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TECHNICAL REPORT

69-36-FL

GROWTH OF PLANT CELL CULTURES

II. Nutrient Requirements and Their Role in the Growth of Suspension Cultures

by

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FOREWORD

This report represents the second phase of the work on culture of cells of higher plants undertaken at Natick under the Unconventional Food program in the Microbiology Division of the Food Laboratory. The objective of this study is to determine whether it would be feasible to use plant cell cultures as a source of human food. The objective of this phase was to investigate the nutrient requirements of suspension cultures, to simplify media, and improve growth rates.

The work covered in this report was performed under Department of the Army Project 1J014501A71C.

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ABSTRACT

A detailed study has been made of some nutrient requirements of several plant cell suspension cultures. These cultures require the usual inorganic salts including inorganic nitrate nitrogen. Hydrolyzed protein or amino acids will serve as the sole source of nitrogen for growth, but organic nitrogen is not required. Urea or ammonia nitrogen in the absence of nitrate will not support growth. Sucrose is an excellent carbon source. Glucose and starch will also support growth of some cultures. Sucrose sterilized by gamma irradiation supports normal growth of these cultures. Growth is retarded on glucose sterilized by gamma irradiation at ambient temperature, but growth is normal on glucose irradiated at -80°C. A few growth factors of low concentration are also required. An auxin (2,4 - dichlorophenoxyacetic acid or naphthaleneacetic acid) and thiamine are required by all cultures. Kinetin (6 furfurylaminopurine) is also required by some cultures. Maximum productivity on this simple medium is about 1.5 grams dry weight per liter of culture per day, equal to, but not significantly greater than productivities obtained on much more complex media.

GROWTH OF PLANT CELL CULTURES

II. NUTRIENT REQUIREMENTS AND THEIR ROLE IN THE GROWTH OF SUSPENSION CULTURES

INTRODUCTION

In our first report (12) we described the isolation of twenty-nine strains of plant cells in culture and the initial studies of their growth on various solid and liquid media. We found that plant cells could be grown on simple defined media for many transfers maintaining growth rates equal to or better than those obtained on complex media containing supplements such as coconut milk. Growth tended to be of zero order with productivity at best about 10 grams dry weight per liter of culture per day. Such growth rates are of the same order of magnitude as are found for higher plants growing naturally under optimum conditions.

This study was aimed toward investigating in more depth the role of the various nutrients in the defined medium with the emphasis on the vitamins and hormones. The primary objective was to establish the minimum nutrient requirements of these plant cell cultures. We also hoped that changes in the nutrient composition might lead to faster growth rates, or greater productivity.

MATERIALS AND METHODS

The methods and cultures used in this study have been described in our first report (12). The basic medium was that of Murashige and Skoog (13) (Table 1) with 3.0% sucrose, and either 0.05 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) referred to as M medium or 0.10 mg/L of naphthaleneacetic acid (NAA) referred to as NAA medium. Suspension cultures were used in all tests. Stock suspension cultures were maintained in shake flasks and transferred (10% v/v) at intervals of about 3 weeks. These cultures were used to inoculate

Table 3 Murashige and Skoog Medium for Growth of Plant Tissue Cultures (13)

Component	mg/Liter
$K NO_3$	1900
$NH_4 NO_3$	1650
$Ca Cl_2 \cdot 2 H_2O$	440
$Mg SO_4 \cdot 7 H_2O$	370
KH_2PO_4	170
$Mn SO_4 \cdot 4 H_2O$	22.3
$Zn SO_4 \cdot 4 H_2O$	8.6
$H_3 BO_3$	6.3
KI	0.83
Molybdic acid	0.25
$Cu SO_4 \cdot 5 H_2O$	0.25
$Co Cl_2 \cdot 6 H_2O$	0.25
Sodium EDTA	37.3
$FeSO_4 \cdot 7 H_2O$	27.8
Inositol	100
Nicotinic acid	0.5
Pyridoxine	0.5
Kinetin (6-furfurylaminopurine)	0.32
Thiamine	0.1-0.5
2,4 dichlorophenoxyacetic acid (2,4-D)*	0.05
Sucrose	30,000
pH 5.5	
Optional additives	
Phytone (BBL) - soy peptone	1000
Tryptone (Difco) - a meat peptone	1000
Glycine	2

* α naphthaleneacetic acid (NAA) 0.1 mg/L can be substituted for 2,4-D.

experiments using a 5 - 10% (v/v) inoculum to give an initial dry weight of 0.5 - 1.0 mg/ml. Cultures were grown on reciprocating or rotary shakers at 26° - 28°C in continuous dark, continuous white fluorescent light of low intensity (840 foot candles) or in light programmed on 16 hours, off 8 hours. Growth is illustrated or expressed as Productivity, mg dry weight increase per ml per day. All weights were measured after drying at 70°C. Other methods are described in the discussions of the various experiments.

PART I. MINERALS, CARBON SOURCES, AND NITROGEN SOURCES IN THE MEDIUM

A. Effects of Calcium, Magnesium, and Phosphate Concentration.

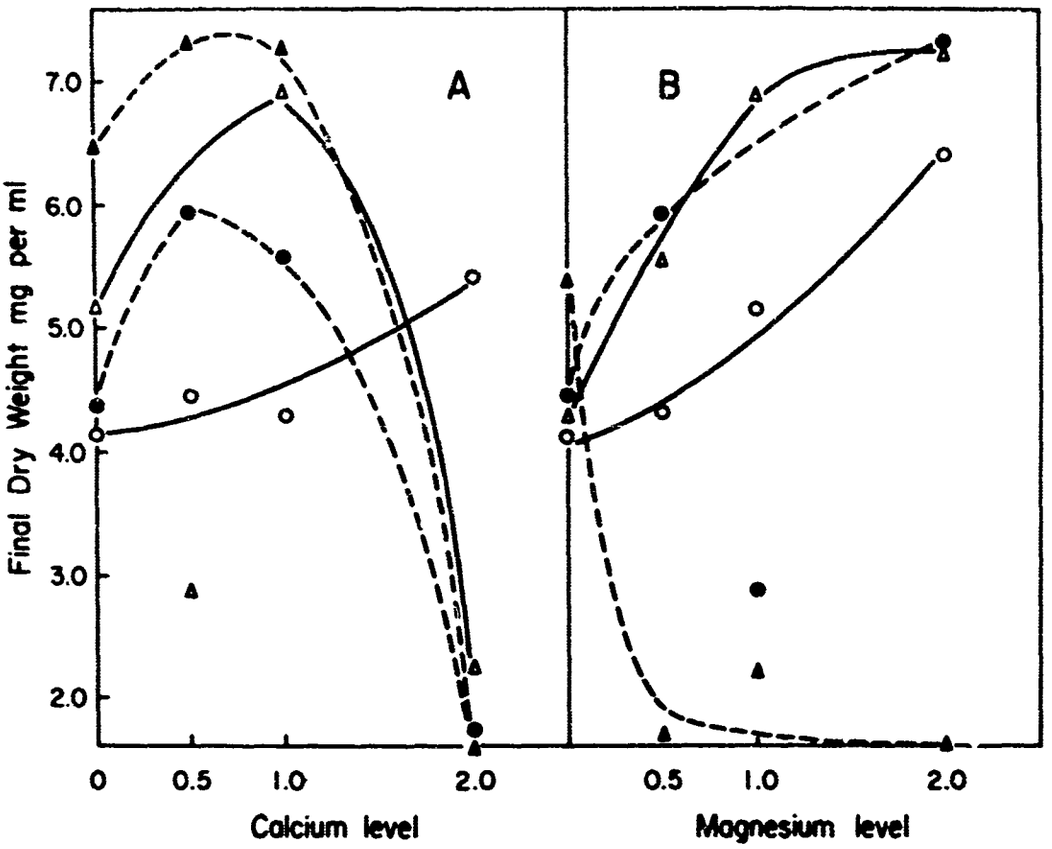
For carrot cells grown in the light of M medium (Fig. 1) the usual calcium level was optimum in the presence of three levels of magnesium. Doubling the calcium level had an unfavorable effect. Doubling the usual magnesium level enhanced growth at or below the usual calcium level. Similar results were found for carrot and bean cells grown in the dark (Fig. 2). A two-or three-fold increase in the phosphate level was somewhat beneficial (Fig. 2).

