UNIVERSITY MICROFILMS, ANN ARBOR, MICH.

L. C. Card no. Mic 58-1016

WHALLEY, Barbara Eloise. ISOLATION, GROWTH, AND DIFFERENTIATION OF TRAGOPOGON TISSUE CULTURES ON DEFINED MEDIA

University of Michigan, Ph. D., 1957
Botany

Negative microfilm received from university.

ISOLATION, GROWTH, AND DIFFERENTIATION OF *TRAGOPOGON* TISSUE CULTURES ON DEFINED MEDIA

by
Barbara E. Whalley

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in the
University of Michigan
1956

Doctoral Committee:

Assistant Professor Alfred S. Sussman, Chairman
Professor Felix G. Gustafson
Associate Professor Robert J. Lowry
Assistant Professor Donald J. Merchant
Assistant Professor Warren H. Wagner, Jr.

ISOLATION, GROWTH, AND DIFFERENTIATION OF *TRAGOPOGON* TISSUE CULTURES ON DEFINED MEDIA

by Barbara E. Whalley

The isolation of *Tragopogon* tissue, its morphology, the development of a defined medium for its growth, and the chemical control of its differentiation in culture form the basis of this dissertation.

Tragopogon was selected because it was one of the plants which produced leaves and roots from root discs; it was therefore suitable for studies concerning the control of differentiation. In addition it had a root sufficiently large to permit the removal of sterile segments.

Continuing cultures of both leaf- and root-derived tissues were established on a modified White's medium in which coconut milk was substituted for 20 percent of the distilled water. The percentage increase in fresh weight was used as a measure of growth and inocula were taken from stock cultures 10 to 20 days old-- that is, in the log phase of growth. Cultures were maintained in the dark at 27°C for 25 days. Under these conditions increases in growth averaging approximately 2000 percent were obtained.

Initially the cultures could not be grown without the addition of coconut milk to the medium. However, by means of the auxanographic technique the types of chemicals

needed for growth were delimited. The Kruskal-Wallis H test, a nonparametric test of significance, was used to assess both simple and interaction effects of the substances investigated. A completely defined medium consisting of a mixture of amino acids and adenine sulphate in addition to the modified White's medium was evolved. Growth obtained on this medium was comparable to that obtained on medium containing coconut milk.

Tissue cultures of both the root and leaf of Tragopogon produced most of the cell types found in the parent plant. At no time, however, did they form organs without the addition of differentiation-inducing factors such as hydrolyzed ribose nucleic acid, adenine sulphate, or diphenyl urea. This is believed to be the first report that diphenyl urea acts as an organizing factor.

After being maintained in culture for several years the root tissue appeared to undergo habituation, although not to indole acetic acid as is the usual case. Habituation resulted in an altered response to diphenyl urea, a compound isolated from coconut milk.

It was found that when a high concentration of tyrosine was used in the medium the formation of the enzyme tyrosinase was induced. The enzyme was extracted and it was shown to act on dihydroxy phenylalanine, a common substrate for this enzyme.

ACKNOWLEDGMENTS

The author wishes to express her indebtedness to the late Professor C. D. LaRue, under whose guidance this study was begun. Professor LaRue's kindly encouragement, his humor, his fund of knowledge, and his love of things botanical provided the inspiration for this study.

The author also wishes to acknowledge her appreciation to Dr. Alfred S. Sussman, who assumed chairmanship of the committee after Dr. LaRue's death. His interest and guidance, his generous and helpful assistance, his astute and critical evaluations shaped the final course of this project.

Special thanks are also extended to James Hardin, Joseph Krakow, Robert Paterson, and Robert Whitmire, who so generously provided photographic equipment used in this study.

Not the least of the author's gratitude is reserved for Bert Forrin, for his introduction to the uses of statistics and for his assistance with the analysis of the data.

Thanks are also due to the members of the committee for their helpful criticisms of the manuscript.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
HISTORICAL REVIEW	4
Use of Coconut Milk	5
Chemical Control of Organ Formation	7
MATERIALS AND METHODS	11
Isolation of the Tissue	11
Preparation of the Basal Medium	12
Measurement of Growth	15
Statistical Methods	16
Nonparametric analysis of variance by ranks	17
Interaction	19
EXPERIMENTAL RESULTS	24
Effect of Environment on Growth and Differentiation in Undefined Media	24
The growth curve	24
Effect of hydrogen ion concentration on growth	24
Morphology of root and leaf tissue grown on 20 percent coconut milk medium	26
The effect of hydrolyzed ribose nucleic acid on differentiation	30

Page

Morphology of leaves and roots formed in culture 36

Effect of hydrolyzed ribose nucleic acid on growth 36

Auxanographic Approach to a Defined Growth Medium 38

Effect of yeast extract 39

Effect of casein hydrolysate 40

Effect of amino acids 43

Effect of Ribose Nucleic Acid Components on Organ Formation 49

Effect of Growth Regulators upon Growth and Differentiation 49

Experiment I 51

Experiment II 55

DISCUSSION 59

SUMMARY 68

LITERATURE CITED 70

APPENDIX I 76

APPENDIX II 77

APPENDIX III 78

APPENDIX IV 80

APPENDIX V 82

APPENDIX VI 84

Page

APPENDIX VII 85

APPENDIX VIII 87

APPENDIX IX 88

LIST OF TABLES

TABLE		Page
I.	Increase in Wet and Dry Weight of Root Tissue	26
II.	Effect of Hydrogen Ion Concentration on Growth	27
III.	Effect of Hydrolyzed RNA on Organ Formation	33
IV.	Effect of Concentration of Hydrolyzed RNA on Growth of Root Tissue	37
V.	Effect of Hydrolyzed RNA in 20 Percent CM Medium on Growth of Root Tissue	38
VI.	Effect of Concentration of Yeast Extract on Growth of Leaf Tissue	39
VII.	Effect of Increasing Concentrations of Casein Hydrolysate (Unsupplemented) on Growth of Root Tissue	41
VIII.	Effect of Increasing Concentrations of Casein Hydrolysate and Ribose Nucleic Acid on Growth of Root Tissue	41
IX.	Effect of Increasing Concentrations of Casein Hydrolysate Plus Cysteine and Tryptophane on Growth of Root Tissue	42
X.	Effect of Increasing Concentrations of Casein Hydrolysate plus Ribose Nucleic Acid plus Cysteine and Tryptophane on Growth of Root Tissue	44

TABLE Page

XI. Effect of Amino Acids on Growth of Root Tissue 45

XII. Effect of Amino Acid Solutions plus Yeast Extract and Ribose Nucleic Acid on Growth of Root Tissue 45

XIII. Composition of Amino Acid Solutions 46

XIV. Differentiation on Media Containing Amino Acids, Ribose Nucleic Acid, and Yeast Extract 48

XV. A Comparison of the Effects of DPU, Kinetin, and Adenine in Two Replications 51

XVI. Simple Effects of DPU, Kinetin, Adenine, and IAA on Growth of Root Tissue 52

XVII. Interaction Effects of DPU, Kinetin, Adenine, and IAA 53

XVIII. Effect of Two Different Concentrations of DPU, Kinetin, and IAA on Growth of Root Tissue 54

XIX. Simple Effects of DPU, Kinetin, Adenine, "B" Solution, and RNA 55

XX. Interaction Effects of DPU, Kinetin, Adenine, "B" Solution, and RNA 57

XXI. Effect of DPU, Kinetin, or RNA in Medium Containing Adenine and "B" Solution 58

XXII. Composition of Optimal Defined Medium, Based on Experiments Reported in Results 61

LIST OF PLATES

PLATE — Page

I. Morphology of Tragopogon Root Tissue Cultures Grown on 20 Percent Coconut Milk Medium 29

II. Morphology of Tragopogon Root Tissue Cultures Grown on 20 Percent Coconut Milk Medium plus Hydrolyzed Ribose Nucleic Acid 32

III. Differentiation of Leaves and Roots from Tragopogon Root Tissue Cultures 35

ILLUSTRATION

Figure | Page

1. The growth of Tragopogon root tissue as a function of time 25

ABBREVIATIONS

CH	Casein hydrolysate
CM	Coconut milk
DPU	1,3-Diphenyl urea
IAA	3-Indoleacetic acid
ns	Not significant
PW	Plain White's

INTRODUCTION

On looking back over the half-century of work which has followed Haberlandt's abortive attempts to grow isolated single cells, the enormity of the problem which he attempted to solve becomes apparent. Not only is there the necessity for a suitable medium, but also there is the necessity for a good choice of experimental plant material. Haberlandt was unfortunate in that he chose to culture green leaf material, thinking that its cells would not require a carbohydrate source.

Subsequent work has shown that the tissues most likely to respond favorably are those from the meristematic regions. This does not mean that tissues from other areas cannot be cultured. In the main, however, it is still not always possible to select a given plant with the certainty of establishing cultures from a given part. This is so despite the development of basic media adequate for the growth of many plant tissues, and the possibility of enhancing or stimulating growth by the addition of growth regulators and metabolites.

Most tissue cultures have been obtained from stems or roots, some from leaves, and a few from other plant parts such as pollen and endosperm. Dicotyledons, whether herbaceous or woody, have been the least refractory, and there is accordingly a long list of these plants reported in culture. Other plant groups have proved more recalcitrant: of the monocotyledons only eight have been reported in culture (16) and success has been even more limited with the gymnosperms and pteridophytes.

The tissue cultures derived from these various sources exhibit important morphological and physiological differences. In the majority of cultures small, scattered clumps of tracheids are formed while other cell types are rare. Only four cultures have been reported in which no cellular differentiation occurs. These are Zea and Asimina, both endosperm-derived; Selaginella from the female gametophyte; and Ginkgo tissue from pollen (47). In marked contrast is the Nicotiana tissue isolated by White (55), which is unique in its ability to produce organs when grown on the surface of solid medium. Because of this special ability to form differentiated cells and to organize them into roots and buds, Skoog and Tsui (43) used this tissue for the study of the chemical control of differentiation. Thus, under conditions which did not ordinarily result in the formation of organs, these could be induced by the addition of a metabolite such as adenine sulphate.

In working with the problems of the chemical control of differentiation the choice of materials is important. It seemed that the use of a tissue other than Nicotiana might be desirable since this tissue responds to other than chemical stimuli and thus a certain amount of nonspecificity of treatment seems to be involved in organ formation. Therefore, one needs to obtain plant material which never produces differentiated organs in culture except in the presence of chemicals which induce their formation. It was perhaps natural, in seaching for a plant with such properties, to select one possessing a propensity for regeneration in its natural environment. The work of Warmke and Warmke (51, 52) demonstrated that root cuttings of Taraxacum regenerated roots and shoots with ease, and Tragopogon, also a member of the Compositae, in the hands of the present investigator demonstrated similar properties and so was chosen for this study. The isolation of the tissue, its morphology,

the development of a synthetic medium, and the chemical control of its differentiation in culture form the basis of this dissertation.

HISTORICAL REVIEW

Since the early literature of plant tissue culture before 1931 and up to 1951 has been reviewed by White (53, 54, 56, 57, 58), and more recently by Gautheret (18), only work pertaining directly to this study will be discussed here.

Most of the work in plant tissue culture would not have been possible without the solution of the two principal problems which occupied earlier workers, including Haberlandt and Robbins (White, 53), namely the choice of suitable plant material and development of a satisfactory culture medium. Culmination of the early work came in 1939 when White (55), Gautheret (10), and Nobécourt (34), all working independently, reported the establishment of tissue cultures having potentially unlimited growth.

In the seventeen years following this significant attainment a large number of plant tissues have been grown successfully in vitro. In 1954 Gautheret (16) published a catalogue of plant tissue cultures that included thirty-seven herbaceous dicotyledons, twenty-one woody dicotyledons, nine monocotyledons, five gymnosperms, and four pteridophytes. These cultures included normal, habituated, and tumor tissues.

The diversity of tissues which can be obtained in culture has been made possible, in part, by the addition to the medium of growth factors, some as yet unknown, contained in substances such as yeast extract and coconut milk.

Use of Coconut Milk

Since the discovery by Van Overbeek, Conklin, and Blakeslee (49) that coconut milk stimulated the growth of young Datura embryos many workers have found it to be an unusually active growth-stimulating substance. Duhamet (3, 4, 5, 6, 7, 8) has reported such an effect on a variety of tissues including those of Scorzonera hispanica, Helianthus tuberosus, Parthenocissus tricuspidata, and Vitis sp. Henderson, Durrell, and Bonner (20) state that the addition of 10 percent coconut milk to the medium causes a striking increase in callus production of Helianthus stems. Indeed, there are tissues cultured that cannot be maintained in the absence of coconut milk in the medium. Morel and Wetmore (31) found that their cultures of the monocotyledon Amorphophallus rivieri were dying after a few months of subculturing. They then transferred these to a medium containing 15 percent coconut milk, and have grown them successfully on this medium for several years. In some cases coconut milk will support the growth of tissues when no other nutrients are added to the medium. Nickel (33) reported that Rumex virus tumor tissue would grow on pure coconut milk plus 1 percent agar; and Henderson, Durrell, and Bonner (20) found it effective in the culture of Helianthus stem callus.

The age of the coconuts from which the milk is taken is apparently not important. Steward and Caplin (45) found that the fluid endosperm was active at all stages of its development when assayed on tissue of Daucus carota, though, in the case of the oldest fruits, an excess of coconut milk depressed growth. Mauney et al. (24) found that the meat contained more of the active factor than did the milk. Most workers have used filtered rather than autoclaved milk, although Nickel (33) found that in the case of

Rumex tissue autoclaved milk was more stimulatory than unautoclaved. This could possibly be due to a high IAA tolerance on the part of the tumor tissue since Van Overbeek (48) has stated that the IAA in coconut milk is much more extractable after autoclaving than before. While this might also mean that it was more available to the tissue, it must not be concluded that the effect of coconut milk on growth is due to its IAA content alone. Caplin and Steward (1) found that the stimulation caused by the addition of coconut milk to the medium was of a different order of magnitude than that caused by the addition of IAA alone. Thus in a 21 day interval 4 mg explants of Daucus carota tissue with IAA showed a mean increase of 8.2 mg, while with coconut milk the increase was 184.0 mg.

Attempts to isolate the active factor or factors from coconut milk have, in the main, been disheartening. For example, Van Overbeek, Sui, and Haagen-Smit (50) attempted to isolate and identify these factors. After concentrating nine liters of milk they finally ended up with a light-brown residue which was 170 times more active than the coconut milk on a dry-weight basis. More recently Mauney et al. (25) attempted similar identification using the meat rather than the milk, since their tests indicated that the meat had greater activity. Their final product was a heat-stable, acid- and alkali-labile, nonvolatile, water-soluble, organic compound which, on a fresh-weight basis, was 4,350 times more active than the starting material. At the same time, Shantz and Steward (38) were analyzing coconut milk and were able to recognize three chemical entities which they isolated in small amounts. These were designated as compounds "A," "B," and "C." They stated that their assay procedure indicated the almost certain occurrence of other active components. Later Steward and Shantz (45) reported that compound "A" had been identified as 1,3-diphenyl urea (DPU). An interesting feature of this

compound is that it must be used with casein hydrolysate in order to be effective. Even with the hydrolysate, compound "A" did not give as good results as whole coconut milk.

