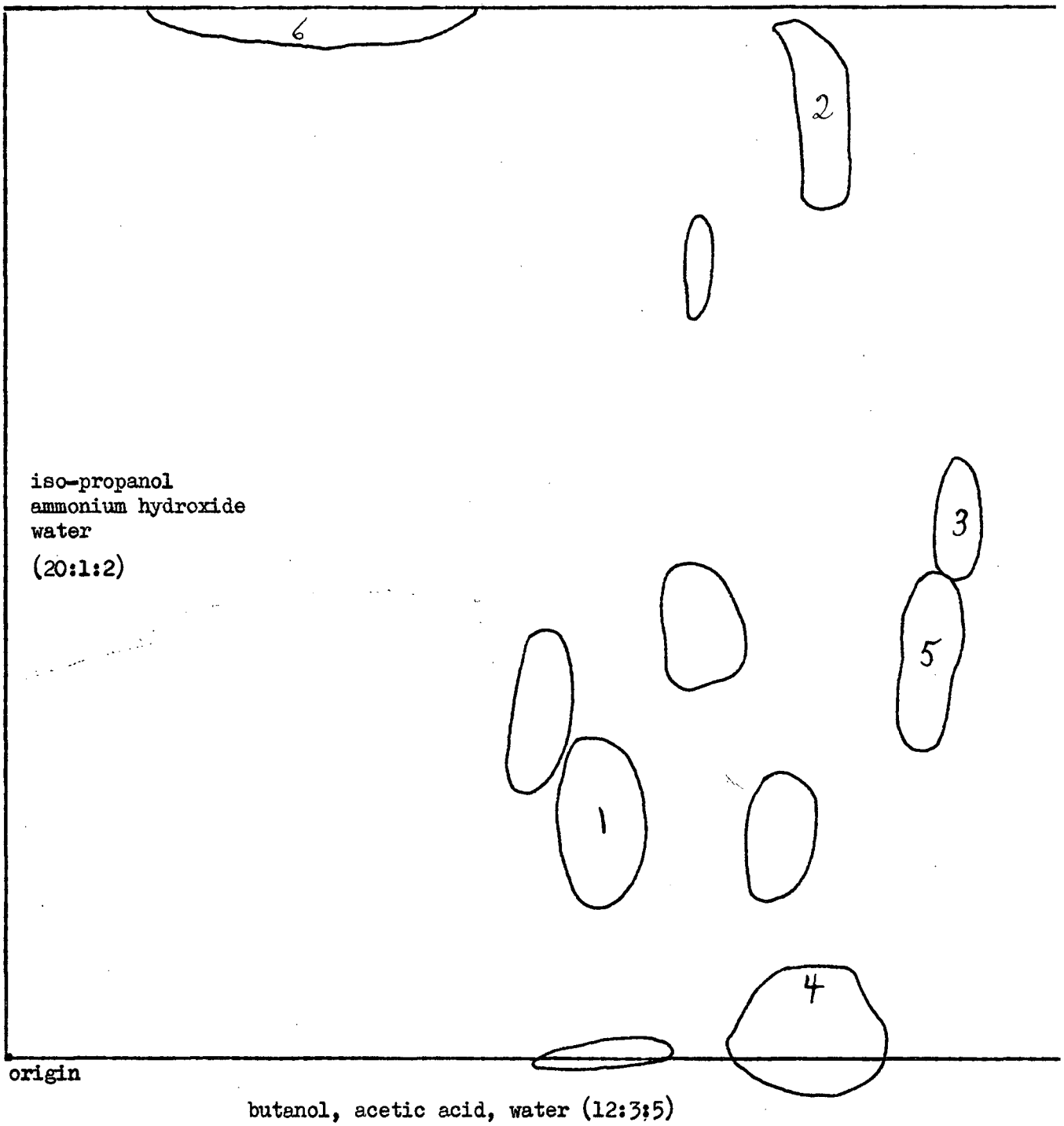
TABLE VI.