B. Carbon Sources.

1. Sucrose compared to glucose, glycerol, and starch.

Sucrose is the most favorable carbon source tested for plant cell cultures although many will grow quite well on glucose (Table 2, Fig. 3). Attempts to replace sucrose with glycerol have not been successful (Table 2, Fig. 3). Bean cultures will grow well on starch, particularly if previously grown on starch (Fig. 4).

Figure 1 Effect of Calcium and Magnesium on Growth of Carrot No. 2

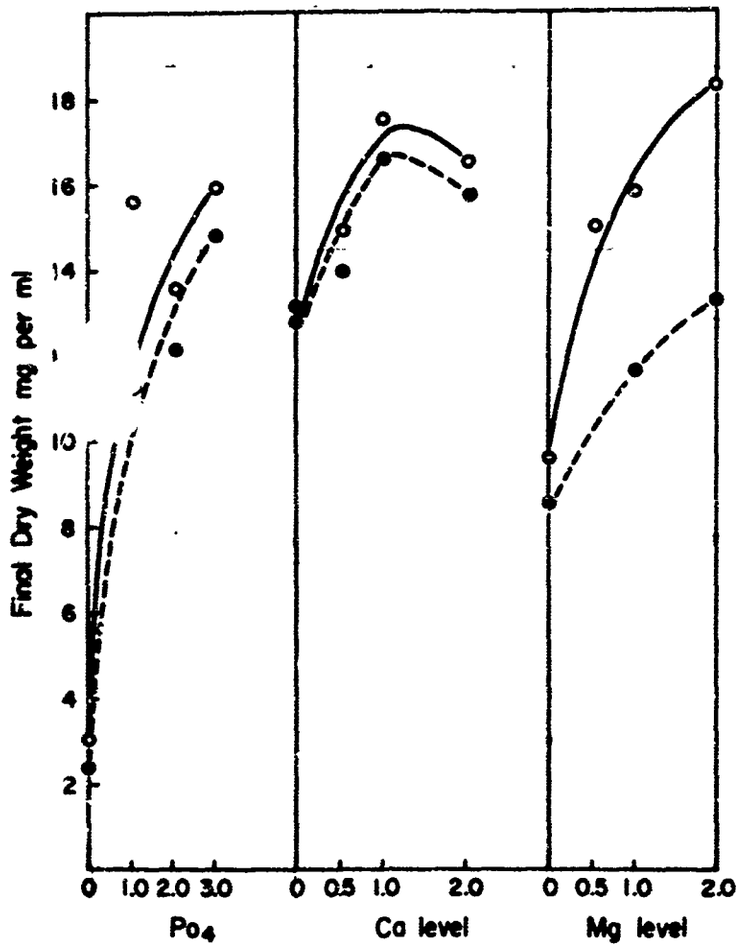


10% Inoculum, cultures grown for 27 days in continuous light on the rotary shaker, 2,4-D 0.05 mg/L

	A	B
○——○	No Mg	Nc Ca
●- - -●	Mg 0.5	Ca 0.5
△——△	Mg 1.0 *	Ca 1.0 *
▲- - -▲	Mg 2.0	Mg 2.0

* 1.0 = usual level in medium (Table 1).

Figure 2 Effect of Calcium, Magnesium, and Phosphate on Growth of Carrot No. 2 and Bean No. 8



10% Inoculum, grown for 21 days in the dark on the rotary shaker. 2,4-D 0.05 mg/L

1.0 = usual level in medium (Table 1).