Another compound, although it has not been isolated from coconut milk, appears to be a factor of physiological importance and therefore will be mentioned here. It was recently isolated by Miller et al. (27), principally from hydrolyzed desoxyribose nucleic acid (DNA) but also from yeast extract (YE). It has been called kinetin because of its apparent primary function in cell division as shown in assays using Nicotiana pith tissue which cannot divide unless supplied this substance. Miller et al. (28) have identified kinetin as 6-furfurylaminopurine, and Skoog (41) has recently published a note to the effect that it has some regulatory effect on the formation and development of roots and buds.

Chemical Control of Organ Formation

Following the establishment of a variety of plant tissue cultures and the development of media on which to grow them the way was open for physiological studies concerning such basic problems as the chemical control of organ formation.

It has often been observed that cultures will produce buds or roots sporadically. Nobécourt (35) reported the production of roots by morphologically undifferentiated tissue of Daucus carota. Several years later (36) he noted the spontaneous appearance of leafy shoots in four Daucus carota cultures which had grown for three years producing only roots. White (54) found that when cultures of Nicotiana were grown on semisolid medium they were undifferentiated; however, when immersed in a liquid medium, they began to produce leafy branches. Similar reports of sporadic bud or root production

have been made by Gautheret (11, 12), Morel (30), and Démétriades (2). Gautheret, in reviewing this phenomenon, pointed out that, while root formation was frequently observed, bud production was found more rarely; in still fewer cases buds and roots were both formed so that whole plants were regenerated. The latter has been found in cultures of Cichorium intybus (endive) and sometimes with Daucus carota.

Interesting though such spontaneous differentiation may be, it is almost to be considered a disadvantage in studies of chemical control of differentiation. On the other hand, it does indicate that many undifferentiated tissues are capable of producing many kinds of cells and of organizing them into complex structures.

Inasmuch as Nicotiana tissue could be grown in an undifferentiated state and had a known potential for producing buds, Skoog (40) used it for his first experiments on the chemical control of differentiation. He found that initiation and development of buds could be completely prevented by the addition of IAA to the medium. Gautheret (12) obtained similar results using cambial tissue from Ulmus. Later experiments by Skoog and Tsui (43), though not always conclusive, indicated that with a higher phosphate and sugar content a higher concentration of IAA was needed to prevent bud formation, and this effect was even more marked with adenine sulphate.

Further experiments were done by Miller and Skoog (26) to substantiate the theory that an interaction exists between adenine sulphate and IAA such that changes in the proportions of these components are reflected by changes in growth and organ formation. For this work they used stem segments rather than tissue cultures. It was found that the addition of as little as 10 µg per

liter of IAA caused complete inhibition of bud formation, and this inhibition could be reversed by the addition of adenine.

Several other compounds were tested, including guanine, cytidine, cytosine, and uridine, but none of these caused an increase in bud formation. Skoog (41) later found that thymine, cytosine, or guanine, used in combination with adenine, enhanced bud production more than did adenine alone.

Wiggans (59) compared the responses of Daucus root discs and Nicotiana stem segments to several metabolites. Root formation could be induced in Daucus discs by either IAA or coconut milk although more IAA was required to stimulate growth in Daucus than was the case with Nicotiana. Callus from the discs was subcultured and grown for four months on adenine sulphate medium without any buds appearing. However, when these calluses were transferred back to the basic medium about 10 percent produced buds. The response to adenine was not well defined since bud formation could not be induced consistently.

Thus far Nicotiana stem segments have been the only tissues in which formation of roots or buds could be clearly controlled by a balance between adenine sulphate and IAA. Wiggans (59) was able to show that IAA caused stimulation of rooting in Daucus tissue, as was Jacquoit (21) using tissue of Ulmus. In neither tissue, however, was it possible to demonstrate a clear-cut response to adenine.

In searching for a tissue other than Nicotiana which could be induced to differentiate by the use of chemicals, members of the Compositae would seem a natural choice. Naylor (32) has shown that Taraxacum root segments kept in moist chambers will proliferate buds from the proximal ends and roots from the distal ends. Warmke and Warmke (51) found that roots could be made to form from both ends by the application of auxin, and that, by lowering

the auxin content below normal, leaves would differentiate at both ends. In later experiments using both Taraxacum and Cichorium, Warmke and Warmke (52) found that the inherent tendency to form roots at the distal end and leaves at the proximal end could not be altered by changing the orientation of the cuttings. Application of auxin to the distal end enhanced root production without interfering with leaf formation at the proximal end. They also found that the auxin content of Cichorium roots was extraordinarily high. Gautheret (13, 14, 15) has cultured root discs of three members of the Compositae, Helianthus tuberosus, Cichorium intybus, and Tragopogon sp. In the absence of auxin Helianthus tuberosus grew feebly; Tragopogon required 40 to 50 days to produce leafy shoots, while Cichorium immediately formed callus, roots, and leafy shoots. When IAA was included in the medium, Helianthus tuberosus and Tragopogon produced abundant callus where they were bathed by the nutrient medium. In the case of Cichorium, IAA increased the volume of callus formed but decreased the number of buds.

Among the plants that responded by differentiating and producing callus from root discs, Tragopogon seemed to offer considerable promise. In addition, it was available on the local market and it had a root sufficiently large that sterile segments could be removed from it. With these facts in mind, experiments were undertaken to accomplish the following ends:

1. To establish continuing cultures.
2. To obtain a defined growth medium.
3. To note the effect of variations in the nutrient and other environmental factors upon growth of this tissue.
4. To study means of inducing differentiation.

MATERIALS AND METHODS

The tissues used in this investigation were isolated from Tragopogan porrifolius (salsify). It has a tap root and a condensed stem which produces a rosette of leaves. The latter are grasslike and grow from the base. Attempts were made to obtain sterile cultures from T. pratensis, a local wild species, but these were unsuccessful because of the small size of the root which allowed complete penetration by fungi and bacteria.

Isolation of the Tissue

The root was cut into small segments 1 to 1.5 cm long. The uppermost segment of the root included the condensed stem region from which all the leaves had been trimmed. Since microorganisms frequently penetrate the outer cortex, this was removed and the segments were then surface-sterilized in 10 percent Chlorox for 20 minutes. The cells killed by this treatment were cut away and the sterile segments were transferred to the appropriate medium.

Extensive callus formed over the surface of these root segments and transfers of this were used in establishing root-derived cultures. The leaves which grew out from the stem region of the uppermost segment also produced callus which was used to establish leaf-derived tissue.

In the attempt to obtain continuing cultures several different media were used: Plain White's (PW), White's with IAA, White's with yeast extract, and finally White's with coconut milk. On all

these media cell proliferation took place, but only on White's with coconut milk could transfers be kept alive.

Preparation of the Basic Medium

The basic medium used in these experiments, referred to throughout the text as "Plain White's," represents a slight modification of White's original medium. Increased numbers of trace elements and of vitamins were used. (The trace elements solution was adapted from Nitsch.) In addition, the quantity of sodium dihydrogen phosphate was ten times greater than that used by White.

The final composition of the medium was as follows:

Major Elements	mg/l
magnesium sulphate	360.0
calcium nitrate .$4H_2O$	200.0
sodium sulphate	200.0
potassium nitrate	80.0
potassium chloride	65.0
sodium dihydrogen phosphate .H_2O	16.5
Trace Elements	**mg/l**
manganese sulphate .$4H_2O$	3.0
zinc sulphate .$7H_2O$	0.5
boric acid	0.5
copper sulphate .$5H_2O$	0.025
sodium molybdate .$2H_2O$	0.025
cobalt chloride	0.025
ferric citrate	10.0
Vitamins + Glycine	
glycine	7.5
niacin	1.25
thiamine HCL	0.25
pyridoxine	0.25
calcium pantothenate	0.25

To the above elements were added 890 ml of double-distilled water, 20 gm of sucrose, and 8 gm of agar.

To avoid weighing out the individual salts and vitamins each time medium was made, concentrated stock solutions of the major elements, trace elements, ferric citrate, and vitamins were prepared as listed below:

Major Elements

magnesium sulphate	14.4 gm
calcium nitrate $.4H_2O$	10.4 gm
sodium sulphate	8.0 gm
potassium nitrate	3.2 gm
potassium chloride	2.6 gm
sodium dihydrogen phosphate $.H_2O$	6.6 gm
double-distilled water	4.0 liters

Nitsch's Trace Elements

sulphuric acid sp. gr. 1.83	0.5 ml
manganese sulphate $.4H_2O$	3000.0 mg
zinc sulphate $.7H_2O$	500.0 mg
boric acid	500.0 mg
copper sulphate $.5H_2O$	25.0 mg
sodium molybdate $.2H_2O$	25.0 mg
cobalt chloride	25.0 mg
double-distilled water	1.0 liter

Ferric Citrate Solution

ferric citrate	2.5 gm
double-distilled water	1.0 liter

Vitamin + Glycine Solution

glycine	450.0 mg
niacin	75.0 mg
thiamine HCL	15.0 mg
pyridoxine	15.0 mg
calcium pantothenate	15.0 mg
double-distilled water	300.0 ml

In making up the final medium the following amounts of the stock solutions were used per liter:

major elements solution	100.0 ml
trace elements solution	1.0 ml
ferric citrate solution	4.0 ml
vitamin solution	5.0 ml

During the attempts to establish the tissue in culture it was found that coconut milk was essential for growth; thus the stock cultures were maintained on Plain White's medium in which coconut milk was substituted for 20 percent of the distilled water. During the early part of this work the coconuts were opened as needed; later, however, fifty to one hundred coconuts were opened at a time and the milk mixed to ensure uniformity, and frozen to ensure a continuing supply. The coconut milk was never autoclaved but was heated to 60°C to precipitate some of the proteins, and these were removed by filtering. This facilitated the Seitz filtering which followed. After passage through the Seitz filter the milk was added by means of a filling apparatus to the rest of the medium which had been autoclaved and cooled.

In some of the experiments the medium was supplemented with 0.01, 0.1, or 0.5 percent ribose nucleic acid. The nucleic acid was hydrolyzed by raising the pH to 12.0 with KOH, autoclaving for 30 minutes, and, after cooling, adjusting the pH to 5.5 with HCL. It was then added to the medium.

The medium was made up and autoclaved in a pyrex leveling bulb and from this was dispensed into 20 ml screw-cap bottles which had been autoclaved at the same time as the medium. About 10 ml were put in each bottle.

Precautions were taken to ensure sterile results, and the amount of contamination acquired during the pouring of the medium was extremely low. A plastic sheet was draped over the working area, forming a tent, open on one side, and this area was sprayed with Steri-Wipe S 300 obtained from the Redox Research Laboratories. Before this spray came on the market the table was simply swabbed with 10 percent Chlorox. The spray was more effective in maintaining sterile conditions and was easier to use.

Measurement of Growth

The percentage increase in fresh weight was used as a measure of growth, and was simply calculated from the following formula:

$$\frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100 = \text{percent increase}$$

While dry weight of tissue cultures generally parallels wet weight, this is not always the case (47). Thus, in the preliminary experiments dry weights were also measured. The factor for the initial dry weight was not calculated at the same time as the dry weights were measured; thus there exists a possibility for error in this factor. Since the final dry weight was known exactly, the relative increase in dry weight was not affected. In computing the factor for the original dry weight, ten inoculi were dried in the usual manner, in an oven maintained at 90°C ± 4°C for 24 hours, cooled in a desiccator, and weighed. The mean percent increase was 2.95. The percentage increase in dry weight was then calculated from the following formula:

$$\frac{\text{(Final dry weight)} - \text{(Initial fresh weight} \times 0.0295)}{\text{(Initial fresh weight} \times 0.0295)} = \text{percent change in dry weight}$$

In setting up experiments to measure the increase in weight two methods were possible. The first was to weigh each inoculum at the beginning and end of the experiment, and the second, described by Straus (47), was to weigh a larger inoculum and then to subdivide it into an arbitrary number of parts, putting each part into a separate bottle of medium, and at the end of the experiment harvesting all the growth from the original inoculum. While there was greater risk of contamination in the second method, it seemed preferable because larger amounts of tissue could be used. This would perhaps not be important if many replicates were used, but because

of the time involved in setting up growth experiments this was not practicable. Throughout most of the experiments inocula were divided into five parts.

Inocula were taken from stock cultures 10 to 20 days old--that is, in the log phase of growth--and were transferred to sterile weighing bottles of known weight. They were weighed in the first experiments using an analytical balance and later on a torsion balance. In determining the amount of growth which occurred in a given period of time, usually 25 days, eight inocula and forty bottles of medium were used for a given treatment.

Statistical Methods

It is difficult to evaluate the significance of treatment effects where the samples used are small and where there is considerable overlap between sample distributions. Obtained differences between sample distributions provide no assurance of true treatment effects; they may result simply from the operation of chance factors. If general conclusions are to be based upon the data it is necessary to guard against uncontrolled sources of variation through the use of appropriate statistical techniques. Only then is it possible to interpret the results obtained with any reasonable degree of confidence.

An investigation of the possibilities of the statistical analysis of these data revealed a number of difficulties. While metric techniques are commonly used for the analysis of physical and biological data, in the present case a number of considerations militated against their use. Thus the data could not readily be subsumed under any existing analysis of variance design. In addition, this technique requires that the distribution of observations within a

sample be normal, that within a single analysis sample means and variances be uncorrelated, and that the variances for all samples be homogeneous.

Unfortunately, the present data did not meet these restrictions. A test for correlation of means and variances was made for one experiment consisting of eleven samples (Appendix VII) and a highly significant correlation was found ($r = 0.98$; $P < 0.001$).

Problems concerning the nature of the parent distribution from which the samples were drawn can, however, be avoided through the use of distribution-free or nonparametric tests. These tests make no assumptions concerning the form of the population distribution. The price of fewer restrictions (or wider generality) is a decrease in the power or sensitivity of the test; that is, for samples of a given size they are less likely to detect a true difference between groups when such a difference exists. For ranked data, however, the decrease in power tends to be slight.

On the basis of these considerations the Kruskal-Wallis H test (23) was selected as an appropriate statistical technique for the analysis of the present data. This test is an analogue of the simple randomized analysis of variance design for use with ordered data.

The H test is only slightly less sensitive than the comparable analysis of variance technique where the assumptions associated with the latter are met. Where 100 cases would be required for the analysis of variance test to detect a true difference at the 1 percent level of confidence, approximately 105 cases would be required by the H test to detect the difference at this level of significance.

Nonparametric analysis of variance by ranks.--The Kruskal-Wallis H test does not require the assumption of normality or of

homogeneity of variance and hence seems appropriate for the present data. Further, there is no necessity that the number of observations in each sample be equal. The test is applied by ranking the observations from the combined groups in order of magnitude, giving the largest observation a rank of one, the next largest a rank of two, et cetera. Ties are given the average of the ranks they would ordinarily occupy. Then H is given by:

$$H = \left[\frac{12}{N(N+1)}\right]\left[\sum_{i}^{k} \frac{R_i^2}{n_i}\right] - 3(N+1)$$

where k = the number of groups

n_i = the number of observations in the i^{th} group

$N = \Sigma n_i$, the total number of observations

R_i = the sum of the ranks for the i^{th} group

A correction for ties in rank is given by:

$$H_{corr} = \frac{H}{1 - \frac{\Sigma T}{N(N^2-1)}}$$

where $T = (t-1)t(t+1)$

t = the number of observations in any one tie

If the samples are drawn from a single population and all n_i's are greater than 3, H is distributed approximately as $\chi 2$ with k-1 degrees of freedom.