RESULTS OF THE UNIFORMLY RING LABELLED PHENYLALANINE-C^{14} FEEDING EXPERIMENTS

COMPOUND ANTICIPATED	LENGTH OF FEEDING (days) 3	6
phenylalanine	+	+
phenethylamine	+	+
phenyllactic acid	-	-
benzoic acid	+	+
tyrosine	-	-
p-hydroxyphenylpyruvic acid	-	-
fumaric acid	+	+
tropic acid	+	+
atropic acid	-	-
phenylacetic acid	-	-
mandelic acid	-	-
succinic acid	-	-
α-phenyl-β-amino propionic acid	-	-
tryptophan	-	-
hyoscyamine	-	+
phenethyl alcohol	-	-
benzyl alcohol	-	-
benzaldehyde	-	-
lactic acid	-	-

FIGURE 6.

DIAGRAM OF TYPICAL AUTORADIOGRAM FROM UNIFORMLY RING LABELLED PHENYLALANINE-C^{14} FEEDING



Note: see following page for identification of compounds.

FIGURE 6. (continued)

COMPOUND NUMBER	IDENTITY
1	phenylalanine
2	phenethylamine
3	benzoic acid
4	fumaric acid
5	tropic acid
6	hyoscyamine

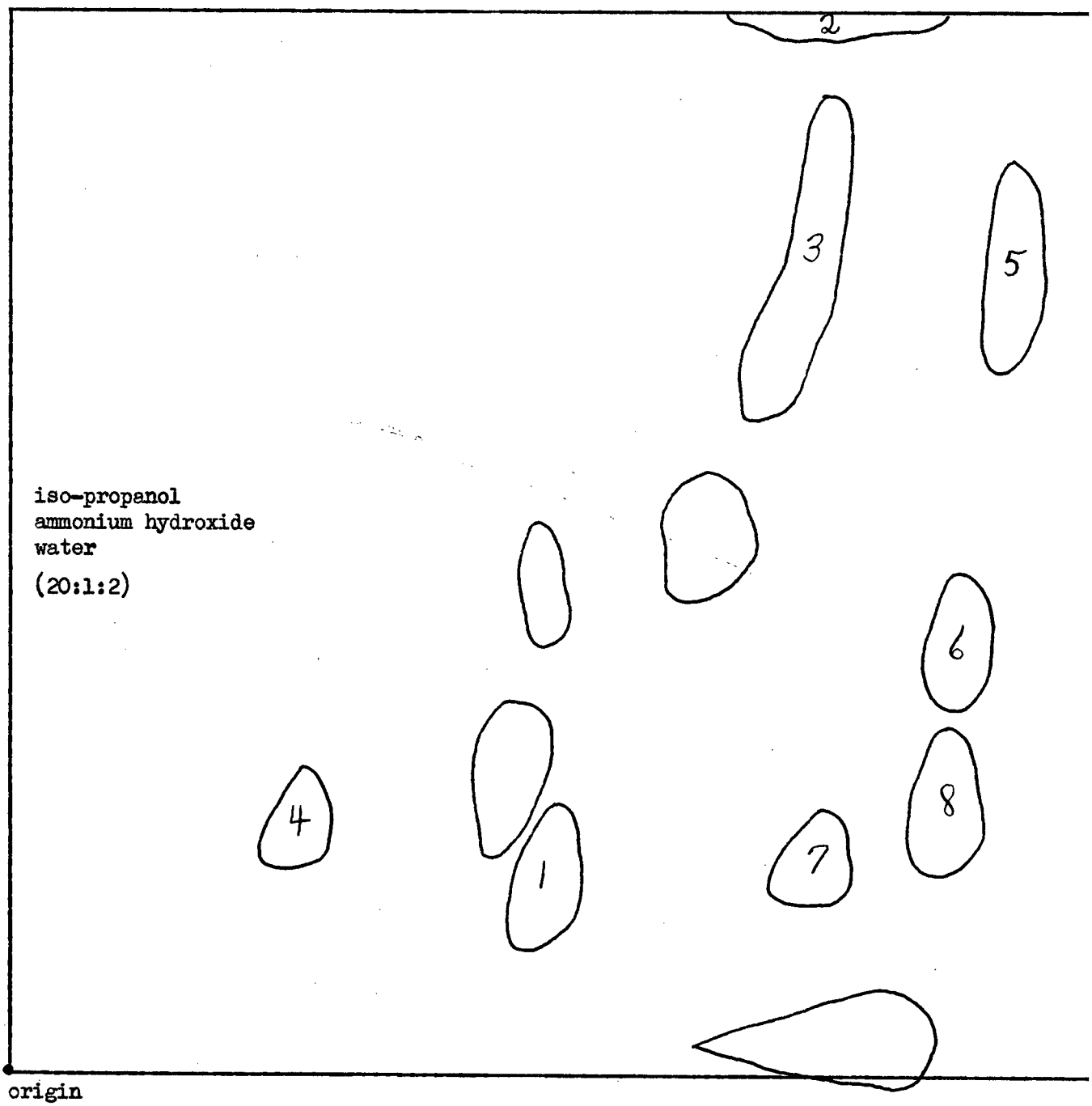
TABLE VII.