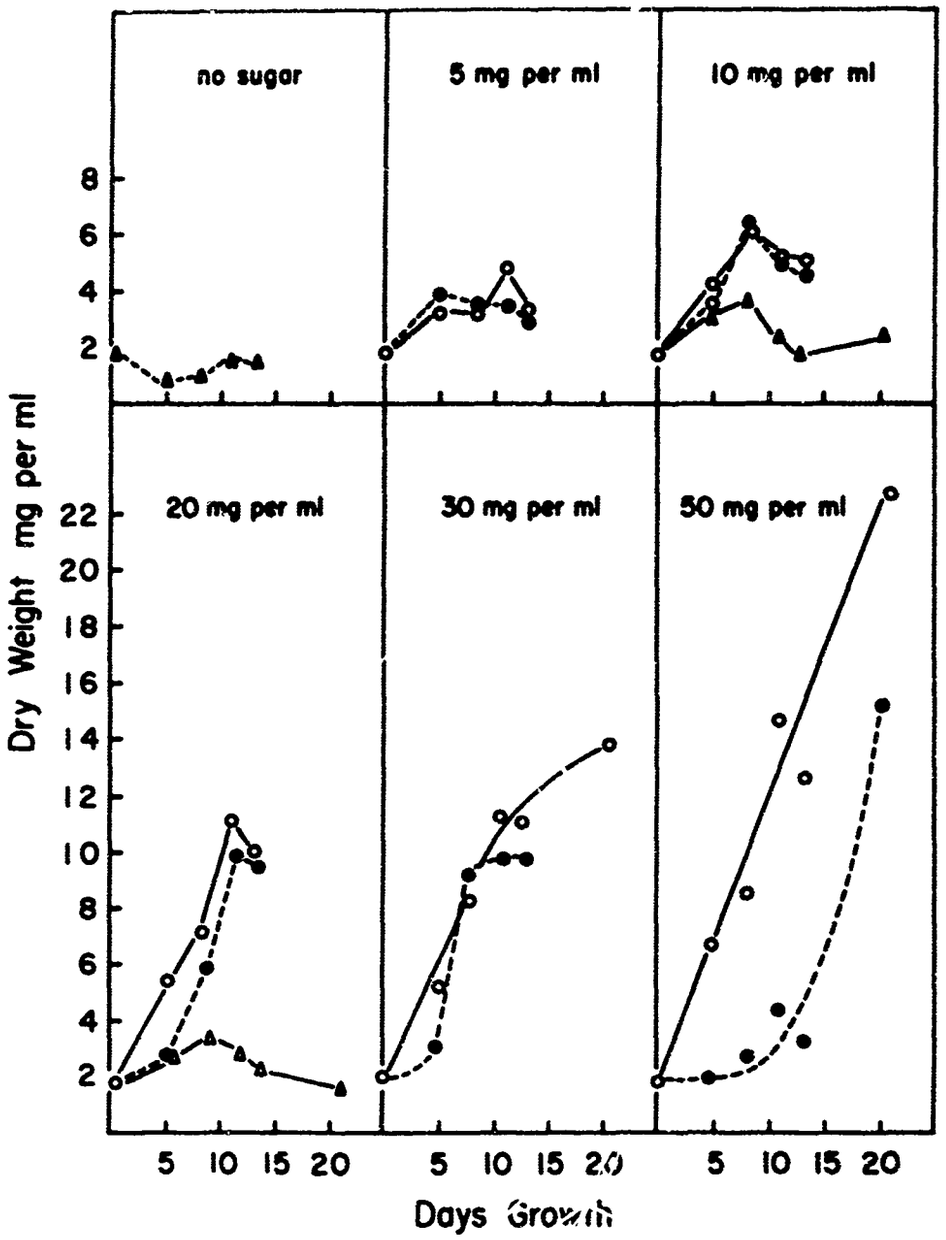
Bean Carrot

Table 2 Growth of Plant Cells on Sucrose, Glucose and Glycerol

Culture Auxin	mg/L	Initial Weight*	Dry weight mg/ml at 21 days on				
			Sucrose		Glucose		Glycerol
		mg/ml	1.5%	3%	1.5%	3%	3%
Carrot No. 18							
2,4-D	0.05	0.8	3.1	10.7	4.9	8.8	
2,4-D	0.10	0.9		7.7			3.1
NAA	0.10	1.1		8.1			3.5
Gibberellic Acid	1.0	1.2		6.2			2.4
Bean No. 8							
2,4-D	0.05	0.7	7.0	11.5	7.6	7.4	
2,4-D	0.10	1.5		17.7			2.4
NAA	0.10	1.3		15.1			3.4
Gibberellic Acid	1.0	1.6		8.0			1.4
Bean No. 12							
2,4-D	0.05	0.4	4.3	7.3	2.9	4.0	
Lettuce No. 7							
2,4-D	0.05	2.0	4.8	13.0	3.5	13.3	
Lettuce No. 13							
2,4-D	0.10	0.9		4.4			1.6
NAA	0.10	0.7		5.3			2.5
Gibberellic Acid	1.0	0.8		6.3			2.6

* 10% Inoculum.

Figure 3 Effect of Carbon Source on Growth of Bean No. 8



10% Inoculum, cultures grown in the dark on the rotary shaker with 0.05 mg 2,4-D and 1.0 g phytone/liter. Sugar concentration indicated on figure.

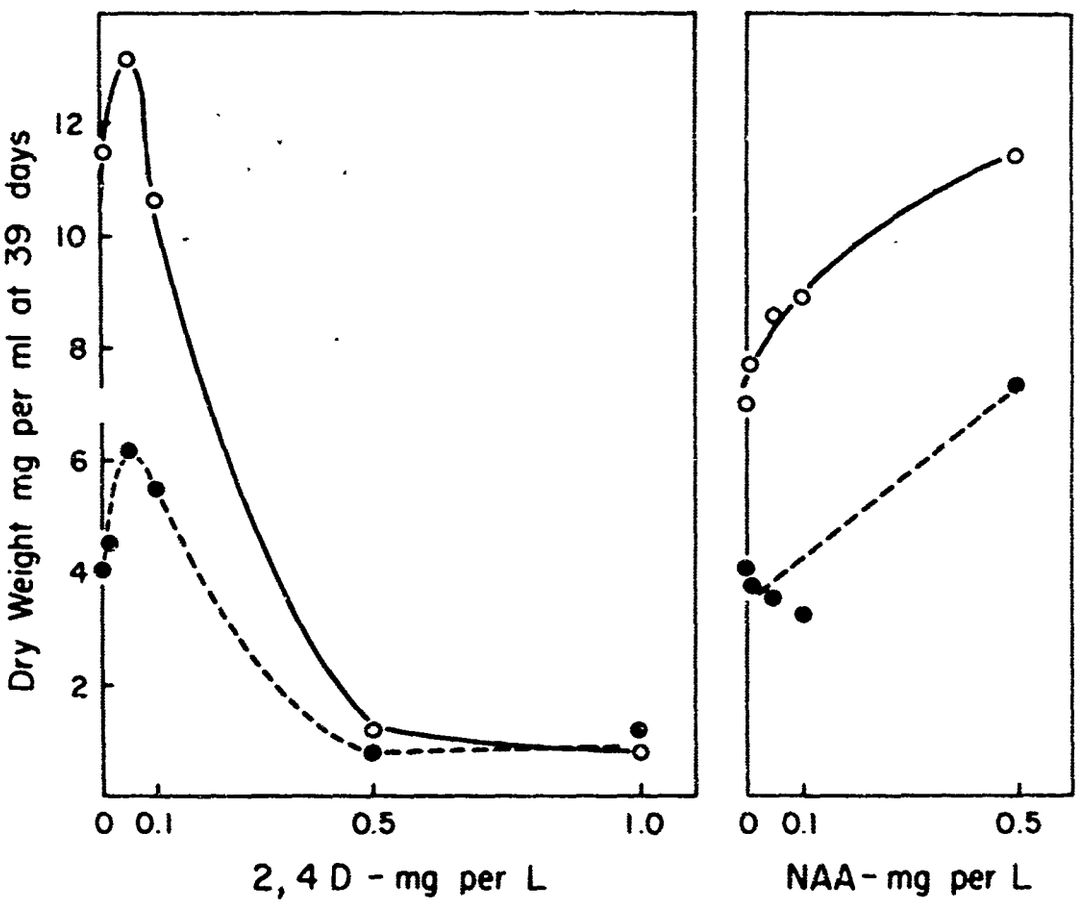
▲ - - - - - - ▲ No sugar added

○ ——— ○ Sucrose

● - - - - - ● Glucose

▲ ——— ▲ Glycerol

Figure 4 Growth of Bean No. 12 on 3% Starch



5% Inoculum, cultures grown for 39 days in the dark on the rotary shaker, 2,4-D 0.05 mg/L.

○——————○ Inoculated from 3% starch culture

●- - - - - - - - -● Inoculated from 3% sucrose culture

2. Effects of Irradiated Sugars.

Irradiated sugars have been reported to be toxic to various living systems, including plant cell cultures (2, 6, 8, 9, 11, 14, 16). When glucose solutions are irradiated with Cobalt 60 at ambient temperature (20°C) pH falls, and ultra-violet absorption at 260 µm develops. When temperature during irradiation is held at -80°C the acidity and ultra-violet absorption do not develop to the same extent. We wished to see whether sugar solutions irradiated at -80°C would be less toxic than sugar solutions irradiated at 20°C.

Murashige medium was prepared omitting sucrose and 20% of the water. Thiamine was at 0.5 mg/L, 0.05 mg/L of 2,4-D was added for bean cultures and 0.10 mg/L of NAA for lettuce cultures. Media was dispensed, 40 ml per 250 ml Erlenmeyer, and the flasks were autoclaved for 20 minutes at 15 pounds pressure. Sucrose (Merck Reagent) 15% and glucose (Merck Reagent) 10% were made up in water and sterilized separately as follows:

	pH after sterilization	
	Glucose	Sucrose
1. Filter through 0.22 micron Millipore filter	6.0	5.6
2. Autoclave 20 minutes at 15 pounds pressure	4.7	4.9
3. Irradiate with Cobalt 60 to 5 megarads at 20°C	3.2	3.7
4. Irradiate with Cobalt 60 to 5 megarads at -80°C	3.7	4.1

To maintain a final pH of 5.0 - 5.5 in all flasks (before inoculation) 2.0 ml of 0.01 N NaOH was added to each flask receiving sucrose irradiated at +20°C and 4.0 ml of 0.01 N NaOH to each flask receiving glucose irradiated at +20°C. No addition of alkali was required for flasks receiving

sugars irradiated at -80°C. 10 ml of sugar solution was added aseptically to each flask to give a final concentration of 2% glucose or 3% sucrose. Flasks were left at room temperature for 24 hours and then inoculated with a suspension culture of bean or lettuce cells. Bean cultures were grown on the rotary shaker in the dark, lettuce cultures were grown on the rotary shaker in continuous light.