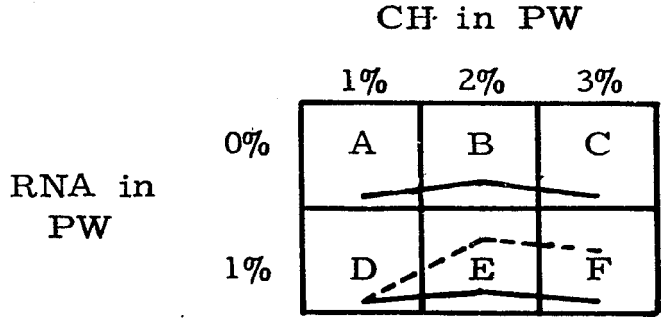
The Kruskal-Wallis technique tests the hypothesis that the k samples come from the same population. If no true differences exist among the samples it would be expected that each sample would have the same sum of ranks. To the degree that the obtained results depart from this expectancy the probability that the k samples were all drawn from a single population decreases. H provides an index of the discrepancy among the sample sums of ranks. When

this discrepancy is large, H is correspondingly large. Assuming that the conditions for the use of the test have been met, the probability of occurrence, by chance, of an H of the obtained magnitude may be assessed by reference to the χ^2 tables. A probability of, say, 0.01 associated with H means that the differences among samples are so large that they would occur by chance only once in every one hundred replications of the experiment. Since this is an event of extremely rare occurrence, the hypothesis that the obtained differences are due to chance may be rejected and the alternative hypothesis that true differences exist becomes tenable. In this situation one would err in rejecting "the hypothesis of no difference" only once in every one hundred experiments.

A stepwise outline of the arithmetic manipulations involved in assessing simple treatment effects by means of the H test will be found in Appendix I.

Interaction.--If the effect of a factor (a chemical) is constant, irrespective of the presence of other factors, there is no interaction; if the effect is altered by the presence of other factors, there is interaction. The Kruskal-Wallis H test is commonly used to provide information concerning main effects (as in a simple randomized design) and there are no set procedures for assessing interaction. Several alternative methods of evaluating interaction effects using the H test are possible, and all are in accord with the meaning of interaction. Two of these procedures have been used: (a) comparison of curves; (b) direct combination of main effects.

Consider first a hypothetical experiment in which the effect of casein hydrolysate (CH) in PW medium is studied both in the presence of RNA and in its absence.



If there is no interaction, the curves in the above diagram, defined by the CH means in the presence and in the absence of RNA, will be parallel, and the difference between the means A and D, B and E, and C and F will be constant. If, on the other hand, the differences between the designated means differ significantly, then interaction is present.

In practice the interaction effect may be estimated by subtracting the A mean from each of the observations in group D, the B mean from each of the observations in group E, and the C mean from each of the observations in group F. If the resulting three distributions are not comparable, then interaction exists and may be estimated by the use of H in the usual fashion. For a numerical example see Appendix II.

In a metric analysis the interaction between CH and RNA, designated CH × RNA, would be identical with the interaction RNA × CH. In assaying interaction by means of the comparison-of-curves technique, using the H test, this is not necessarily the case. Ordering the data places a monotonic but nonlinear transformation upon it and this is sufficient to produce the above-mentioned apparently incongruous result. However, where interaction occurs, both estimates are likely to be significant (though not numerically identical), and where no interaction exists both estimates will be clearly non-significant.

Consider next a hypothetical experiment in which interaction is studied by direct combination of main effects:

DPU in PW	kinetin in PW: 0%	kinetin in PW: 2%
0%	PW + e A	PW + K + e B
2%	PW + DPU + e C	PW + DPU + K + DPU × K + eD

The effects which contribute to the obtained distribution in each group are indicated in the accompanying diagram. To be more explicit, consider group D. Each value (percent increase) in this distribution includes the following effects: the effect of the PW medium, the effect of DPU, the effect of kinetin, the DPU × kinetin interaction, and error. This may be written symbolically in the following form:

$$X = PW + DPU + K + (DPU \times K) + e$$

Estimates of the simple DPU and kinetin effect may be obtained by subtracting the mean of group A from the means of groups B and C, respectively. The addition of the summed kinetin and DPU effects to each of the observations in group A provides the expected distribution for the combination of kinetin and DPU in PW in the absence of interaction. Thus each value in the expected distribution takes the form:

$$X = PW + DPU + K + e$$

This expected distribution may be compared directly with the obtained distribution for group D by means of the H test in the usual way. As is apparent from the above formulae, a significant difference between these groups is attributable to the interaction effect.

Whether interaction results in more or less growth than could be expected on the basis of a simple additive effect of the two chemicals is indicated by the relative magnitude of the means of the expected and obtained distributions. A numerical example is provided in Appendix III.

It must be noted that this test for interaction (and, indeed, the comparison of slopes test) assumes a simple additive combination of effects including the interaction effect. If this model is not appropriate, the tests of interaction are of course inappropriate. One, however, is forced to be arbitrary in this regard. Current statistical techniques for the estimation of interaction are all based on such a model, and if the test is to be made the model must at least be tentatively accepted.

When data are pooled in a main-effects test, considerable distortion is introduced if the numbers within the pooled groups are not equal. Unfortunately, in many cases there were unequal numbers in the samples involved. To minimize distortion the numbers of observations in the smaller groups were increased by including the group mean the requisite number of times. An example of this procedure is given in Appendix IV.

The evaluation of interaction effects serves two major purposes. First it provides additional information available in the data, and second it affords justification for the pooling of data where no significant interaction exists. When pooling is permissible it is valuable since it results in an increase in the power of the test for main effects and in the greater reliability of the test. In addition, the evaluation of interaction permits one to determine whether the effect of a chemical is uniform in a variety of media or whether its effect varies with the composition of the media. One may also determine whether the effects of two chemicals

are simply additive or whether there is an increased facilitation or inhibition of growth that would not have been predicted on the basis of the simple effects of each chemical. Where two chemicals are used in combination a significant effect might be attributable to any one of seven conditions; for example, in the combination of adenine and DPU a significant effect might be due to an adenine effect only, a DPU effect only, both an adenine and DPU effect, an adenine effect plus an adenine-DPU interaction (no simple DPU effect), a DPU effect plus an adenine-DPU interaction (no simple adenine effect), an adenine effect plus a DPU effect plus an adenine-DPU interaction, or an adenine-DPU interaction (but no simple adenine or DPU effect). Testing for both simple effects and interaction effects permits one to indicate which one of these conditions is responsible for the effect obtained.

EXPERIMENTAL RESULTS

Effect of Environment on Growth and Differentiation in Undefined Media

The growth curve.--Before beginning the experimental work it was necessary to determine the log phase of growth. Several growth curves were plotted showing the log percentage increase in wet weight of both leaf- and root-derived tissues. These had been grown on coconut milk medium, and since they were done at different times the milk was from different fruits.

Examination of the data indicates that while typical growth curves were obtained the amount of growth varied with the quality of the coconut milk used. The lag phase was very brief, usually lasting not more than two days, and maximum growth was obtained between 20 and 25 days. Comparison of wet and dry weight curves (Fig. 1) indicates that water uptake was more rapid than dry-weight increase. The original data from which these curves were drawn are included in Table I.

Effect of hydrogen ion concentration on growth.--In general the pH of the medium is not a critical factor in maintaining plant tissue cultures, since most cultures can be grown successfully over a wide range of pH values.

Because of the natural buffering system present in coconut milk (CM) the pH of the medium to which it is added is automatically adjusted to 5.5. While good growth was obtained on 20 percent

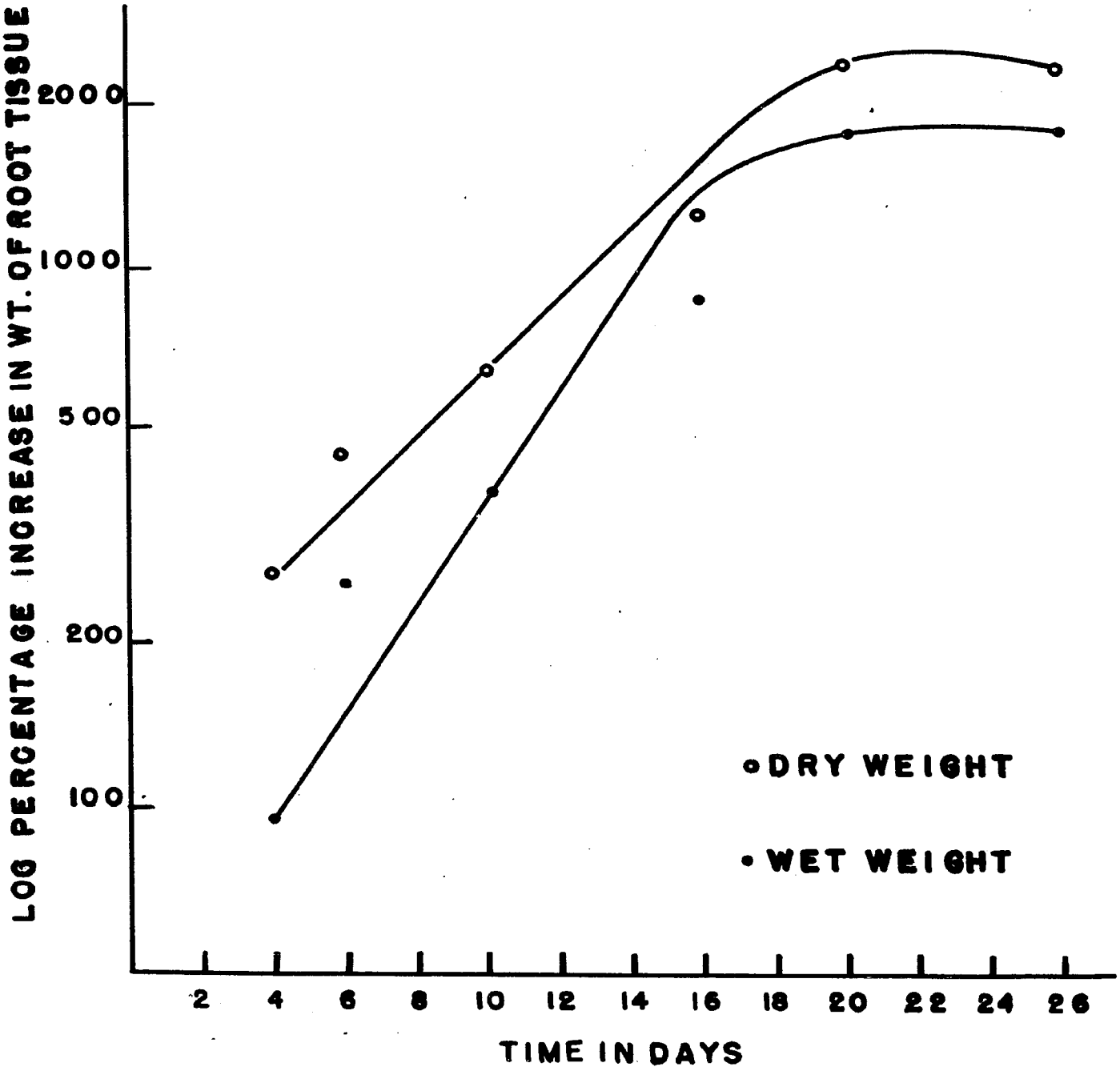


Figure 1. The growth of Tragopogon root tissue as a function of time.

TABLE I.

INCREASE IN WET AND DRY WEIGHT OF ROOT TISSUE
(cultures were maintained in the dark at 27°C)

Inoculum Wt. (grams)	Time (days)	Final Wet Wt. (grams)	Final Dry Wt. (grams)
1.6891	4	3.2897	0.1456
1.5382	6	5.5939	0.2512
1.8068	10	8.7694	0.3932
2.1786	16	20.6223	0.8422
1.5590	20	29.4330	1.1460
1.7312	26	32.3933	1.2470

CM medium at this pH, it was nonetheless considered advisable to determine the optimum pH for growth.

The pH was determined using a Beckman Model G pH meter. The hydrogen ion concentration of the coconut milk was adjusted with KOH or HCl to the required value. Table II indicates that, while the over-all test was not significant when the extremes were compared, a significant difference was found. Despite the superiority of the lowest value, all experiments were carried out in the range 5.0-6.0 as a matter of convenience.

Morphology of root and leaf tissue grown on 20 percent coconut milk medium.--No morphological differences were observed between the leaf- and root-derived tissues. Thus an inoculum from either tissue during the log phase produced loosely adhering cells which, by slight shaking in water, could be separated into a mixture of single cells and small clumps of cells. These varied in shape from spherical to elongate forms somewhat like young pollen tubes

TABLE II.

EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH
(cultures were maintained in the dark at 27°C for 25 days)

Medium Used	pH	Mean Pct. Inc. Wet Wt.	n_i	H	P
PW + 20 percent CM	4.0	1016.8	6	6.21	<.10 (ns)
	5.5	751.8	6		
	6.2	840.5	6		
	7.0	751.9	6		
PW + 20 percent CM	4.0	1016.8	6	8.29	<.01
	7.0	751.9	6		

(Plate I, Fig. 1). Although the illustrations in Plate I are from root-derived tissue, they are identical with what was obtained from leaf-derived tissue.

Twenty to thirty days after inoculation the callus mass was quite solid due to the presence of scattered clumps of vascular tissue. Unlike the majority of tissue cultures reported in the past, these contained most of the cell types found in the parent plant. A cross section through a typical vascular area (Plate I, Fig. 2) showed the xylem towards the inside. The most conspicuous cells of the xylem were the vessel elements which had simple perforations on the end walls and secondary wall thickenings ranging from reticulate to pitted. Fibers and tracheids were also found but these were most easily observed in macerated material. The phloem was outside the xylem and contained long, articulated anastomosing laticifers. The sieve-tube elements had deposits

PLATE I

MORPHOLOGY OF TRAGOPOGON ROOT TISSUE CULTURES GROWN ON 20 PERCENT COCONUT MILK MEDIUM

Figure 1. Cells produced during the log phase of growth and separated by gentle shaking in water. Dilute safranin was added to increase contrast and some cells stained more deeply than others. X125.

Figure 2. Section through an older culture showing compact tissue and a nodule of differentiated cells. Stained with Delafield's hematoxylin and safranin. X125.