RESULTS OF THE TRYPTOPHAN-(2-INDOLYL)-C^{14} FEEDING EXPERIMENT

COMPOUND ANTICIPATED	LENGTH OF FEEDING (days) 21
tryptophan	+
tryptamine	+
indole-3-aldehyde	+
indole acetonitrile	-
alanine	-
skatole	-
α-phenyl-β-amino propionic acid	+
atropic acid	+
tropic acid	+
phenylalanine	-
tryptophanol	-
hyoscyamine	-
indole-3-DL lactic acid	+
indole-3-acetic acid	+
indole-3-propionic acid	-
5-hydroxyindole acetic acid	-
indole-3-acetamide	-
lactic acid	-

FIGURE 7.

DIAGRAM OF TYPICAL AUTORADIOGRAM FROM TRYPTOPHAN-(2-INDOLYL)-C^{14} FEEDING



Note: see following page for identification of compounds.

FIGURE 7. (continued)

COMPOUND NUMBER	IDENTITY
1	tryptophan
2	tryptamine
3	indole-3-aldehyde
4	α-phenyl-β-amino propionic acid
5	atropic acid
6	tropic acid
7	indole-3-lactic acid
8	indoleacetic acid

TABLE VIII.

SOLVENT SYSTEMS EMPLOYED

SOLVENT SYSTEM NUMBER	VERTICAL SOLVENT		HORIZONTAL SOLVENT	
1.	iso-propanol	200	butanol	120
	ammonium hydroxide (28%)	10	acetic acid (glacial)	30
	water	20	water	50
2.	ethanol	16	n-propanol	5
	ammonium hydroxide (28%)	1	eucalptol	5
	water	3	formic acid	2
			water	sufficient to saturate
3.	butanol	120	butanol	7
	acetic acid (glacial)	30	1N sodium acetate	120
	water	50	1N hydrochloric acid	60

TABLE IX.

CONFIRMATION OF THE IDENTIFICATION OF SELECTED COMPOUNDS FROM TABLES THREE TO SEVEN

TENTATIVE IDENTIFICATION FROM SOLVENT SYSTEM #1	SOURCE OF THE COMPOUND	SOLVENT SYSTEM FOR RE-CHROMATOGRAPH	CONFIRMATION
α-phenyl-β-amino propionic acid	tryptophan-3-C^{14} seven day feeding	2	+
tropic acid	indole acetic acid-2-C^{14} 120 minute infiltration	2	+
hyoscyamine	indole acetic acid-2-C^{14} 30 minute infiltration	3	+
fumaric acid	phenylalanine-3-C^{14} seven day feeding	2	+
fumaric acid	uniformly ring labelled phenylalanine-C^{14} six day feeding	2	+
lactic acid	tryptophan-3-C^{14} seven day feeding	2	-
lactic acid	phenylalanine-3-C^{14} seven day feeding	2	-
phenylacetic acid	phenylalanine-3-C^{14} twelve day feeding	2	+
tropic acid	tryptophan-(2-indolyl)-C^{14} twenty-one day feeding	2	+
atropic acid	tryptophan-(2-indolyl)-C^{14} twenty-one day feeding	2	+

TABLE X

DISTRIBUTION OF THE C^{14}-LABEL IN THE DEGRADATION PRODUCTS OF TROPIC ACID DERIVED FROM THE TRYPTOPHAN-(2-INDOLYL)-C^{14} FEEDING

COMPOUND	% YIELD OF THE REACTION	COUNTS PER MINUTE	COUNTS PER MINUTE CORRECTED TO 100% YIELD
carbon dioxide	76.87	52.5	68.25
styrene	76.87	65.0	84.5
benzoic acid	86.4	0.0	0.0

Note: The number of counts per minute was corrected to compensate for background radiation and for the efficiency of the apparatus. The scintillation fluid used was Liquiflour* and the samples were counted using a Nuclear-Chicago Unilux liquid scintillation counter.