We did not find irradiated sucrose toxic to bean (Fig. 5) or lettuce (Fig. 6) cultures, regardless of the temperature of irradiation. In comparison to filtered or autoclaved glucose, glucose irradiated at 20°C was toxic to bean (Fig. 7) and lettuce (Fig. 8) cultures, but glucose irradiated at -80°C was not toxic.

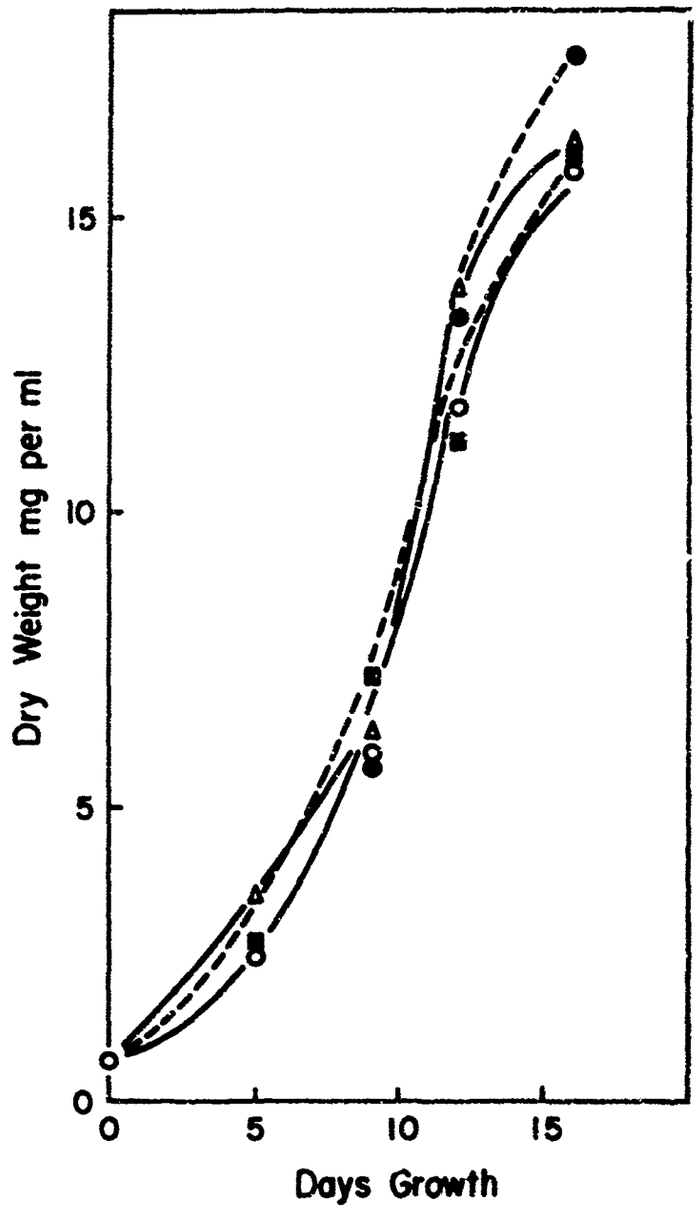
C. Nitrogen Sources.

Murashige medium contains per liter, 1650 mg NH_4NO_3 (578 mg N), and 1900 mg KNO_3 (266 mg N, 360 mg K) (Table 1). With 3% sucrose as a carbon source this gives a C/N ratio of 15 on a weight basis, 17.4 on a molar basis. If plant cells are ever used for food in a limited environment it would be desirable to use sewage as a source of nutrients. Therefore, we are interested in the possibility of using urea or hydrolyzed protein as a nitrogen source. Our previous studies had shown that addition of Phytone was beneficial to young growing cultures, but did not increase growth rate after the lag phase.

1. Effects of amino acids.

Dougall (4) has reported that certain amino acids at low concentrations inhibit growth and amino acid biosynthesis in suspension cultures of Paul's Scarlet Rose. We have tested 8 amino acids added singly or all together at 10^{-4} M to bean (Fig. 9) and lettuce (Fig. 10) cultures and have

Figure 5 Effect of Irradiated Sucrose on Bean No. 8



10% Inoculum, 3% sucrose, 0.05 mg 2,4-D/L.

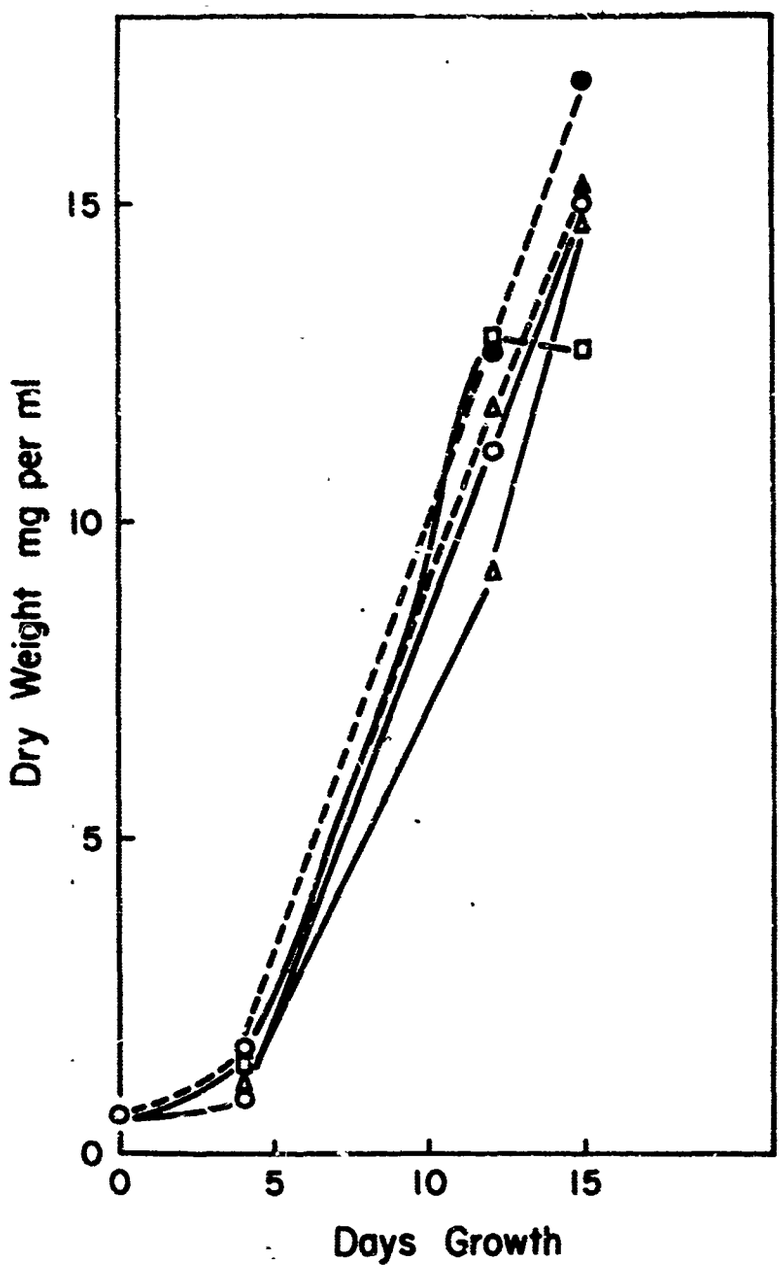
△——————△ Sugar autoclaved separately

■- - - - - - - -■ Sugar autoclaved in medium

○——————○ Sugar irradiated to 5.0 megarads with Cobalt 60 at 20°C

●- - - - - - - -● Sugar irradiated to 5.0 megarads with Cobalt 60 at -80°C

Figure 6 Effect of Irradiated Sucrose on Lettuce No. 13



5% Inoculum, 3% sucrose, 0.10 mg NAA/L.