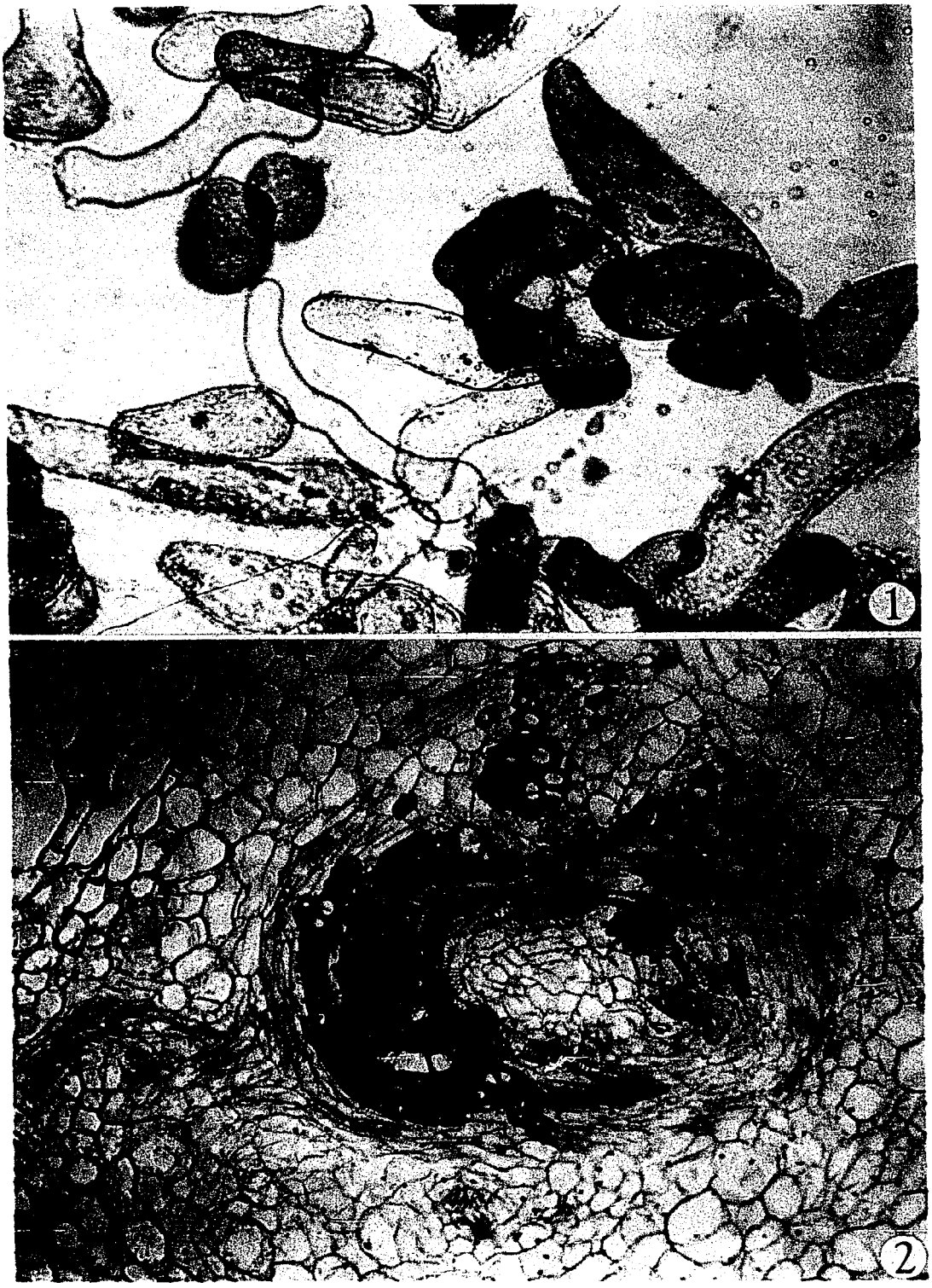


PLATE I

of callose encasing the connecting strands through the sieve plates, as was seen in freehand sections stained with IKI and aniline blue. Since the connecting strands stained bright blue under these conditions it was considered that they were indeed callose.

When the tissues were grown in the light they developed chlorophyll and became bright green in color. The ability to form chlorophyll is not unique as it has been reported for other tissues such as Daucus carota, Parthenocissus quinquefolia, and Helianthus tuberosus (17).

The effect of hydrolyzed ribose nucleic acid (from yeast) on differentiation.--The unusual amount of cellular differentiation which occurred in the stock cultures suggested that organ differentiation might be obtained through use of chemical stimuli. Because of the marked success of Skoog and Tsui (43), using adenine sulphate to regulate bud production in Nicotiana stem segments, it seemed reasonable to turn first to the nucleic acids and their derivatives as likely agents in organ formation. Accordingly, 0.05 g/l of hydrolyzed ribose nucleic acid (RNA) was added to Plain White's medium and to Plain White's plus 20 percent coconut milk. Results of this experiment are shown in Table III.

There was no difference in the response of root- and leaf-derived tissues, and in both cases the cultures became very nodular in appearance. Microscopic examination of serial sections indicated larger areas of differentiated cells than were ever observed in stock cultures. In addition there was greater organization in these areas and a definite tendency toward radial alignment. A large mass of xylem occupied the center of each such area and was surrounded by phloem cells and laticifers (Plate II, Figs. 1 and 2).

PLATE II

MORPHOLOGY OF TRAGOPOGON ROOT TISSUE CULTURES GROWN ON 20 PERCENT COCONUT MILK MEDIUM PLUS HYDROLYZED RIBOSE NUCLEIC ACID

Figures 1 and 2. Sections through older cultures showing radial alignment of cells in vascular nodules and increase in amount of differentiation as compared with cultures grown in the absence of RNA (Plate I, Fig. 2). Stained with Bismarck Brown and Orange G. ×125.

Figure 3. Section through young root formed in culture. Stained with Delafield's hematoxylin. ×125.

Figure 4. Cross section through young leaf showing one vascular strand. Stained with Delafield's hematoxylin. ×125.



PLATE II

TABLE III

EFFECT OF HYDROLYZED RNA ON ORGAN FORMATION

Tissue Origin	Total Samples	Organs Formed			Pct. Diff.
		Leaves	Roots	Leaf + Root	
	Media: PW + 20 pct. CM + 0.5 g/l RNA				
Leaf	20	5	0	1[a]	30
Root	14	4[b]	0	1	35
	Media: PW + 0.5 g/l RNA				
Leaf	24	2	3[c]	1[d]	25
Root	18	2	2	0	22

[a]The leaf and root were attached instead of being separate, as was the case with all other samples which differentiated.

[b]One of these cultures produced three leaves.

[c]One of these cultures produced two roots.

[d]One of these cultures produced two leaves.

After a period of about 40 days, 22 to 35 percent of the cultures had formed small leaves and roots. These developed from nodules which tended to be discrete from the tissue mass. Leaves and roots formed on different parts of the same tissue culture but, with one exception, leaf and root were never formed from the same nodule (Table III). The macroscopic appearance of similar leaves and roots are shown in Plate III, Figs. 5 and 6.

PLATE III

DIFFERENTIATION OF LEAVES AND ROOTS FROM TRAGOPOGON ROOT TISSUE CULTURES

Figure 1. Leaves differentiated on medium containing "B" solution of amino acids and adenine sulphate.

Figure 2. Small leaves formed on medium containing "B" solution of amino acids, 0.1 percent yeast extract, and 0.1 percent ribose nucleic acid.

Figure 3. Leaf formed on medium containing "B" solution of amino acids and 1,3-diphenyl urea.

Figure 4. Stock culture of Tragopogon showing growth on 20 percent coconut milk medium.

Figures 5 and 6. Roots and small leaf formed on medium containing 20 percent coconut milk and 0.01 percent ribose nucleic acid.

Figure 7. Leaves formed on medium containing "B" solution of amino acids, 0.1 percent yeast extract, and 0.1 percent ribose nucleic acid.

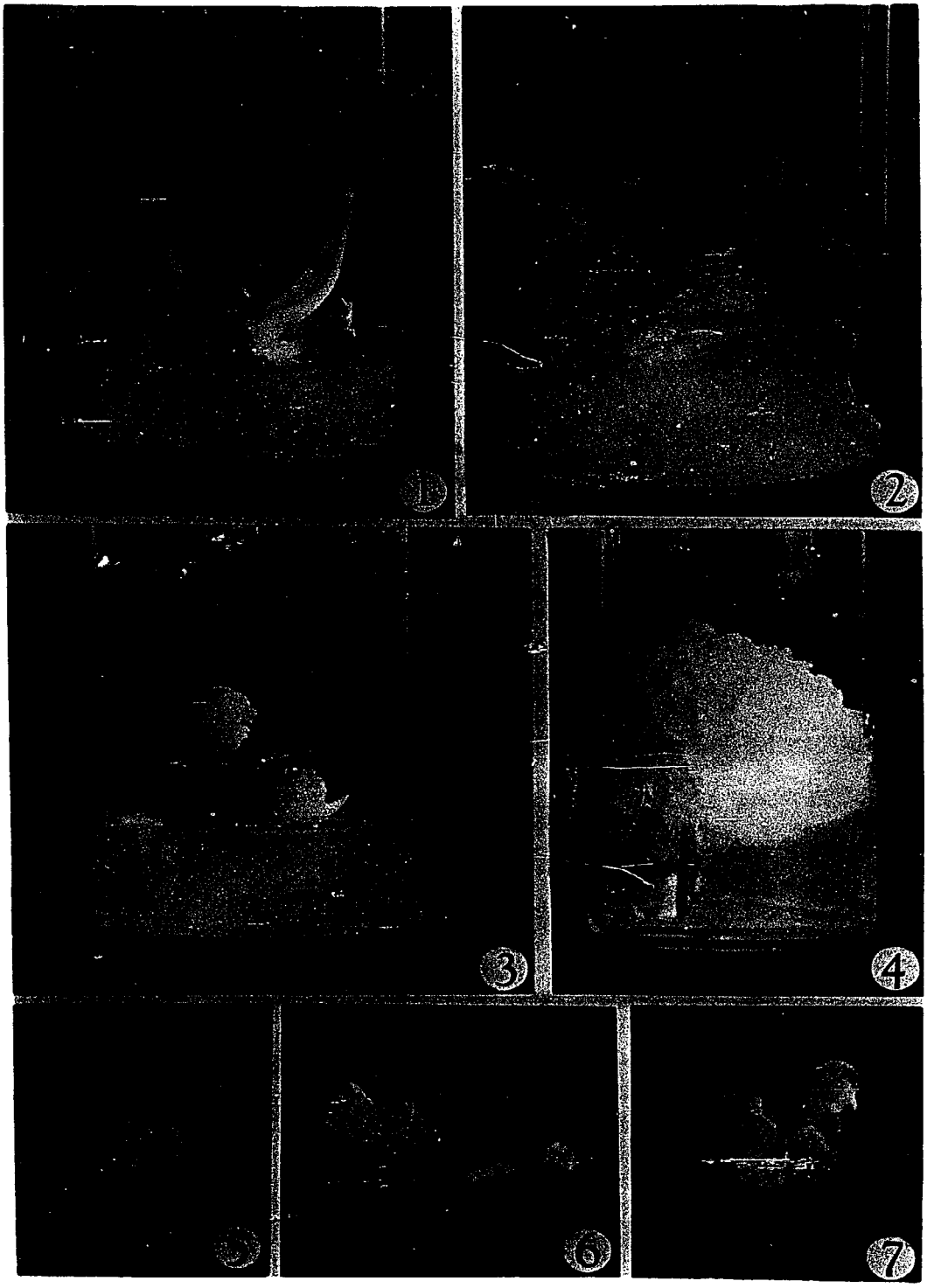


PLATE III

Morphology of leaves and roots formed in culture.--The leaves were very small, the longest being only 1 cm. Despite the small size, however, the organization was definitely leaflike. When grown in the light, both the tissue and the leaves became green in color, and in the light or dark stomata formed on the surface. While the smallest leaves had only one vascular strand (Plate II, Fig. 4), it was more common to find three parallel strands in the larger leaves. Surrounding the vascular tissue was a broad cortex extending out to the epidermis.

The roots varied in length from 1 to 3 cm, and frequently developed club-shaped tips as they became aged. In cross section they showed the normal structure of young roots. An endodermis enclosed the vascular cylinder, and around this a broad cortex extended out to the epidermis (Plate II, Fig. 3).

The gross morphology of roots and leaves formed on a variety of media may be seen in Plate III, Figs. 1 to 7.

Because RNA had such a marked effect on the organization and differentiation of Tragopogon tissue cultures, it was of interest to know the effect of concentration upon growth. This may be seen in Table IV.

Effect of hydrolyzed ribose nucleic acid on growth.--There was no significant difference in the amount of growth on medium containing 0.01, 0.1, or 0.5 percent of RNA, but on the medium containing 1.0 percent the tissue died. Dying tissue was recognized by its dark and often soggy appearance and by its decrease in wet weight. In the two lower concentrations the growth was pale yellow in color and nodular in appearance. However, when RNA was used at the 0.5 percent level the tissue appeared to be unhealthy because of the dark pigmentation which accompanies deterioration of the

TABLE IV

EFFECT OF CONCENTRATION OF HYDROLYZED RNA ON GROWTH OF ROOT TISSUE
(cultures were maintained in the dark at 27°C for 25 days)

Pct. RNA Used	Percent Increase Wet Wt.	H	df	P
0.01	64.6	3.6	3	<.20 (ns)
0.1	52.9			
0.5	97.7			
1.0	died			

cultures. From these results it seemed that the best concentration to use in future experiments lay between 0.01 and 0.1 percent.

It was also of interest to ascertain the effect of RNA on growth in combination with coconut milk. From Table V it may be seen that there was no significant difference in the amount of growth obtained on 20 percent CM medium and on 20 percent CM with 0.01 percent RNA added.

The results of these preliminary experiments may be summarized as follows: The optimal duration of growth experiments was 25 days, since maximum growth was reached in that time. The optimal pH of the growth medium was about 4.0, but a pH in the range 5.0-6.0 gave satisfactory results and was more convenient to use. When RNA was added to a PW or PW + CM medium it appeared to act as an organizing principle rather than a growth factor, since leaves and roots were formed without accompanying stimulation of growth.

TABLE V

EFFECT OF HYDROLYZED RNA IN 20 PERCENT CM MEDIUM ON GROWTH OF ROOT TISSUE
(cultures were maintained in the dark at 27°C for 25 days)

Medium Used	Pct. Increase Wet Wt.	H	df	P
PW + 20 percent CM + 0.01 percent RNA	805.4	0.92	1	<.50 (ns)
PW + 20 percent CM	705.2			

Auxanographic Approach to a Defined Growth Medium

Because it is generally unacceptable to use an undefined medium for experimental work and because the quality of the CM was very variable, it was particularly desirable to obtain a completely defined medium capable of supporting adequate growth. To this end the following experiments were carried out.

The auxanographic approach described by Pontecorvo (37) was used in order to delimit in a broad way the types of chemicals required for growth. This method was used originally in determining the nutritional requirements of microorganisms. Arrays of substances were applied to seeded agar plates, and by successive steps the field of search was progressively restricted. In the present study several undefined media were used as sources of certain classes of compounds. Hydrolyzed RNA was used to furnish purines, pyrimidines, and their derivatives; casein hydrolysate

(vitamin-free) furnished amino acids; and yeast extract supplied all the above plus vitamins, fatty materials, and other substances.