* Liquiflour is the trade name for a PPO:POPOP:toluene mixture manufactured by Pilot Chemicals, Inc.

DISCUSSION OF THE RESULTS

The results shown in tables three to seven indicate that there are no intermediates common to both pathways previously outlined for the biogenesis of tropic acid. This does not however, eliminate the possibility of producing radioactive tropic acid from tryptophan-3-C^{14}, indoleacetic acid-2-C^{14}, or tryptophan-(2-indolyl)-C^{14} by participation of only the C^{14} -carbon atom in the form of a bicarbonate ion. This could be produced by the degradation of the labelled precursor. Leete and Louden[25] have shown that phenylalanine-1-C^{14} is incorporated into tropic acid, and that the label is almost entirely on the carboxy carbon of tropic acid. The mechanism proposed by these workers is that of an intra-molecular shift, where carbon atom number one of the phenylalanine moves up the chain and becomes attached to carbon atom number three, thus producing a 2-phenyl propionic acid structure. The net result of this reaction is the same as that found in red clover by Grisebach and Doerr[26]. The biosynthesis of 7-hydroxy-4'-methoxy-isoflavone in red clover apparently involves an identical shift of the side chain of phenylalanine to give a 2-phenyl propionic acid structure. There is no evidence to support the participation of a bicarbonate ion in this reaction in red clover, however, it is interesting to note that this type of shift has been found in a plant other than Datura stramonium even if the actual mechanisms should prove to be different. The reaction in Datura stramonium can apparently utilize carbon dioxide or bicarbonate ion from sources other than carbon atom number one of phenylalanine. This is shown by the fact that phenlyacetic acid-2-C^{14} is an efficient precursor of tropic acid. It is possible therefore that bicarbonate produced from the degradation of tryptophan-3-C^{14}, indoleacetic acid-2-C^{14}, (by side chain oxidation), and tryptophan-(2-indolyl)-C^{14}, (by conversion of N-formy kynurenine to kynurenine), could be incorporated into the carboxy carbon of tropic acid even though the bulk of the tropic acid atom would have been derived from phenylalanine. If this supposition were true, then feeding sodium becarbonate-C^{14} should give rise to radioactive tropic acid with the activity concentrated largely in the carboxy carbon. Leete and Louden[25] have also used this material in their feeding experiments and they found that the tropic acid produced using sodium bicarbonate-C^{14} as precursor was randomly labelled rather than carboxy labelled. Because of this, one is led to the conclusion that since tryptophan-3-C^{14}, as shown by Goodeve[14], labelled only the carboxy carbon of tropic acid

it must act not via a bicarbonate ion but rather by a unique and independent pathway as illustrated by Goodeve in figure one.