▲ - - - - - - - - ▲ Sugar sterilized by Millipore filtration

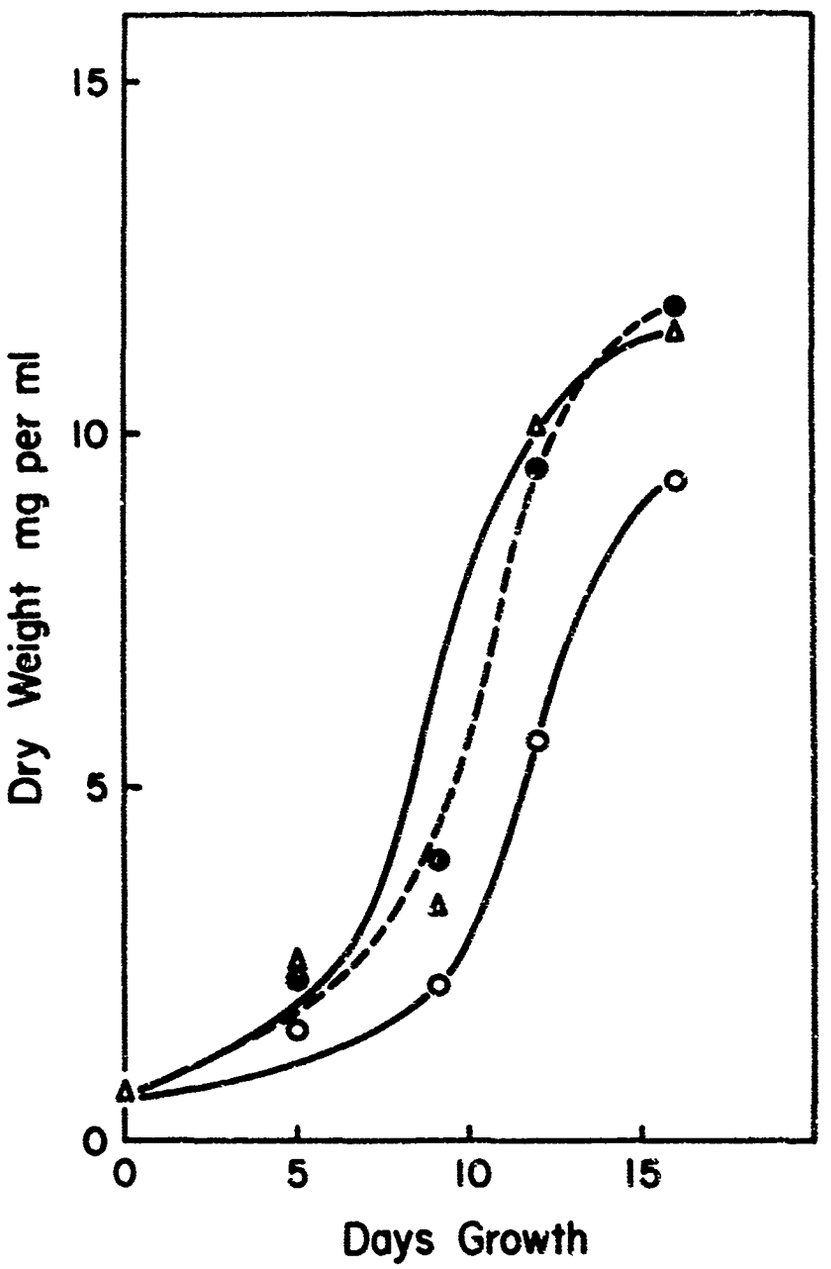
△ ——————— △ Sugar autoclaved separately

□ ——————— □ Sugar autoclaved, 1.0 g phytone/L

○ ——————— ○ Sugar irradiated to 5.0 megarads with Cobalt 60 at 20°C

● - - - - - - - - ● Sugar irradiated to 5.0 megarads with Cobalt 60 at -80°C

Figure 7 Effect of Irradiated Glucose on Bean No. 8



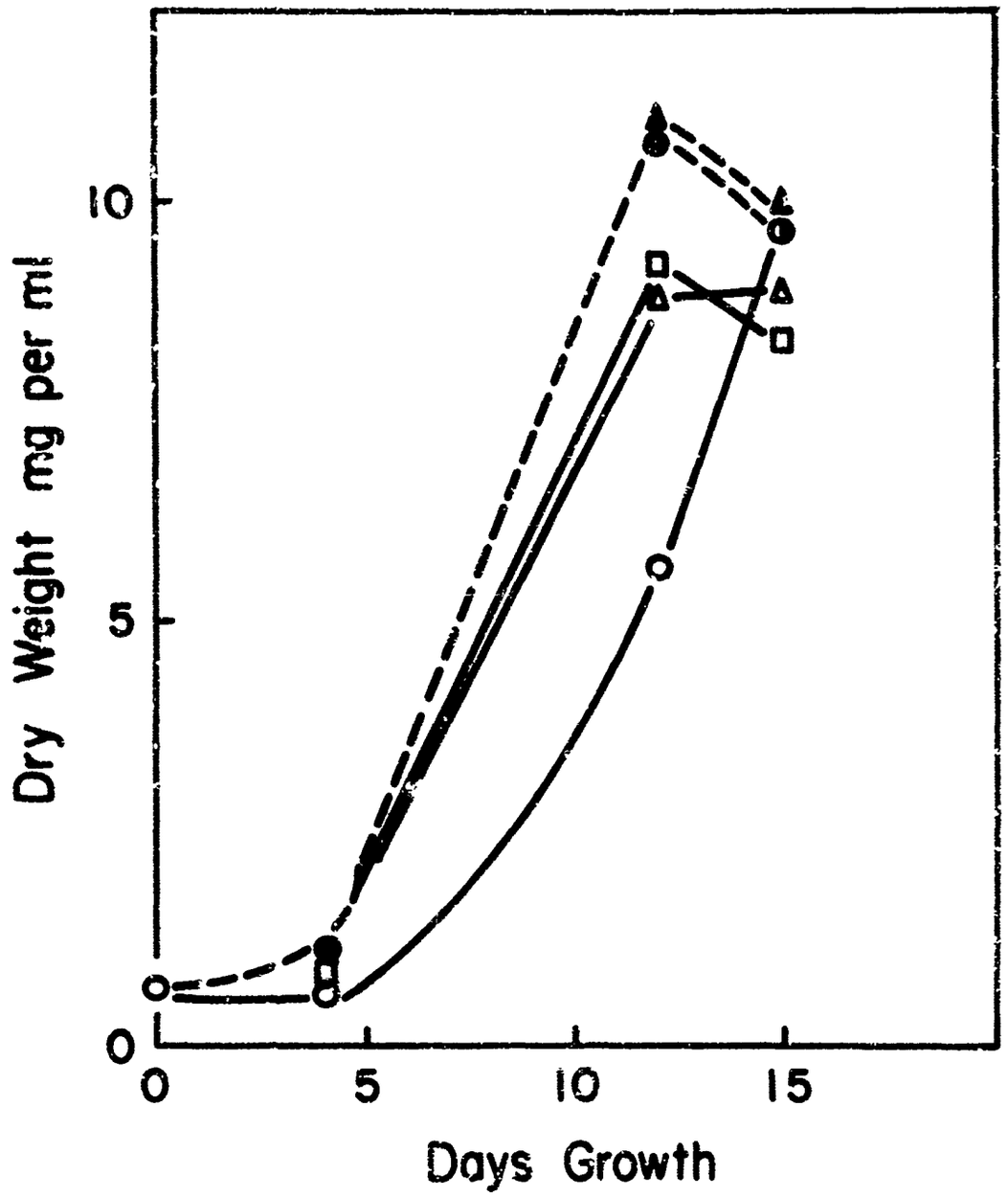
10% Inoculum, 2% glucose, 0.05 mg 2,4-D/L.