Effect of yeast extract.--Yeast extract (YE) was added to the basal medium in three concentrations. As may be seen in Table VI, there was no significant difference in the amount of growth obtained on medium containing the two higher concentrations of YE. However, that containing the lowest concentration gave significantly poorer growth. In all three cases the tissue was hard, and yellow in color, and had turned dark brown on the side next to the agar. Thus, when added alone to the basal medium, YE was unable to support good growth of the tissue.

TABLE VI

EFFECT OF CONCENTRATION OF YEAST EXTRACT ON GROWTH OF LEAF TISSUE
(cultures were maintained in the dark at 27°C for 25 days)

Medium Used	Additives	Mean Pct. Increase Wet Wt.	n_i	H	P
PW	0.01% YE	16.2	5	9.98	<.01
	0.1% YE	122.9	5		
	0.5% YE	75.1	5		
PW	0.1% YE	122.9	5	1.29	<.30 (ns)
	0.5% YE	75.1	5		

Effect of casein hydrolysate.--Casein hydrolysate (CH) was available in both acid- and enzyme-hydrolyzed forms, and since the latter had a lower ash and salt content it was thought that it would be preferable. Because the amount of cysteine and tryptophane present in this material is low, the medium was supplemented with 16 mg/l of each. When 0.5 and 1.0 percent of CH were used alone the medium proved unsatisfactory and the tissue died. Therefore, acid-hydrolyzed CH, alone and in combination with cysteine, tryptophane, and RNA, was used with the results provided in Tables VII, VIII, IX, and X.

As may be seen in Table VII, increasing concentrations of CH proved inhibitory. The treatment differences shown in this table were altered by the addition of 0.01 percent RNA to the medium ($H = 13.11$; $P < 0.01$). Because of this interaction which occurred between CH and RNA, no pooling of the data was possible.

The effect of adding RNA to the CH media is shown in Table VIII. It was found that, when added to a 0.5 percent CH medium, the effect of RNA was of doubtful significance. However, in a 1.0 percent CH medium RNA stimulated growth, and in a 2.0 percent CH medium increasing concentrations of RNA produced a monotonic increase in growth. It would thus appear that RNA counteracted the inhibitory effect of increasing concentrations of CH. It had little or no effect at the lowest concentration of CH but a marked effect at the highest concentration.

It was found that when 16 mg/l of both cysteine and tryptophane were added to the CH media significant interaction occurred ($H = 7.50$*; $P < 0.01$), again making the pooling of data unfeasible.

As shown in Table IX, cysteine and tryptophane had an inhibitory effect when added to the 1.0 percent CH medium. No significant effect was noted when these compounds were added to the

TABLE VII

EFFECT OF INCREASING CONCENTRATIONS OF CASEIN HYDROLYSATE (UNSUPPLEMENTED) ON GROWTH OF ROOT TISSUE
(cultures were maintained in the dark at 27°C for 25 days)

Medium Used	Additives	Mean Pct. Increase Wet Wt.	n_i	H	P
PW	0.5% CH	233.2	5	13.32*	<.01
	1.0% CH	95.0	5		
	2.0% CH	28.5	6		

*Throughout the text this symbol indicates a maximum value for H; that is, no overlap of sample distributions occurred.

TABLE VIII

EFFECT OF INCREASING CONCENTRATIONS OF CASEIN HYDROLYSATE AND RIBOSE NUCLEIC ACID ON GROWTH OF ROOT TISSUE
(cultures were maintained in the dark at 27°C for 25 days)

Media Used	Additives	Mean Pct. Increase Wet Wt.	n_i	H	P
PW + 0.5% CH	Basal med.	233.2	5	6.30	<.05
	0.01% RNA	165.7	6		
	0.1% RNA	237.1	6		
PW + 1.0% CH	Basal med.	95.0	5	8.83	<.02
	0.01% RNA	226.9	6		
	0.1% RNA	198.5	6		
PW + 2.0% CH	Basal med.	28.5	6	14.73	<.001
	0.01% RNA	67.3	6		
	0.1% RNA	172.1	6		

Basal medium = medium used with no additive.

TABLE IX

EFFECT OF INCREASING CONCENTRATIONS OF CASEIN HYDROLYSATE PLUS CYSTEINE AND TRYPTOPHANE ON GROWTH OF ROOT TISSUE
(cultures were maintained in the dark at 27°C for 25 days)

Media Used	Additives	Mean Pct. Increase Wet Wt.	n_i	H	P
PW + 1.0% CH	Basal med.	95.0	5	5.77	<.02
	Cysteine and tryptophane	29.7	5		
PW + 2.0% CH	Basal med.	28.5	6	0.00	ns
	Cysteine and tryptophane	27.7	6		

Basal medium = medium used with no additive.

2.0 percent CH medium. Thus it would appear that cysteine and tryptophane in the concentrations used tended to enhance the inhibitory effect of CH. That this effect was not demonstrable when 2.0 percent CH was used may have been the result of an asymptotic inhibition of growth.

The presence of a significant first-order interaction between CH and RNA and between CH and cysteine and tryptophane suggested the likelihood of a second-order interaction among these factors. This was borne out by the data. Thus the addition of 0.01 percent RNA as well as cysteine and tryptophane to the CH media significantly altered the simple effect of CH ($H = 13.32^{*}$; $P < 0.001$). An interaction effect was also noted when the concentration of RNA was

increased to 0.1 percent with the concentration of cysteine and tryptophane held constant ($H = 15.18^{*}$; $P < 0.001$).

As indicated in Table X, a marked decline in growth was noted when cysteine and tryptophane were added to a 0.5 percent concentration of CH supplemented by 0.01 or 0.1 percent RNA. When the concentration of CH was increased to 1.0 percent there was still inhibition with the lower concentration of RNA. With 0.1 percent RNA, however, this trend was reversed and growth was somewhat facilitated. With cysteine and tryptophane present in a 2.0 percent CH medium, increasing concentrations of RNA produced increased growth. It would seem that the addition of cysteine and tryptophane to a CH medium or to a CH + 0.01 percent RNA medium led to a relative inactivation of these compounds. With the higher concentration of RNA, however, the RNA effect predominated. This again suggests that a balance between the components of RNA and amino acids must be maintained for maximum growth.

Effect of amino acids.--In view of the previous experiments it seemed likely that a defined medium might be obtained through the judicious combination of amino acids, nucleic acids or their derivatives, and possibly vitamins. Accordingly, two solutions of amino acids designated "A" and "B" were arbitrarily chosen. Their composition and the amounts used in 500 ml. of medium are given in Table XIII. The results of an experiment in which these factors were used alone and combined with RNA and YE are presented in Tables XI and XII.

The differences between the "A" and "B" solution media were constant, irrespective of the presence of RNA. Thus, in the absence of interaction between these factors ($H = 0.21$; $P < 0.90$) it was possible to obtain a better estimate of the difference

TABLE X

EFFECT OF INCREASING CONCENTRATIONS OF CASEIN HYDROLYSATE PLUS RIBOSE NUCLEIC ACID PLUS CYSTEINE AND TRYPTOPHANE ON GROWTH OF ROOT TISSUE
(cultures were maintained in the dark at 27°C for 25 days)

Media Used	Additives	Mean Pct. Increase Wet Wt.	n_i	H	P
PW + 0.5% CH	Basal medium	233.2	5		
	0.01% RNA + cysteine and tryptophane	50.0	5	10.08	<.01
	0.1% RNA + cysteine and tryptophane	42.3	6		
PW + 1.0% CH	Basal medium	95.0	5		
	0.01% RNA + cysteine and tryptophane	57.7	6	14.20*	<.001
	0.1% RNA + cysteine and tryptophane	144.4	6		
PW + 2.0% CH	Basal medium	28.5	6		
	0.01% RNA + cysteine and tryptophane	57.0	5	13.43	<.001
	0.1% RNA + cysteine and tryptophane	187.1	6		

*Represents a maximum value for H.

Basal medium = medium used with no additive.

TABLE XI

EFFECT OF AMINO ACIDS ON GROWTH OF ROOT TISSUE
(cultures were maintained in the dark at 27°C for 25 days)

Media Used	Additives	Mean Pct. Increase Wet Wt.	n_i	H	P
PW, 0.5% RNA (pooled data)	Sol. "A"	94.0	16	19.50	<.001
	Sol. "B"	234.6	16		
PW, 0.5% RNA (pooled data)	Sol. "A"	94.0	16	0.87	<.50 (ns)
	Sol. "A" + "B"	102.0	16		
PW, 0.5% RNA (pooled data)	Sol. "B"	234.6	16	15.19	<.001
	Sol. "A" + "B"	102.0	16		

TABLE XII

EFFECT OF AMINO ACID SOLUTIONS PLUS YEAST EXTRACT AND RIBOSE NUCLEIC ACID ON GROWTH OF ROOT TISSUE
(cultures were maintained in the dark at 27°C for 25 days)

Media Used	Additives	Mean Pct. Increase Wet Wt.	n_i	H	P
PW + 0.1% RNA + 0.1% YE	Sol. "A"	598.7	8	9.60*	<.01
	Sol. "B"	229.6	6		
PW + Sol. "A"	Basal medium	80.0	6	9.60*	<.01
	0.1% RNA + 0.1% YE	598.7	8		
PW + Sol. "B"	Basal medium	202.5	8	0.23	<.90 (ns)
	0.1% RNA + 0.1% YE	229.6	6		

*Represents a maximum value for H.
Basal medium = medium used with no additive.

TABLE XIII

COMPOSITION OF AMINO ACID SOLUTIONS

Amino Acids in Solution	Molarity of Medium		Molarity of Stock Solutions	
	Exp. I	Exp. II	Exp. I	Exp. II
	Solution "A"			
L-leucine	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
DL-phenylalanine	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
L-tryptophane	2.5×10^{-4}	2.5×10^{-4}	3×10^{-3}	5×10^{-3}
DL-valine	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
L-proline	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
Hydroxy L-proline	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
DL-threonine	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
Glycine	1.6×10^{-3}	1×10^{-3}	2×10^{-2}	2×10^{-2}
DL-aspartic acid	5×10^{-4}	5×10^{-4}	5×10^{-3}	1×10^{-2}
DL-lysine	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
	Solution "B"			
DL-isoleucine	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
DL-methionine	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
L-tyrosine[a]	5×10^{-4}	2×10^{-4}	6×10^{-3}	4×10^{-3}
L-alanine	8.3×10^{-4}	7.4×10^{-4}	1×10^{-2}	1.5×10^{-2}
L-glutamic acid	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
DL-serine	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
L-arginine	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
L-histidine	5×10^{-4}	5×10^{-4}	6×10^{-3}	1×10^{-2}
L-cystine[a]	5×10^{-4}	4.5×10^{-5}	6×10^{-3}	9×10^{-4}

[a]This compound did not go entirely into solution in the first experiment, hence the concentration was lowered in the second experiment.

between the "A" and "B" effect by pooling the results. As shown in Table XI, it was found that the medium containing solution "B" gave much better growth than that containing solution "A" or solution "A" + "B." A comparison of the amount of growth produced on medium containing solution "A" and medium containing solution "A" + "B" indicated that there was no difference between these two solutions. Thus it might be inferred that solution "A" had a negating effect on the stimulating properties of solution "B."

When YE as well as RNA was added to the amino acid solutions the growth properties of the "A" solution were altered, while those of the "B" solution remained unchanged. Because of this interaction ($H = 9.60^{*}$; $P < 0.01$), data were not pooled.

It was found that the combination of "A" solution with YE and RNA resulted in a marked increase in growth (Table XII). Up to this point this was the most growth obtained on a medium other than coconut milk. Yeast extract and RNA added to the "B" solution had no effect. Furthermore, this was the only combination in which the "A" solution gave better growth than the "B." It will be shown later, however, that a totally defined medium gave even more growth.

After the tissue had grown on media containing the "B" solution, a blackening of the agar was observed. Since this solution contained tyrosine, a test was made for tyrosinase activity. A gram of tissue was ground in cold M/10 phosphate buffer at pH 6.7 and was added to 1/20M Dopa after centrifugation to remove cell debris. At the end of ten minutes a dark color had formed. When the preparation was boiled no color developed, so it was concluded that the activity was due to an enzyme, probably tyrosinase.

It was considered of interest to grow cultures on these media for longer than 25 days in order to observe whether organ

differentiation occurred. Consequently, the cultures not to be used in the growth experiment were transferred to a "light room" ten days after inoculation. They were maintained here under conditions of constant light (GE cool white bulbs, 200 foot-candles), temperature (20°C), and humidity (50 to 70 percent) for 86 days. The results are presented in Table XIV. No differentiation occurred on a medium containing RNA + "A" solution, while considerable differentiation took place on those containing RNA + "B" solution.

TABLE XIV

DIFFERENTIATION ON MEDIA CONTAINING AMINO ACIDS, RIBOSE NUCLEIC ACID, AND YEAST EXTRACT

Media Used	Total Samples	Organs Formed			Pct. Diff.
		Leaf	Root	Leaf + Root	
PW + 0.5% RNA + "A" solution	6	0	0	0	0.0
PW + 0.5% RNA + "B" solution	7	5	0	0	71.4
PW + 0.5% RNA + "A" + "B" solution . . .	7	5	1	0	85.7
PW + 0.1% RNA + 0.1% YE + "A" solution	11	0	0	0	0.0
PW + 0.1% RNA + 0.1% YE + "B" solution	18	1	0	1[a]	11.1

[a]This culture formed four leaves and two roots.

Effect of Ribose Nucleic Acid Components on Organ Formation

Since it had been shown that RNA induced organ formation in Tragopogon, as did one of its components, adenine sulphate, it was considered of importance to determine if other components of RNA would act in a similar manner. Accordingly, an experiment was set up in which White's plus 20 percent coconut milk was used as the basic medium. The effect of each of the following substances at concentrations of 50 and 100 mg/l was observed. In each case fifty to sixty cultures were kept for 60 days in the dark at 27°C.

adenine	adenylic acid
cytosine	adenosine triphosphate
uracil	cytidylic acid
adenosine	uridylic acid
cytidine	

Adenine sulphate was the only one of these compounds to induce organ formation. In all cases growth was good, though slight browning of the cultures was observed at the higher concentration.

Effect of Growth Regulators upon Growth and Differentiation

Reports on the isolation of two chemicals, DPU (46) and kinetin (27), which acted as cell division factors were considered to be of special interest. Accordingly, factorial experiments were designed to test their effect upon Tragopogon root tissue. The first of these, designated as experiment I, contained all the first-order interactions of IAA, DPU, kinetin, and adenine. This was performed in December, 1955, with the results shown in Tables

XVI and XVII. The second experiment, referred to as experiment II, contained all the first-order interactions of DPU, kinetin, adenine, and the two amino acid solutions previously designated "A" and "B," and was performed in March, 1956. In the latter experiment, however, the concentrations of some of the components of the amino acid solutions were altered as described in Table XIII.

Where experiments or parts of experiments are duplicated, it is common practice to pool the data, since this increases the number of observations and hence the power of the test and the reliability of the statistic. In doing this, one must be certain that the treatment effects are uniform over replications. To ascertain whether this were the case, a comparison was made of the effects of the chemicals common to both experiments. From Table XV it may be seen that, although the adenine effect remained constant, the effect of kinetin and DPU was not the same in both experiments. This necessitated separate statistical analysis.