The results of the tryptophan-3-C^{14} feeding experiment are shown in table number three. All of the compounds found in the pathway shown in figure one are found in this table with the exception of indolepyruvic acid. Indolepyruvic acid, as well as being somewhat unstable, is also a very labile compound metabolically. Because of these two factors it is not surprising that it does not appear in the extract. The sequence of appearance of the intermediates of this pathway generally follows that outlined by Goodeve in figure one. The only compound which appears to be out of sequence is indoleacetic acid. It is probable that indoleacetic acid would remain at quite a low and carefully regulated level in root tissue since it has hormonal activity and generally inhibits the growth of root tissue. Quite likely the formation of indoleacetic acid is the rate determining step in the biogenesis of tropic acid from tryptophan. When indoleacetic acid-2-C^{14} is fed, tropic acid appears labelled after only nine days compared to twenty-one days when tryptophan-3-C^{14} is used as the precursor. The sequence of the appearance of intermediates between indoleacetic acid and tropic acid is quite clearly shown in table five. Indole-3-aldehyde appears after three days of metabolism, α-phenyl-β-amino propionic acid and atropic acid both appear on the sixth day, and tropic acid finally appears on the ninth day. All of these are in the sequence shown in figure one. The results of the twenty-one day feeding experiment using tryptophan-(2-indolyl)-C^{14} shown in table seven, indicate that the same radioactive intermediates between tryptophan and tropic acid are formed with this compound as had previously been found with the tryptophan-3-C^{14} twenty-one day feeding experiment. Conventional cleavage of the indole nucleus of tryptophan would result in the removal of carbon atom number two of the indole nucleus in the form of formate. Since it is this carbon which carries the C^{14} label in tryptophan-(2-indolyl)-C^{14}, it was particularly valuable to feed this material and to see that the label was retained in tropic acid. It would be anticipated that this material would give rise to tropic acid specifically labelled in the hydroxymethyl carbon.

Both the phenylalanine-3-C^{14} and the uniformly ring labelled phenylalanine-C^{14} feeding experiments shown in tables four and six respectively, indicate that tropic acid is synthesized within three days. The amount of isotope ut-

ilized for tropic acid production is estimated at approximately thirty per cent, as judged by the intensity of the spot on the autoradiogram. It is evident that the ring of phenylalanine is incorporated into tropic acid as well as the side chain. The speed with which the root tissue forms labelled tropic acid from the ring labelled phenylalanine would indicate that the C^{14} is incorporated without degradation of the phenyl ring. The mechanism for the biogenesis of tropic acid from phenylalanine as shown by Underhill and Youngken Jr. in figure two may well be correct in essence. Certainly the scheme is difficult to prove without the isolation of the actual enzymes involved since most of the intermediates would remain bound to the enzyme or would be unstable and difficult to isolate. The only discrepancy in this scheme is the fact that the carbon atom number one of phenylalanine is shown to be given off as carbon dioxide. The results of Leete and Louden[25] indicate that this is not the case since carbon atom number one of phenylalanine is incorporated into the carboxy group of tropic acid. This incorporation was found to be higher than that which would be expected if this carbon atom were cleaved and allowed to enter the general metabolic pool of one carbon fragments and subsequently recovered. It would seem therefore that the mechanism of this reaction is indeed that of an intra-molecular shift where carbon atom number one of phenylalanine is not lost but rather retained to form the carboxy carbon of tropic acid. It is interesting to notice that phenylacetic acid, shown as an alternate precursor for tropic acid in figure two, is not produced from phenylalanine-3-C^{14} until the seventh day. This acid therefore cannot be considered to be on the pathway between phenylalanine and tropic acid. The absence of labelled atropic acid in these feeding experiments with phenylalanine offers further circumstantial evidence for the existence of two separate and distinct pathways for the biogenesis of tropic acid in Datura stramonium root tissue.

The results of the feeding experiments show that labelled hyoscyamine appears from three to six days after the biogenesis of labelled tropic acid when phenylalanine-3-C^{14}, indoleacetic acid-2-C^{14}, and uniformly ring labelled phenylalanine-C^{14}, are used as precursors. It would be expected that labelled hyoscyamine would therefore be produced from tryptophan-3-C^{14} and tryptophan-(2-indolyl)-C^{14} if the feeding experiments were continued for a period of twenty-four to twenty-seven days.