△——△ Sugar autoclaved separately

○——○ Sugar irradiated to 5.0 megarads with Cobalt 60 at 20°C

●- - -● Sugar irradiated to 5.0 megarads with Cobalt 60 at -80°C

Figure 8 Effect of Irradiated Glucose on Lettuce No. 13



5% Inoculum, 2% glucose, 0.10 mg NAA/L.

▲ - - - - - - - ▲ Sugar sterilized by Millipore filtration

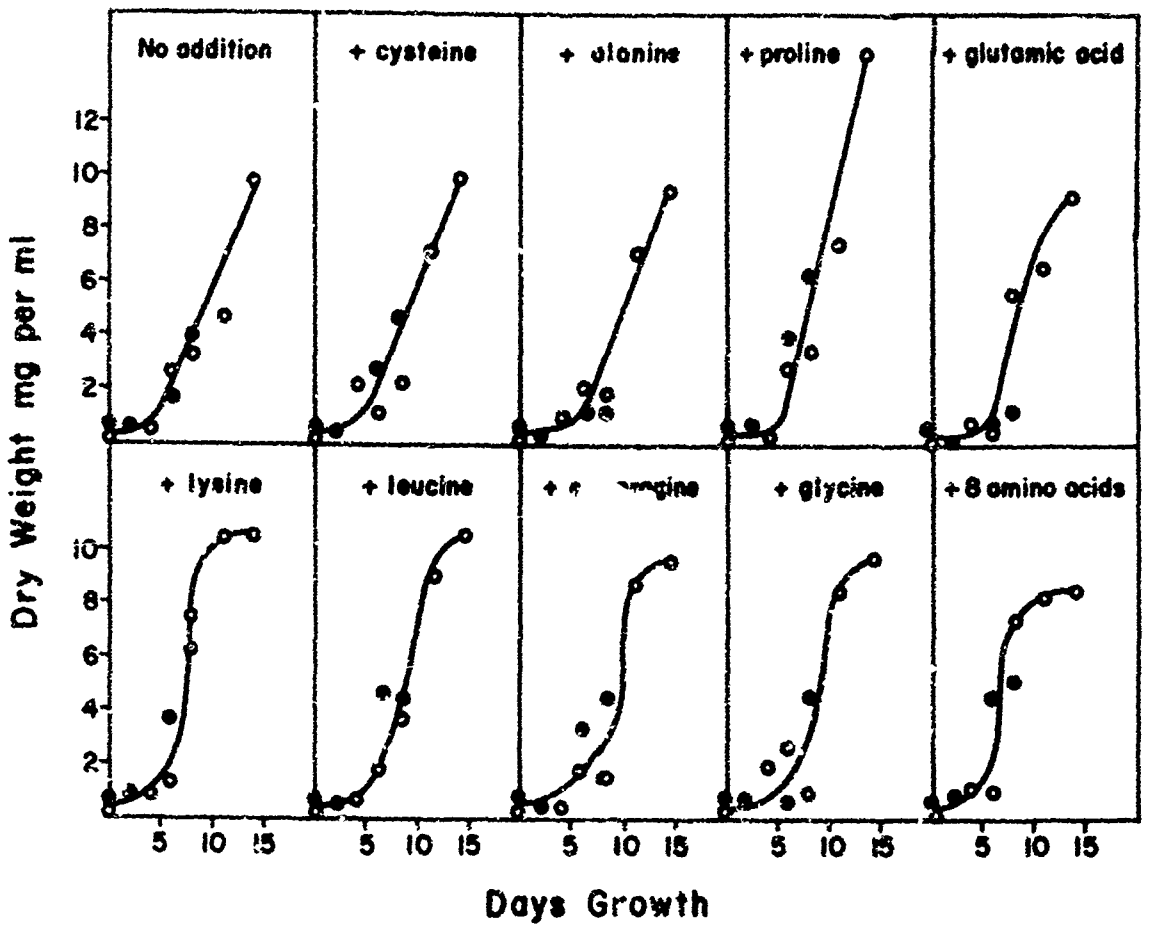
△ ——————— △ Sugar autoclaved separately

□ ——————— □ Sugar autoclaved, 1.0 g phytone/L.