These results were not entirely unexpected, since the phenomenon of habituation is already familiar to people working with tissue cultures. In this case a tissue which has required the presence of IAA in the medium since its original isolation undergoes some change such that IAA is no longer required. In the case of the Tragopogon tissue slight changes in its gross morphology had been noted in the time between these experiments. Stock cultures of the tissue appeared to have fewer differentiated areas, and the entire tissue seemed to be less firm and more watery than it had been previously. Since the addition of IAA to the medium had not been required, this change could not be directly attributed to the usual habituation phenomenon. However, as a result of these two experiments and their statistical analysis, it was apparent that the change in the tissue resulted in an altered response to certain chemicals.

TABLE XV

A COMPARISON OF THE EFFECTS OF DPU, KINETIN, AND ADENINE IN TWO REPLICATIONS

Media Used	Interaction	H	P
PW, K, Adenine (pooled data)	DPU × Replication DPU effect I DPU effect II	12.22	<.001
PW, DPU, Adenine (pooled data)	Kinetin × Replication Kinetin effect I Kinetin effect II	10.02	<.01
PW, DPU, Kinetin (pooled data)	Adenine × Replication Adenine effect I Adenine effect II	1.57	<.30 (ns)

As noted previously, it was necessary to consider the results of these two experiments separately. In each case both simple effects and interaction effects of the different treatments have been analyzed.

Experiment I.--The simple effects of the substances used in the first experiment may be seen in Table XVI. It was found that DPU, kinetin, and IAA all had an inhibiting effect upon growth, while adenine had no effect. That DPU and kinetin did not stimulate growth when added to the basal medium was not surprising in view of the reports that each required CH in order to act.

TABLE XVI

SIMPLE EFFECTS OF DPU, KINETIN, ADENINE, AND IAA ON GROWTH OF ROOT TISSUE
(cultures were maintained in the dark at 27°C for 25 days; the concentrations used were 2 mg/l of DPU, kinetin, and IAA, and 100 mg/l of adenine)

Media Used	Additives	Mean Pct. Increase Wet Wt.	n_i	H	P
PW, IAA (pooled data)	Basal medium	127.9	16	8.77	<.01
	DPU	58.4	16		
PW	Basal medium	159.1	8	4.77	<.05
	Kinetin	63.1	6		
PW, IAA (pooled data)	Basal medium	127.9	16	1.60	<.30 (ns)
	Adenine	94.6	16		
PW, DPU, Adenine (pooled data)	Basal medium	121.4	24	7.12	<.01
	IAA	65.7	24		

Basal medium = medium used with no additives.

The interaction effects of these substances are given in Table XVII. It was found that IAA interacted with kinetin, giving more growth than would be expected on the basis of a simple additive effect. It did not, however, interact with adenine or DPU. Considering the simple inhibitory effects of DPU and kinetin, one would expect this combination to be lethal, but as a result of interaction some growth occurred. Similarly, only a small amount of growth might be expected from the combination of DPU with adenine or of kinetin with adenine; this was not the case, however, as the interaction of these factors produced a striking increase in growth.

TABLE XVII

INTERACTION EFFECTS OF DPU, KINETIN, ADENINE, AND IAA

Interaction	Mean Pct. Increase Wet Wt.	n_i	H	P
DPU × Kinetin				
Expected distribution, no interaction	-20.7	8	10.57	<.01
Obtained distribution	54.0	8		
DPU × Adenine				
Expected distribution, no interaction	46.1	8	9.55*	<.01
Obtained distribution	534.4	6		
DPU × IAA				
Expected distribution, no interaction	12.8	8	0.48	<.50 (ns)
Obtained distribution	41.4	7		
Kinetin × Adenine				
Expected distribution, no interaction	33.9	8	11.26*	<.001
Obtained distribution	581.7	8		
Kinetin × IAA				
Expected distribution, no interaction	0.6	8	4.92	<.05
Obtained distribution	97.3	8		
Adenine × IAA				
Expected distribution, no interaction	67.4	8	1.02	<.50 (ns)
Obtained distribution	59.2	6		

*Represents a maximum value for H.

The calculation of the expected distribution is explained in "Statistical Methods."

Since kinetin and DPU had been recently isolated, little was known concerning the effect of concentration of these chemicals. While the 2 mg concentration was selected on the basis of Miller's work, it was decided to investigate the effect of a higher concentration of these factors as well as IAA.

From Table XVIII, it may be seen that increasing the concentration of IAA increased its inhibitory effect. With kinetin and DPU such an effect was not demonstrable. As was shown in Table XVI, a concentration of 2 mg/l of kinetin or DPU was inhibitory. However, the data lead one to suspect that the trend was reversed at the higher concentration of these compounds.

TABLE XVIII

EFFECT OF TWO DIFFERENT CONCENTRATIONS OF DPU, KINETIN, AND IAA ON GROWTH OF ROOT TISSUE
(cultures were maintained in the dark at 27°C for 25 days)

Medium Used	Additives	Mean Pct. Increase Wet Wt.	n_i	H	P
PW	Basal medium	159.1	8	9.55	<.01
	IAA 2 mg	96.6	8		
	IAA 10 mg	45.8	8		
PW	Basal medium	159.1	8	3.92	<.20 (ns)
	DPU 2 mg	75.3	8		
	DPU 10 mg	135.0	8		
PW	Basal medium	159.1	8	4.65	<.10
	Kinetin 2 mg	63.1	6		
	Kinetin 10 mg	120.1	5		

Basal medium = medium used with no additive.

Experiment II.--The simple effects observed in experiment II are presented in Table XIX. Here it was found that DPU, kinetin, and RNA were without effect on growth, while adenine and the "B" solution caused stimulation. Because of the change believed to have taken place in the tissue, direct comparisons with earlier experiments are not possible. It is noted, however, that the changed tissue growing on the modified "B" solution grew better than the unchanged tissue on the unmodified "B" solution. It was also of interest to note that the modified "B" solution contained less tyrosine, and no darkening of the agar occurred as a result of growth on medium containing this solution.

TABLE XIX

SIMPLE EFFECTS OF DPU, KINETIN, ADENINE, "B" SOLUTION, AND RNA
(cultures were maintained in the dark at 27°C for 25 days)

Media Used	Additives	Mean Pct. Increase Wet Wt.	n_i	H	P
PW, Kinetin (pooled data)	Basal medium	78.0	16	2.06	<.20 (ns)
	DPU	97.8	16		
PW, DPU, Adenine (pooled data)	Basal medium	264.0	24	1.09	<.30 (ns)
	Kinetin	219.7	24		
PW, Kinetin (pooled data)	Basal medium	78.0	16	23.64*	<.001
	Adenine	549.9	16		
PW, RNA (pooled data)	Basal medium	59.4	14	19.61	<.001
	"B" solution	387.1	14		
PW, "B" solution (pooled data)	Basal medium	248.8	14	1.67	<.20 (ns)
	RNA	197.6	14		

*Represents a maximum value for H.

Basal medium = medium used with no additive.

In this experiment, as in the previous one, many interaction effects were noted. These are presented in Table XX. The interaction of DPU with adenine resulted in less growth than would be expected on the basis of a simple additive effect, while the combination of DPU with "B" solution or RNA enhanced growth. DPU did not interact with kinetin. The interaction effect of kinetin with "B" solution proved inhibitory, and no interaction occurred between kinetin and adenine. The interaction effect between adenine and "B" solution was marked, and resulted in the most growth yet obtained on a completely defined medium: indeed, an amount of growth comparable to that obtained on the coconut milk medium. No interaction occurred between "B" solution and RNA.

In both experiment I and experiment II cultures were observed for differentiation. This occurred as expected in media containing RNA or adenine sulphate. In experiment I, however, one culture growing on DPU medium produced a small leaf, and again in experiment II, two cultures on DPU medium produced leaves (Plate III, Fig. 3). Since this chemical has never been reported to be a differentiation factor, these results were considered of interest.

Part of experiment II involved some second-order interactions, and these are presented in Table XXI. It was found that when medium containing adenine and "B" solution was supplemented with DPU or RNA growth was inhibited, while the addition of kinetin had no effect.

In this experiment, as in the previous one, many interaction effects were noted. These are presented in Table XX. The interaction of DPU with adenine resulted in less growth than would be expected on the basis of a simple additive effect, while the combination of DPU with "B" solution or RNA enhanced growth. DPU did not interact with kinetin. The interaction effect of kinetin with "B" solution proved inhibitory, and no interaction occurred between kinetin and adenine. The interaction effect between adenine and "B" solution was marked, and resulted in the most growth yet obtained on a completely defined medium: indeed, an amount of growth comparable to that obtained on the coconut milk medium. No interaction occurred between "B" solution and RNA.

In both experiment I and experiment II cultures were observed for differentiation. This occurred as expected in media containing RNA or adenine sulphate. In experiment I, however, one culture growing on DPU medium produced a small leaf, and again in experiment II, two cultures on DPU medium produced leaves (Plate III, Fig. 3). Since this chemical has never been reported to be a differentiation factor, these results were considered of interest.

Part of experiment II involved some second-order interactions, and these are presented in Table XXI. It was found that when medium containing adenine and "B" solution was supplemented with DPU or RNA growth was inhibited, while the addition of kinetin had no effect.

TABLE XX

INTERACTION EFFECTS OF DPU, KINETIN, ADENINE, "B" SOLUTION, AND RNA

Interaction	Mean Pct. Increase Wet Wt.	n_i	H	P
DPU × Kinetin				
Expected distribution, no int. . .	129.1	7	2.94	<.10 (ns)
Obtained distribution	69.7	7		
DPU × Adenine				
Expected distribution, no int. . .	639.4	7	8.97*	<.01
Obtained distribution	329.4	6		
DPU × "B" solution				
Expected distribution, no int. . .	470.8	7	9.75*	<.01
Obtained distribution	905.9	7		
DPU × RNA				
Expected distribution, no int. . .	91.9	7	8.97*	<.01
Obtained distribution	228.0	6		
Kinetin × Adenine				
Expected distribution, no int. . .	593.3	7	2.12	<.20 (ns)
Obtained distribution	509.8	7		
Kinetin × "B" solution				
Expected distribution, no int. . .	424.6	7	10.50*	<.001
Obtained distribution	85.7	8		
Adenine × "B" solution				
Expected distribution, no int. . .	945.0	7	8.97*	<.01
Obtained distribution	1269.5	6		
Adenine × RNA				
Expected distribution, no int. . .	556.1	7	10.50*	<.001
Obtained distribution	146.0	8		
"B" solution × RNA				
Expected distribution, no int. . .	387.4	7	0.07	<.80 (ns)
Obtained distribution	352.8	6		

*Represents a maximum value for H.

The calculation of the expected distribution is explained in "Statistical Methods."

TABLE XXI

EFFECT OF DPU, KINETIN, OR RNA IN MEDIUM CONTAINING ADENINE AND "B" SOLUTION
(cultures were maintained in the dark at 27°C for 25 days)

Medium Used	Additives	Mean Pct. Increase Wet Wt.	n_i	H	P
PW + Adenine + "B" solution	Basal medium	1269.5	6	6.50	<.02
	DPU	893.7	6		
PW + Adenine + "B" solution	Basal medium	1269.5	6	0.0	(ns)
	Kinetin	1291.9	8		
PW + Adenine + "B" solution	Basal medium	1269.5	6	7.50*	<.01
	RNA	711.9	5		

*Represents a maximum value for H.

Basal medium = medium used with no additive.

DISCUSSION

This study was undertaken with the intent of achieving, in vitro, further chemical control of differentiation. It was necessary, however, first to isolate a suitable tissue and second to obtain a completely defined medium on which to grow it.

Tragopogon porrifolius was selected because its roots possessed good powers of regeneration and in addition were sufficiently large that sterile segments could be obtained. The size of the root was an important factor in the choice of T. porrifolius since it was found impossible to obtain sterile segments from Taraxacum, Cichorium, or indeed from another species of Tragopogon. The difficulty in culturing the latter plants seemed to be due to the presence of endogenous contaminants which made it impossible to achieve sterility.

While Tragopogon appeared to be the most convenient member of the Compositae to use, it does not necessarily follow that it is the most suitable for differentiation studies. Gautheret (13) compared the growth of root slices of Helianthus tuberosus, Tragopogon, and Cichorium and found that the Helianthus gave feeble growth with no organ formation, while the Tragopogon formed callus, and, at the end of 40 to 60 days, leafy shoots; at the end of the first day the Cichorium had produced little roots, callus, and leafy stem. Naylor (32) studied root cuttings of Taraxacum kept in moist chambers and found that at the end of two or three days shoots had formed at the proximal ends and roots at the distal ends.

Although some of these plants have been grown in culture and have proliferated rapidly, undefined media and varying growth conditions have been used. In addition, these cultures were not studied for their ability to differentiate. For these reasons comparison with Tragopogon porrifolius is rendered difficult. What can be said, however, is that the latter plant in culture offers many advantages, and it has proved to be a useful tool in this research.

In developing a defined medium, an auxanographic (37) approach was used. This deliminted in a broad way the types of chemicals which were needed for growth. By this means it became apparent that a balance between amino acids, RNA derivatives, and possibly other factors was needed for optimal growth. The amino acids were selected arbitrarily, and, while the growth obtained using the "B" solution in combination with 100 mg/l adenine sulphate was comparable to that obtained using a coconut milk medium, it might be even further improved through extension of these studies. The composition of the defined medium is summarized in Table XXII.

Basal media such as White's or Gautheret's on which tissue cultures are grown are composed of major elements, trace elements, vitamins, and a carbohydrate. Frequently, cultures require the addition of such factors as IAA, YE, or CM. For example, cultures of Cichorium were grown by Gautheret on basal media alone. Most cultures of monocotyledons require the addition of both an auxin and CM to the medium. However, no generalizations could be made as to the growth requirements in defined media of higher plant tissue cultures.

It was found that when the "B" solution contained a high concentration of tyrosine the tissue growing on media containing this solution produced the enzyme tyrosinase. This was considered

TABLE XXII

COMPOSITION OF OPTIMAL DEFINED MEDIUM, BASED ON EXPERIMENTS REPORTED IN RESULTS

PW Medium (see pages 12-13)

Amino Acids	Molarity of Medium
DL-isoleucine	5×10^{-4}
DL-methionine	5×10^{-4}
L-tyrosine	2×10^{-4}
L-alanine	7.4×10^{-4}
L-glutamic acid	5×10^{-4}
DL-serine	5×10^{-4}
L-arginine	5×10^{-4}
L-histidine	5×10^{-4}
L-cystine	4.5×10^{-5}

Adenine Sulphate 100 mg/l

to be of special interest since the study of induced enzymes has thus far been almost exclusively limited to the lower organisms--the bacteria, yeasts, and fungi. There have been a few exceptions to this, including the induction of the formation of tryptophane oxidase following the administration of tryptophane to rats or rabbits (22), and Galston (9) has reported on the induction of indole acetic acid oxidase in higher plants.

That the formation of tyrosinase by Tragopogon tissue cultures is truly an induced enzyme phenomenon would be upheld

according to the interpretation of Monod (29). He defines the phenomenon as L'induction de la biosynthèse des enzymes sous l'influence de substances spécifiques. In many cases the inductor is also a substrate of the enzyme, but this need not be, and to make clear the distinction he suggests the term "inductor-substrate." It would be of considerable interest to follow up this observation both from the standpoint of induced enzymes in general and because of the wide distribution of tyrosinase in plants.