The results of degradation studies done on the tropic acid isolated from the tryptophan-(2-indolyl)-C^{14} twenty-one day feeding experiment are shown in table X. These results show that tryptophan-(2-indolyl)-C^{14} gave rise to tropic acid which is not only labelled on carbon atom number three, but also on carbon atom number one, the carboxy carbon. The benzoic acid portion of the molecule had very little if any activity. It was anticipated that tryptophan-(2-indolyl)-C^{14} would produce tropic acid labelled specifically on carbon atom number three. The fact that carbon atom number one carried forty-four percent of the total C^{14} found in the sample of tropic acid is not at all surprising. Judging from the intensity of the radioactive spots on the autoradiograms produced, it can be said that approximately ninety percent of the tryptophan-(2-indolyl)-C^{14} was degraded via the formylkynurenine/kynurenine pathway. This of course gave rise to a free formyl-C^{14} group. First thoughts on this reaction would lead one to believe that this free formyl-C^{14} group should produce uniform labelling of the tropic acid molecule, just as Leete and Louden[25] obtained when they fed bicarbonate-C^{14} to whole plants. The difference of course lies in the fact that isolated root tissue, which was used in these experiments, does not carry on the process of photosynthesis. The work done by Leete and Louden[25] therefore is not comparable, since in their experiments using whole plants, the bicarbonate-C^{14} would be fixed into sugars by photosynthesis. These sugars, especially the trioses and heptoses, serve as direct precursors for the aromatic amino acids. This therefore leads to uniform labelling of both phenylalanine and tryptophan and ultimately to the production of uniformly labelled tropic acid. This is the result obtained by Leete and Louden[25]. The work done with isolated root tissue in this laboratory did lead to quite a different result. Carbon atom number three of the tropic acid is labelled by the scheme outlined by Goodeve in figure one. This accounts for fifty-six percent of the total label found on the tropic acid isolated. Forty-four percent of the C^{14} was found on carbon atom number one and this presumably was due to the free formyl-C^{14} group produced from the tryptophan-(2-indolyl)-C^{14}. This free formyl-C^{14} molecule would participate in the biogenesis of tropic acid from phenylacetic acid and in so doing would specifically label carbon atom number one. It is my suggestion that the tropic acid produced

from tryptophan-(2-indolyl)-C^{14} is really a mixture of tropic acid-1-C^{14}, derived from a phenylacetic acid skeleton with the C^{14} label originating from formate-C^{14}, and of tropic acid-3-C^{14} derived from tryptophan via the pathway outlined by Goodeve.[14] The benzoic acid derived from the degradation of tropic acid carried no radioactivity. This is in complete agreement with the hypothesis given above.

This work done on the position of the C^{14} label in the tropic acid produced from tryptophan-(2-indolyl)-C^{14} offers direct proof for the independent existence of two separate pathways for the biogenesis of tropic acid in Datura stramonium.

CONCLUSIONS

The purpose of this investigation was to determine whether tryptophan and phenylalanine acted as precursors for tropic acid via two distinctly separate mechanisms or whether these amino acids formed some common intermediate which in turn was responsible for the formation of tropic acid. From the results of the work performed in this investigation, it seems that there are indeed two separate mechanisms responsible for the biogenesis of tropic acid in Datura stramonium root tissue.

Judging by the speed at which tropic acid is produced and by the relative amount of tropic acid produced, one would conclude that the pathway from phenylalanine is the more important pathway of the two for the biogenesis of tropic acid. One should bear in mind however, that the results of this investigation are based on what may well be to the plant a highly abnormal situation, that of isolated tissue culture. Caution must certainly be used in applying the results of tissue culture experiments to the whole organism. Although plant tissue culture offers many advantages, especially that of reproducability of experimental conditions, recent investigations show that the individual tissue may not behave in the same way, with the same biochemical and physiological pathways as the entire organism.

The emphasis placed by isolated root tissue on the biogenesis of tropic acid from tryptophan is certainly not great. This low emphasis may well be an artifact produced by the experimental method. The biosynthesis of indoleacetic acid in root tissue does not begin to compare in quantity produced to that of the aerial portions of the plant. This could mean that if tryptophan were fed to intact plants, it would be transported to the aerial portions of the plant for conversion to indoleacetic acid which would then travel basipetally in constant supply to the root tissue. Certainly when indoleacetic acid is fed to root tissue it acts as a far better precursor for tropic acid than the amino acid tryptophan. Further studies on the intact plant may show that tropic acid is one of the major metabolites of indoleacetic acid, and that the biogenesis of tropic acid is the major mechanism for the degradation or detoxification of this plant hormone. In any event, the biogenesis of tropic acid from tryptophan definitely does occur in isolated root tissue.

Two pathways exist for the biogenesis of tropic acid in Datura stramonium

root tissue grown under aseptic conditions in liquid nutrient media. These two pathways, one from tryptophan and the other from phenylalanine, have been shown to be separate, independent, and distinct.

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