○ ——————— ○ Sugar irradiated to 5.0 megarads with Cobalt 60 at 20°C

● - - - - - - - ● Sugar irradiated to 5.0 megarads with Cobalt 60 at -80°C

Figure 9 Effect of Added Amino Acids on Bean No. 8

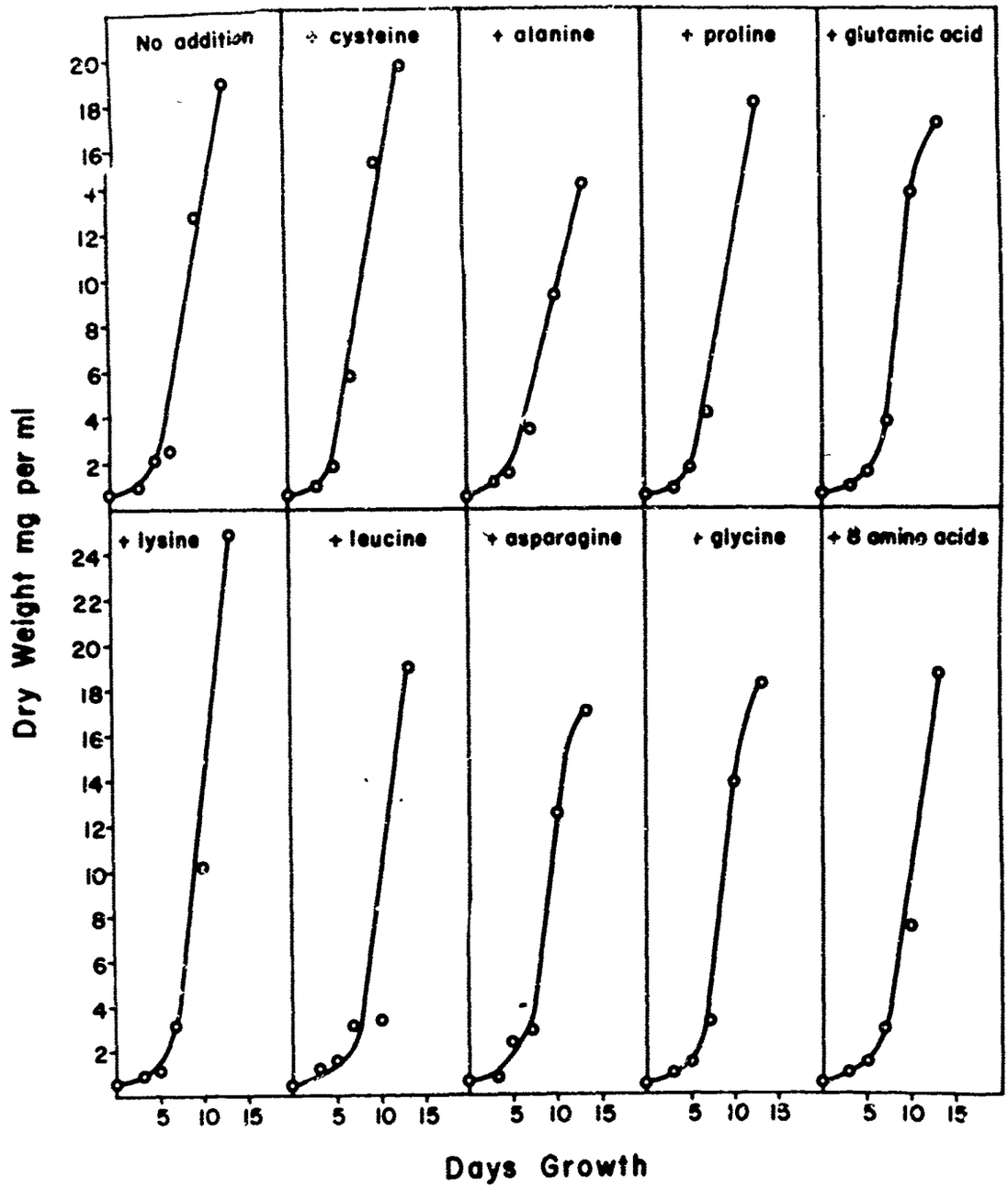


5% Inoculum, grown on rotary shaker in programmed light, 2,4-D 0.05 mg/L, glycine present in all cultures at 0.26 x 10^{-4} M, other amino acids at 10^{-4} M as noted.

● Experiment 1

○ Experiment 2

Figure 10 Effect of Added Amino Acids on Lettuce No. 13



5% Inoculum, grown in programmed light on the rotary shaker, NAA 0.1 mg/L, amino acids at 10^{-4} M as noted.

found no inhibition of rate or extent of growth or change in duration of the lag phase. In our tests addition of low levels of amino acids was neither favorable nor unfavorable.

2. Effect of nitrate, ammonia and urea.

For bean cells on M medium (Table 3) omission of KNO_3 had no effect and omission of NH_4NO_3 was somewhat beneficial. However, substitution of urea for either or both was toxic. Growth was less than when nitrogen was omitted. Substitution of Phytone for NH_4NO_3 or KNO_3 or both increased growth markedly.

For lettuce cells on NAA medium (Table 4) similar results were obtained. Omission of KNO_3 decreased growth, but omission of NH_4NO_3 was somewhat beneficial. Substitution of urea for either or both was toxic. Substitution of NH_4Cl for either NH_4NO_3 or KNO_3 reduced growth only slightly, but substitution of NH_4Cl for both was toxic.

In the next test with lettuce cells on M medium (Table 5a) we maintained KNO_3 at the usual level. Omission of NH_4NO_3 caused only a slight decrease in growth. Urea at 100 mg per liter had no effect in the presence of NH_4NO_3, but was slightly beneficial if NH_4NO_3 were omitted. Urea at 500 mg or more per liter was toxic. Transfers from these cultures to NAA medium (Table 5b) gave good growth (with KNO_3 present) in the presence or absence of NH_4NO_3 with urea at 0 to 400 mg per liter. There was no evidence that urea or NH_4NO_3 was beneficial. Transfers from these cultures to NAA medium with NH_4NO_3 omitted in all (Table 5c) gave good growth with KNO_3 at the usual or one-half level, with urea at 0, 50 or 100 mg per liter. Urea increased growth with one-half level KNO_3, but not with full KNO_3. Addition of KCl had

Table 3 Effect of Nitrogen Sources on Bean No. 8

Variation	pH 14 days	dry wt mg/ml	Productivity mg/ml per day
Control = M	5.4	5.4	0.4
Omit KNO_3*	5.5	5.4	0.4
Omit NH_4NO_3	5.7	7.9	0.5
Omit both*	5.5	2.6	0.2
Urea substituted for NH_4NO_3	7.3	0.7	0.01
Urea substituted for KNO_3*	5.9	1.3	0.08
Urea substituted for both*	7.5	0.6	0.01
Phytone substituted for NH_4NO_3	6.1	14.2	1.0
Phytone substituted for KNO_3*	5.4	9.2	0.6
Phytone substituted for both*	5.6	13.7	0.9

10% Inoculum, initial wt 0.5 mg/ml, grown on rotary shaker for 14 days in the dark, 0.05 mg 2,4-D/L.

Control - 1650 mg NH_4NO_3 + 1900 mg KNO_3

Urea - 2510 mg substituted for NH_4NO_3, 1260 mg for KNO_3 to give equal N

Phytone - 3600 mg substituted for NH_4NO_3, 1800 mg for KNO_3 to give equal N

* 1200 mg KCl/L added.

Table 4 Effect of Nitrogen Sources on Lettuce No. 13

Variation	pH 11 days	dry wt mg/ml	Productivity mg/ml/day
Control = M	5.5	14.9	1.3
Omit KNO_3*	4.0	3.5	0.2
Omit NH_4NO_3	5.2	16.6	1.4
Omit both*	4.0	3.6	0.2
Urea substituted for NH_4NO_3	8.2	1.3	0.03
Urea substituted for KNO_3	7.1	0.9	0
Urea substituted for both*	8.2	1.1	0.01
NH_4Cl substituted for NH_4NO_3	5.5	13.1	1.1
NH_4Cl substituted for KNO_3*	5.6	12.2	1.0
NH_4Cl substituted for both*	3.8	1.8	0.07

10% Inoculum, initial wt 1.0 mg/ml, grown on the reciprocal shaker in programmed light for 11 days, 0.10 mg NAA/L.

Control - 1650 mg NH_4NO_3 + 1900 mg/L KNO_3

Urea - 2510 mg substituted for NH_4NO_3, 1150 mg/L for KNO_3 to give equal N

NH_4Cl - 2200 mg substituted for NH_4NO_3, 1040 mg/L for KNO_3 to give equal N

* 1440 mg KCl/L added.

Table 5 Growth of Lettuce No. 13 on Urea
a. First transfer

NH_4NO_3 mg/L	Urea mg/L	pH 14 days	dry wt. mg/ml	Productivity mg/ml per day
	0	5.8	10.4	0.7
	100	5.7	12.4	0.8
0	500	8.2	0.7	0
	1000	8.3	0.8	0
	2000	8.1	0.6	0
	0	5.2	11.9	0.8
	100	5.6	11.4	0.7
1650	500	5.9	1.8	0.05
	1000	6.8	0.7	0
	2000	7.2	0.6	0

First transfer.

10% Inoculum, initial wt 1.0 mg/ml, grown on the reciprocal shaker in programmed light for 14 days, 2,4-D 0.05 mg/L, KNO_3 at 1900 mg/L.

Table 5 Growth of Lettuce No. 13 on Urea
b. Second transfer

NH_4NO_3	Urea	Initial weight		pH 14 days	dry wt	Productivity	Reducing sugar
mg/L	mg/L	mg/ml			mg/ml	mg/ml per day	mg/ml
	0	1.0	a*	6.6	15.5	1.0	0.21
	50	1.3	b	5.8	15.1	1.0	0.05
0	100	1.3	b	5.4	14.3	0.9	0.10
	200	1.3	b	5.3	13.2	0.9	0.11
	400	1.3	b	5.7	13.4	0.9	0.04
	0	1.2	c	5.9	11.5	0.7	0
	50	1.1	d	6.1	13.0	0.9	0
1650	100	1.1	d	6.0	13.0	0.9	0
	200	1.1	d	6.0	13.9	0.9	0
	400	1.1	d	6.2	11.3	0.7	0

Second transfer (from Table 5a) from:

* a 0 NH_4NO_3 0 Urea
b 0 NH_4NO_3 100 Urea
c 1650 NH_4NO_3 0 Urea
d 1650 NH_4NO_3 100 Urea

10% Inoculum, grown on the reciprocal shaker in programmed light for 14 days, NAA at 0.10 mg/L, KNO_3 at 1900 mg/L in all cultures.