As was pointed out in the text, a comparison of the effects of DPU, kinetin, and adenine in two different experiments indicated that the response to DPU and kinetin had altered. Thus, in experiment I, DPU and kinetin both inhibited growth, while in experiment II they had no effect on growth. In addition, changes had been noted in the morphology of the tissue during the time between these replications. It appeared to have fewer differentiated areas and to be less firm and more watery than usual.

That tissues do undergo certain changes is a well-known phenomenon. However, these changes have always been associated with a response to IAA. Gautheret (17) has described in detail the habituation accoutumance) of Daucus and other tissues. In the case of Daucus, root discs showed but little growth unless the medium contained IAA. A concentration of 1×10^{-5}M was optimal at the outset, but after a few transfers the optimal dose became fixed around 1×10^{-8}M to 1×10^{-7}M. At first the tissue was compact and produced numerous roots, but after eight to ten transfers some colonies had completely lost the power to form roots and at the same time they had become friable and translucent in appearance. When these were transferred to media devoid of IAA they continued to grow and proliferate as rapidly as in the presence of this

compound. Moreover, when grown on media containing various concentrations of IAA, no difference in growth resulted.

Gautheret attempted to interpret the phenomenon on the basis of both anatomical and physiological observations. He found that vascular elements were less frequently found in habituated tissue than in normal tissue, and that the translucent appearance was due to the intercellular spaces being filled with fluid rather than with air as in the case of normal tissue. As for the physiology of this change, he proposed two theories for investigation: the first, that habituated cells were modified in such a way as to be truly insensitive to IAA and capable of dividing without it; and the second, that the modifications resulted in an increase in their power to elaborate IAA such that its exogenous supply was unnecessary. Kulescha (24) found that habituated tissues contained more auxin than normal tissues and so supported Gautheret's second theory. This did not explain why habituated and tumor tissue were able to elaborate more auxin than normal tissue. Henderson and Bonner (19), studying normal and crown gall tissue, invoked the intervention of a substance inhibiting the enzymatic transformation of tryptophane to IAA. As Gautheret suggests, however, it might possibly be an enzyme which destroys IAA. He found, in studying habituation in a number of tissues, that it was manifested in varying degrees and was not an "all or none" phenomenon.

The morphological changes which occurred in the Tragopogon tissue of the present study are not unlike those described by Gautheret for habituated tissue of Daucus and other plants. However, since this tissue never required IAA above that supplied in coconut milk, this change cannot be related to a sudden ability to elaborate this compound. DPU, on the one hand, is present in coconut milk in small amounts, and therefore the change in response to this

factor, discussed previously, could be a habituation phenomenon. On the other hand, the change in response to kinetin cannot be considered under the same heading because the tissue was not exposed regularly to this compound. Up to this point it has been isolated from herring sperm and from yeast extract (Skoog, 42).

Gautheret felt that the term accoutumance first used in the French literature might better be changed to anergie, a word coined from two Greek words meaning loss of action. The English term "habituation," a translation of accoutumance, might be further extended in the present case, to mean a change in action rather than just loss of action. This suggestion is made because in some cases the alterations discussed could be due to the acquisition of some property rather than a loss as is implicit in Gautheret's definition.

A phenomenon which has received but little attention in nutritional studies is interaction or synergism. This was described by Steward and Caplin (44), who found that tissue cultures derived from Solanum tuber would not grow on a coconut milk medium, nor would they survive on media containing 2,4-D alone. If these factors were combined, however, they acted to stimulate growth markedly.

In the present study it was found that when two factors were added together to the basal medium the amount of growth which resulted was frequently more, or less, than would be expected on the basis of a simple additive effect. This was attributed to interaction. For example, in experiment II the interaction of kinetin with "B" solution resulted in much less growth than would be expected on the basis of a simple additive effect, and conversely the interaction of adenine with "B" solution resulted in more growth.

These findings have given a quantitative expression to synergism and open the way for detailed and critical examination of other

cases. The difficulties involved in the interpretation of interactions can largely be circumvented by the use of techniques like those developed in this investigation..

The study of the chemical control of differentiation received its initial impetus from the work of Skoog and Tsui (43), using adenine sulphate and naphthalene acetic acid on Nicotiana tissue. On the whole, tissue cultures have not proved to be as amenable as stem segments or root discs for such studies, though they are perhaps more desirable since after many transfers there is little or no carry-over of such substances as IAA from the original plant. Skoog and Tsui (43) used tissue cultures of Nicotiana and found that, when 10 mg/l of adenine sulphate was added to the medium and twenty-one cultures were observed, seven of these formed a total of fourteen buds. At this time Skoog switched to the use of stem segments rather than tissue cultures, and used these for all his subsequent studies.

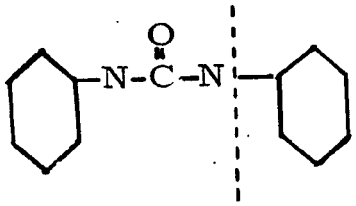
Miller and Skoog (26) found that the addition of IAA to the medium caused the complete inhibition of buds, and this effect could be reversed by the addition of the appropriate concentration of adenine sulphate. These experiments were of 35 days' duration, and in this time the mean number of buds produced by the control segments was 3.2; where 40 mg/l adenine sulphate was added to the medium the mean number of buds was 5.4. If 5 µg IAA was added to the medium 75 mg/l adenine was required to obtain the same number of buds as the control segments.

From these observations it is apparent that a balance must be maintained between the IAA and adenine levels if buds are to be induced. In principle, then, normal tissues to which IAA must be supplied externally should be induced to produce buds more readily

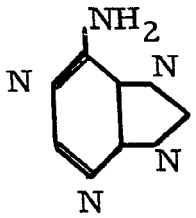
than tumor tissue or habituated tissue in which the IAA content is high.

In view of Skoog's results, it was not surprising that Tragopogon produced organs when exposed to adenine or, indeed, to RNA. While the RNA components which were added separately to the medium did not induce organ formation it is likely that certain combinations might prove effective. Skoog (41) found that the number of buds induced in Nicotiana stem segments by adenine could be increased through the combination of adenine with guanine, thymine, or cytosine. Uracil was not effective. It is clear, therefore, that herein lies another important area of future investigation since recent advances in our knowledge of nucleic acid chemistry have expanded the list of precursors and components of these substances. It is likely that combinations of these will prove to be important in the induction of differentiation.

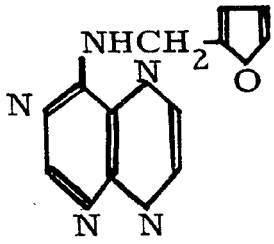
It is of interest that, in the present study, leaf formation was induced by DPU, a compound isolated by Shantz and Steward (38) from coconut milk. The formula of this compound and that of adenine and kinetin are given as follows:



DPU



Adenine



Kinetin

If a break were to occur separating the guanidylic group and one phenyl ring in DPU, a possible precursor for a purine or pyrimidine might result. That this happens is suggested by the fact that only DPU and purine derivatives have thus far been found to induce differentiation. To our knowledge this is the first reported case

wherein a compound other than a purine or purine-containing substance like kinetin has been reported to have such activity. The extension of these findings to related compounds should disclose interesting facets of the specificity involved in the action of substances that induce differentiation.

wherein a compound other than a purine or purine-containing substance like kinetin has been reported to have such activity. The extension of these findings to related compounds should disclose interesting facets of the specificity involved in the action of substances that induce differentiation.

SUMMARY

1. Tissue cultures were established from both the root and the leaf of Tragopogon porrifolius.

2. These cultures were well suited to the study of differentiation since they produced all the cell types found in the parent plant.

3. At no time did they form organs without the addition of differentiation-inducing factors.

4. Initially the cultures could not be grown without the addition of coconut milk to the medium.

5. By means of the auxanographic technique the types of chemicals needed for growth were delimited and a defined medium was evolved.

6. In addition to the Plain White's medium, this consisted of a mixture of amino acids and adenine sulphate.

7. After being maintained in culture for several years the root tissue appeared to undergo habituation, although this was not to IAA as is the usual case.

8. This phenomenon resulted in an altered response to diphenyl urea, a factor isolated from coconut milk.

9. The statistical technique used to analyze the data made possible an evaluation of interaction effects as well as simple effects.

10. By this means it was possible to give quantitative expression to synergism, a type of interaction which has received but little attention in nutritional studies.

11. Differentiation of roots and leaves could be induced by the addition of adenine sulphate, hydrolyzed ribose nucleic acid, or diphenyl urea to the medium.

12. This is believed to be the first report that DPU acts as an organizing factor.

13. When a high concentration of tyrosine was used in the media it induced the formation of the enzyme tyrosinase.

LITERATURE CITED

1. Caplin, S. M., and Steward, F. C. 1948. Effects of coconut milk on the growth of explants from carrot root. Science. 108:655-657.
2. Démétriades, S. D. 1953. Sur un cas de différentiation des tissus de Crown-Gall de Scorsonère cultivés in vitro. Compt. Rend. Soc. Biol. 147:1713-1715.
3. Duhamet, L. 1949. Action du lait de Coco sur la croissance des tissus du tubercule de Topinambour cultivés in vitro. Compt. Rend. Acad. Sci. 229:1353-1355.
4. _____. 1950. Action du lait de Coco sur la croissance des tissus de Parthenocissus tricuspidata cultivés in vitro. Compt. Rend. Soc. Biol. 144:59.
5. _____. 1950. Action du lait de Coco sur la croissance des tissus de Crown-Gall de Scorsonère cultivés in vitro. Compt. Rend. Acad. Sci. 230:770-771.
6. _____. 1951. Action du lait de Coco sur la croissance des cultures des tissus de Crown-Gall de Vigne, de Tabac, de Topinambour et de Scorsonère. Compt. Rend. Soc. Biol. 145:1781-1783.
7. _____. 1951. Action du lait de Coco sur les tissus accoutumés aux hétéro-auxines de Scorsonère, de Tabac et de Vigne. Compt. Rend. Soc. Biol. 145:1783-1785.
8. _____. 1951. Action comparée du lait de Coco sur une souche de tissus de Carotte et sur des fragments prélevés sur des racines. Compt. Rend. Soc. Biol. 145:1841.

9. Galston, A. W., and Dalberg, L. Y. The adaptive formation and physiological significance of indole-acetic acid oxidase. Amer. Jour. Bot. 41:373-380.

10. Gautheret, R. J. 1939. Sur la possibilité de réaliser la culture indéfinie des tissus de tubercules de Carotte. Compt. Rend. Acad. Sci. 208:118-120.

11. _____. 1940. Recherches sur le bourgeonnement du tissu cambial d'Ulmus campestris cultivé in vitro. Compt. Rend. Acad. Sci. 210:632-634.

12. _____. 1940. Nouvelles recherches sur le bourgeonnement du tissu cambial d'Ulmus campestris cultivé in vitro. Compt. Rend. Acad. Sci. 210:744-746.

13. _____. 1941. Recherches sur la croissance de fragments de tissus de quelques végétaux apartenant a la famille des Composées. Compt. Rend. Acad. Sci. 212:1098.

14. _____. 1941. Sur le repiquage des cultures de tissus d'Endive, de Salsifis et de Topinambour. Compt. Rend. Acad. Sci. 213:317.

15. _____. 1941. Caractères anatomiques et cytologiques de tranches d'Endive, Salsifis et Topinambour cultivées in vitro. Compt. Rend. Soc. Biol. 135:1161.

16. _____. 1954. Catalogue des cultures de tissus végétaux. Rev. Gén. Bot. 61:672-702.

17. _____. 1955. Sur la variabilité des propriétés physilogiques des cultures de tissus végétaux. Rev. Gén. Bot. 62:5-112.

18. _____. 1955. The nutrition of plant tissue cultures. Ann. Rev. Plant Physiol. 6:433-484.

19. Henderson, J. H., and Bonner, J. 1952. Auxin metabolism in normal and crown-gall tissue of sunflower. Amer. Jour. Bot. 39:445-451.

20. Henderson, J. H., Durrell, M. E., and Bonner, J. 1952. The culture of normal sunflower stem callus. Amer. Jour. Bot. 39:467-473.

21. Jacquoit, C. 1951. Action du méso-inositol et de l'adenine sur la formation de bourgeons par le tissu cambial d'Ulmus campestris cultivé in vitro. Compt. Rend. Acad. Sci. 233:815-817.

22. Knox, W. E., and Mehler, A. H. 1951. The adaptive increase of the tryptophan peroxidase-oxidase system of liver. Science 113:237-238.

23. Kruskal, W. H., and Wallis, W. A. 1952. Use of ranks in one-criterion variance analysis. Jour. Amer. Stat. Assoc. 47:583-621.

24. Kulescha, Z. 1954. Croissance et teneur en auxine de divers tissus normaux et tumoraux. Anné Biol. 30:319-327.

25. Mauney, J. R., Hillman, W. S., Miller, C. O., Skoog, F., Clayton, R. A., and Strong, F. M. 1952. Bioassay, purification and properties of a growth factor from coconut. Physiol. Plantarum 5:485-497.

26. Miller, C. O., and Skoog, F. 1953. Chemical control of bud formation in tobacco stem segments. Amer. Jour. Bot. 40:768-773.

27. Miller, C. O., Skoog, F., VonSaltza, M. H., and Strong, F. M. 1955. Kinetin, a cell division factor from deoxyribonucleic acid. Jour. Amer. Chem. Soc. 77:1392.

28. Miller, C. O., Skoog, F., Okumura, F. S., VonSaltza, M. H., and Strong, F. M. 1955. Structure and synthesis of kinetin. Jour. Amer. Chem. Soc. 77:2662.

29. Monod, J., et Cohn, M. 1952. La biosynthese des enzymes (Adaptation Enzymatique). Advances in Enzymology 13: 67-119.

30. Morel, G. 1950. Sur la culture des tissus de deux Monocotyledones. Compt. Rend. Acad. Sci. 230:1099-1101.

31. Morel, G., and Wetmore, R. H. 1951. Tissue culture of Monocotyledons. Amer. Jour. Bot. 38:138-140.

32. Naylor, E. 1941. The proliferation of dandelions from roots. Bull. Torrey Bot. Club. 68:351-358.

33. Nickel, L. G. 1950. Effect of coconut milk on the growth in vitro of plant virus tumor tissue. Bot. Gaz. 112:225-228.

34. Nobécourt, P. 1939. Sur la pérennité et l'augmentation de volume des cultures de tissus végétaux. Compt. Rend. Soc. Biol. 130:1271.

35. _____. 1939. Sur les radicelles naissant des cultures de tissus du tubercule de carotte. Compt. Rend. Soc. Biol. 130:1271.

36. _____. 1947. Apparition de pousses feuillées sur des cultures de tissus de Carotte. Compt. Rend. Soc. Biol. 141:590.

37. Pontecorvo, G. 1949. Auxanographic techniques in biochemical genetics. Jour. Gen. Microbiol. 3:122-126.

38. Shantz, E. M., and Steward, F. C. 1952. Coconut milk factor: the growth promoting substances in coconut milk. Jour. Amer. Chem. Soc. 74:6133.

39. Skoog, F. 1939. Experiments on bud inhibition with indole-3-acetic acid. Amer. Jour. Bot. 26:702-707.

40. Skoog, F. 1944. Growth and organ formation in tobacco tissue cultures. Amer. Jour. Bot. 31:19-24.

41. _____. 1954. Substances involved in normal growth and differentiation of plants. Brookhaven Symposia in Biology No. 6:1-21.

42. _____. 1955. Initiation of cell division and organ formation in plant tissues. Jour. Cell. and Comp. Physiol. 46:365.

43. Skoog, F., and Tsui, C. 1948. Chemical control of growth and bud formation in tobacco stem segments and callus cultured in vitro. Amer. Jour. Bot. 35:782-787.

44. Steward, F. C., and Caplin, S. M. 1951. A tissue culture from potato tuber: The synergistic action of 2,4-D and of coconut milk. Science 113:518.

45. _____. 1952. Investigations on the growth and metabolism of plant cells. IV. Evidence on the role of the coconut milk factor in development. Ann. Bot. 16:491-504.

46. Steward, F. C., and Shantz, E. M. 1955. The growth of tissue cultures by cell division and cell enlargement: The effect of certain specified substances. A.I.B.S. Meeting.

47. Straus, J. 1953. Studies on maize endosperm-derived tissue grown in vitro; culture requirements, morphology, and cytology. Thesis, University of Michigan.

48. Van Overbeek, J. 1942. Hormonal control of embryo and seedling. Cold Spring Harbor Symposia on Quantitative Biol. 10:126-134.

49. Van Overbeek, J., Conklin, M. E., and Blakeslee, A. F. 1942. Cultivation in vitro of small Datura embryos. Amer. Jour. Bot. 29:472-477.

50. Van Overbeek, J., Sui, R., and Haagen-Smit, A. J. 1944. Factors affecting the growth of Datura embryos in vitro. Amer. Jour. Bot. 31:219-224.

51. Warmke, H. E., and Warmke, G. L. 1946. The role of auxin in the differentiation of roots and leaves in Taraxacum. Amer. Jour. Bot. 31:827.

52. _____. 1950. The role of auxin in the differentiation of root and shoot primordia from root cuttings of Taraxacum and Cichorium. Amer. Jour. Bot. 37:272-280.

53. White, P. R. 1931. Plant tissue cultures. The history and present status of the problem. Arch. fur. exp. Zellf. 10:501-508.

54. _____. 1936. Plant tissue cultures. Bot. Rev. 2:419-437.

55. _____. 1939. Controlled differentiation in a plant tissue culture. Bull. Torrey Bot. Club 66:507-513.

56. _____. 1942. Plant tissue cultures. Ann. Rev. Biochem. 11:615-628.

57. _____. 1946. Plant tissue cultures II. Bot. Rev. 12:521-528.

58. _____. 1951. Nutritional requirements of isolated plant tissues and organs. Ann. Rev. Plant Physiol. 2:231-244.

59. Wiggans, S. C. 1954. Growth and organ formation in callus tissues derived from Daucus carota. Amer. Jour. Bot. 41:321-326.

APPENDIX I

KRUSKAL-WALLIS H TEST--TEST FOR SIMPLE EFFECT
(this example was taken from the data of experiment I)

Kinetin Effect			
Pct. Increase Wet Weight PW Medium	Rank	Pct. Increase Wet Weight PW+K Medium	Rank
32.1	12	148.3	5
162.2	4	30.2	14
295.1	1	43.3	11
94.1	7	68.1	9
100.9	6	57.9	10
243.7	3	30.9	13
83.1	8		
259.4	2		
R_i =	43		62
R_i^2 =	1849		3844
n_i =	8		6
R_i^2/n_i =	231.1		640.6

$$H = \left[\frac{12}{n(n+1)}\right]\left[\sum_i^k \frac{R_i^2}{n_i}\right] - 3(n+1)$$

$$H = 12/(14\times15) \times 871.7 - 45 = 4.77 \text{ (1 df)}$$

$$P < 0.05$$

APPENDIX II

KRUSKAL-WALLIS H TEST--TEST FOR INTERACTION USING THE COMPARISON OF CURVES TECHNIQUE

This example was taken from the data of the casein hydrolysate experiment. The raw data may be seen in Appendix V. The following distributions were obtained by subtracting each of the means in row 1 of the raw data from each of the observations in the corresponding column of row 2 of the raw data.

Interaction Studied: CH × RNA

(.01% RNA + .5% CH) - .5% CH	Rank	(.01% RNA + 1% CH) - 1% CH	Rank	(.01% RNA) + 2% CH) - 2% CH	Rank
-103.7	14	48.4	8	44.4	9
-109.4	15	179.4	2	54.3	7
- 75.8	16	142.3	4	16.4	13
-119.6	17	88.7	5	78.8	6
+ 44.7	10	152.3	3	24.0	11
-141.3	18	180.8	1	21.0	12
R_i =	90		23		58
R_i^2 =	8100		529		3364
n_i =	6		6		6
R_i^2/n_i =	1350.0		88.1		560.6

$$H = \left[\frac{12}{n(n+1)}\right]\left[\sum_i^k \frac{R_i^2}{n_i}\right] - 3(n+1)$$

$$H = 12/(18 \times 19) \times 1998.7 - 57 = 13.1 \text{ (2 df)}$$

$$P < 0.01$$

APPENDIX III

KRUSKAL-WALLIS H TEST--TEST FOR INTERACTION USING THE DIRECT COMBINATION OF MAIN EFFECTS TECHNIQUE

This example was taken from the data of experiment I. The raw data may be seen in Appendix VII. The expected distribution was determined in the following way: The DPU effect is given by subtracting the control mean from the DPU mean (75.3 - 159.1 = -83.8). Similarly, the kinetin effect is given by subtracting the control mean from the kinetin mean (63.1 - 159.1 = -96.0). These effects summed (-179.8) are then added to each of the observations in the control group to give the expected distribution without interaction for the combination of DPU and kinetin in PW medium.

Interaction Studied: DPU × Kinetin			
Expected Distribution, No Interaction	Rank	Obtained Distribution	Rank
-147.7	12	18.7	6
- 7.6	13	66.5	4
+115.3	1	62.6	7
- 65.7	14	112.8	2
- 78.9	15	33.4	8
+ 63.9	5	33.3	9
- 96.7	16	45.0	10
+ 79.6	3	59.6	11
R_i =	89		57
R_i^2 =	7921		3249
n_i =	8		8
R_i^2/n_i =	990.1		406.1

APPENDIX III (Continued)

$$H = \left[\frac{12}{n(n+1)}\right]\left[\sum_{i}^{k}\frac{R_i^2}{n_i}\right] - 3(n+1)$$

$H = 12/(16\times17) \times 1396.2 - 51 = 10.57$ (1 df)

$P < 0.01$

APPENDIX IV

KRUSKAL-WALLIS H TEST--EXAMPLE OF POOLING OF DATA

This example was taken from the data of experiment II. The raw data may be seen in Appendix IX. Since it had been established that the effect of adenine was uniform in both a PW medium and a PW plus kinetin medium, it was possible to pool the data for these media to test for the adenine effect. To prevent distortion attributable to unequal numbers of observations for each medium used, it was necessary to add the mean observation for the group in two instances (note observations marked "a"). This procedure results in an unbiased value for H.

Pooled Adenine Effect				
	PW	Rank	PW + Adenine	Rank
	147.3	18	622.0	6
	66.5	25	637.5	5
	73.6	23	710.5	2
	45.7	29	484.4	12
	110.1	19	481.1	13
	52.5	26	602.4	7
	38.9	30	493.4	11
	76.3[a]	22	688.2	3
Mean =	76.3		590.0	

APPENDIX IV (Continued)

	PW + Kinetin	Rank	PW + Kinetin + Adenine	Rank
	156.0	17	435.6	15
	69.5	24	507.4	10
	38.0	31	663.8	4
	31.5	32	177.9	16
	49.5	27	555.3	8
	102.2	20	752.1	1
	48.6	28	477.0	14
	91.1	21	509.8[a]	9
Mean =	79.6		509.8	
R_i =		392		136
R_i^2 =		153664		18496
n_i =		16		16
R_i^2/n_i =		9604.0		1156.0

Substituting in the formula given for H,

$$H = 12/(32 \times 33) \times 10760.0 - 99 = 23.6640$$

$$P < 0.001$$

APPENDIX V

RAW DATA FOR CASEIN HYDROLYSATE EXPERIMENT

Basal Media	Casein Hydrolysate		
	0.5%	1.0%	2.0%
PW	165.4	111.7	22.3
	185.2	116.7	37.3
	209.3	107.2	47.9
	315.6	64.8	27.9
	290.5	74.7	11.3
			25.8
Mean	233.2	95.0	28.5
PW + 0.01% RNA	129.5	143.4	73.3
	123.8	274.4	82.8
	157.4	237.3	44.9
	113.6	183.7	107.3
	277.9	247.3	52.5
	91.9	275.8	49.5
Mean	165.7	226.9	67.3
PW + 0.1% RNA	316.9	240.7	163.2
	139.4	243.9	225.2
	203.9	221.3	163.2
	223.0	199.4	152.7
	257.2	100.7	189.9
	282.3	185.3	139.4
Mean	237.1	198.5	172.2

APPENDIX V (Continued)

Basal Media	Casein Hydrolysate		
	0.5%	1.0%	2.0%
PW + 0.01% RNA + Cysteine and Tryptophane	42.9	63.8	66.1
	23.3	60.3	81.9
	43.2	58.1	43.4
	95.5	49.0	35.2
	45.1	57.3	58.5
		55.4	
Mean	50.5	57.7	57.0
PW + 0.1% RNA + Cysteine and Tryptophane	91.6	155.0	275.8
	31.6	171.4	154.7
	33.7	118.5	177.7
	53.2	145.8	203.5
	21.5	142.8	180.0
	22.6	162.9	130.9
Mean	42.3	144.4	187.1
PW + Cysteine and Tryptophane	cont.	21.9	30.7
		34.0	37.4
		1.8	34.3
		19.1	16.6
		71.6	20.8
			26.4
Mean		29.7	27.7

APPENDIX VI

RAW DATA FOR AMINO ACID EXPERIMENT OF PAGE 43

Basal Media	Solution "A"	Solution "B"	Solution "A" + "B"
PW	120.9	222.3	89.6
	110.7	249.6	101.5
	63.3	135.9	79.4
	68.7	199.5	105.9
	36.8	202.1	88.1
		150.2	115.4
		175.3	130.7
		285.0	103.9
Mean	80.0	202.5	101.9
PW + 0.5% RNA	231.9	160.2	94.4
	143.4	365.2	191.6
	139.5	287.6	83.7
	70.1	210.0	243.6
	32.1	152.1	
	120.0	188.8	
		391.5	
		378.5	
Mean	108.1	266.7	102.2
PW + 0.1% RNA + 0.1% YE	605.7	337.5	cont.
	482.3	272.4	
	660.5	165.8	
	791.5	168.6	
	652.5	163.0	
	576.9	270.6	
	522.0		
	498.2		
Mean	598.7	229.6	

APPENDIX VII

RAW DATA FOR EXPERIMENT I

Basal Media	PW	IAA	DPU	Kinetin	Adenine
PW	32.1	107.5	25.1	148.3	48.6
	162.2	100.5	127.6	30.2	78.4
	295.1	105.1	75.1	43.3	173.0
	94.1	65.6	102.8	68.1	174.5
	100.9	151.8	47.0	57.9	173.1
	243.7	148.2	169.8	30.9	131.8
	83.1	50.4	41.8		
	259.4	44.5	18.8		
Mean	159.1	96.6	75.3	63.1	129.9
PW + IAA			5.3	107.5	27.2
			34.3	120.5	29.7
			68.6	97.3	62.6
			27.0	138.9	143.0
			52.7	107.4	33.7
			37.1	39.9	58.8
			65.0	12.5	
				156.0	
Mean			41.4	97.3	59.2

APPENDIX VII (Continued)

Basal Media	PW	IAA	DPU	Kinetin	Adenine
PW + DPU				18.7	405.3
				66.5	365.4
				62.6	596.5
				112.8	440.2
				33.4	337.7
				33.3	1061.4
				45.0	
				59.6	
Mean				54.0	534.4
PW + Kinetin . . .					691.5
					339.7
					315.1
					629.2
					685.0
					843.0
					817.7
					332.1
Mean					581.7

APPENDIX VIII

RAW DATA FOR EXPERIMENT I
(concentration of IAA, DPU, and kinetin--10 mg/l)

Basal Medium	IAA	DPU	Kinetin
PW	34.0	64.8	189.2
	17.1	84.2	122.5
	46.5	158.7	1.3
	39.9	246.4	172.4
	93.2	174.2	114.9
	5.4	90.0	
	82.6	219.5	
	47.3	41.9	
Mean	45.8	135.0	120.1

RAW DATA FROM EXPERIMENT II
(SECOND-ORDER INTERACTIONS)

Basal Medium	RNA	DPU	Kinetin
PW + Adenine + "B" solution . . .	732.1	907.2	1091.2
	573.2	708.7	1010.0
	810.5	768.2	1209.3
	757.2	979.8	1969.8
	686.8	1245.0	1241.1
		753.6	639.1
			1626.2
			1548.8
Mean	711.9	893.7	1291.9

APPENDIX IX

RAW DATA FOR EXPERIMENT II

Basal Media	PW	Adenine	K	DPU	"A" solution	"B" solution	RNA
PW	147.3	622.0	156.0	101.9		401.3	16.4
	66.5	637.5	69.5	143.8	died	609.1	75.9
	73.6	710.5	38.0	164.7		245.6	44.0
	45.7	484.4	31.5	126.9		577.4	33.6
	110.1	481.1	99.5	159.8		275.2	
	52.5	602.4	102.2	99.2			
	38.9	493.4	48.6	74.3			
		688.2	91.1	136.4			
Mean	76.3	590.0	79.6	125.8		421.3	42.4
PW + Adenine . .			435.6	286.1	146.9	1337.5	127.9
			507.4	296.0	60.7	1061.4	98.4
			663.8	372.5	201.6	1418.4	186.1
			177.9	421.5	175.1	1319.5	150.0
			555.3	451.4		1433.7	199.1
			752.1	149.1		1046.6	88.3
			477.0				182.0
							136.8
Mean			509.8	329.4	146.0	1269.5	146.0
PW + Kinetin . . .				149.3	died	84.8	died
				12.0		32.1	
				22.5		197.1	
				116.3		58.2	
				112.6		61.9	
				65.1		163.5	
				10.5		38.6	
						49.8	
Mean				69.7		85.7	

APPENDIX IX (Continued)

Basal Media	PW	Ade-nine	K	DPU	"A" solu-tion	"B" solu-tion	RNA
PW + DPU					died	805.0	182.8
						1056.3	284.1
						1080.2	175.0
						978.6	281.1
						1021.3	248.8
						721.8	196.2
						678.3	
Mean						905.9	228.0
PW + "A" solution						most died	most died
PW + "B" solution							390.4
							383.1
							556.1
							331.3
							370.1
							87.2
Mean							352.8