Table 5 Growth of Lettuce No. 13 on Urea
c. Third transfer

KNO_3 mg/L	Urea mg/L	KCl mg/L	Initial weight		pH 14 days	dry wt mg/ml	Productivity mg/ml per day	Reducing sugar mg/ml
	0	0	1.6	a	5.7	15.0	1.0	0.7
1900	50	0	1.5	b	5.5	13.8	0.9	0.2
	100	0	1.4	c	5.7	11.2	0.7	0.1
	0	0	1.6	a	5.8	11.7	0.7	11.2
950	50	0	1.5	b	5.6	13.6	0.9	6.0
	100	0	1.4	c	5.7	16.5	1.1	6.9
	0	720	1.6	a	5.7	10.8	0.7	10.0
950	50	720	1.5	b	5.3	13.8	0.9	9.7
	100	720	1.4	c	5.3	13.0	0.8	10.5
0	100	720	1.6	a	5.2	13.0	0.1	7.8

Third transfer (from Table 5b) from:

a	0 NH_4NO_3	0 Urea
b	0 NH_4NO_3	50 Urea
c	0 NH_4NO_3	100 Urea

10% Inoculum, grown in the rotary shaker in programmed light for 14 days, NAA at 0.10 mg/L, NH_4NO_3 omitted in all cultures.

little effect. If KNO_3 was omitted and replaced with KCL growth was poor. In other tests (data not shown) KNO_3 could not be replaced by urea plus increased levels of KH_2PO_4. With the usual level of KNO_3 and good growth, little reducing sugar remains in the medium (Table 5b, c). With KNO_3 reduced to half (Table 5c) despite good growth, considerable reducing sugar remains in the medium even if K level is raised by addition of KCl. Whether this is an effect on metabolism of sugar, formation of a reserve polysaccharide, or quantity or activity of invertase has not been determined.

3. Conclusions.

Tentative conclusions from these studies are: (a) The nitrogen level in Murashige medium is greater than required for good growth of plant cell cultures on 3.0% sucrose. With KNO_3 present NH_4NO_3 is not beneficial and omission of NH_4NO_3 does not decrease growth even after three transfers, (b) Plant cell cultures will grow vigorously on NH_4NO_3, KNO_3, or hydrolyzed protein (Phytone) as the sole source of nitrogen, (c) Urea will not serve as the sole source of nitrogen for plant cell cultures, and is toxic at 500 mg or more per liter. With reduced nitrogen in the medium, 50 - 100 mg per liter of urea will increase growth, (d) In limited tests NH_4Cl will not serve as sole source of nitrogen, and (e) Amino acids added to the basic medium neither increase nor decrease growth of plant cell.

PART II. GROWTH FACTORS IN THE MEDIUM

Murashige (M) medium contains 6 organic growth factors in low concentration: (Table 1), Auxin - 2,4 dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid is substituted for the 2,4-D in NAA medium, Cytokinin - kinetin, thiamine, inositol, pyridoxine HCl and nicotinic acid (Table 1). These growth factors are omitted on "minimal" medium as noted in the tables.

We investigated these growth factors to see which were required, and also to see whether changes in them might result in increased growth.

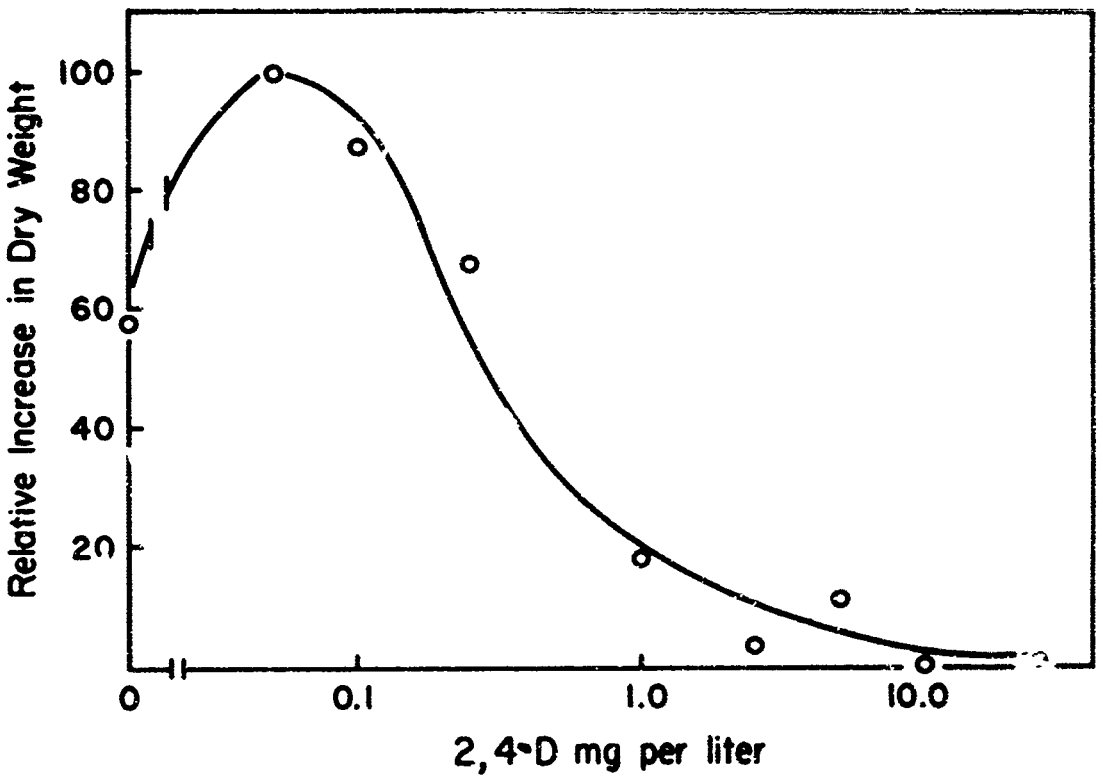
A. Growth Factors for Bean.

Bean cultures grow well with 2,4-D as auxin (Fig. 4, 11). A concentration of 0.05 mg/L is optimum. Concentrations of 1.0 mg/L or above are toxic. NAA and Indoleacetic Acid support growth, but are less favorable. Gitberellic Acid is toxic (Table 6). Bean cultures will grow for a few transfers with no auxin, but then die (data not shown).

Bean No. 8 was grown with 2,4-D, but omitting the other growth factors one at a time and inoculated (10% v/v) from M medium. After 3 weeks growth, cultures were transferred to fresh medium of the same composition for a total of five transfers. Cultures without thiamine died after two or three transfers, but cultures without other single growth factors retained full growth even after five transfers. In the five transfers any growth factor originally present was diluted 10^5. Phytone (which contains some thiamine) or tryptone will not maintain bean cultures in the absence of added thiamine (Table 7). Bean cells grow well with thiamine and 2,4-D as the only growth factors, but growth is increased at increased thiamine levels up to 1.0 mg/L, or on the full Murashige medium (Table 8). Bean cells will grow on medium without thiamine if yeast extract, a rich source of thiamine, is present (Table 8).

Bean No. 8 was grown on a minimal medium omitting all growth factors except 2,4-D and thiamine and adding the 5 other growth factors in M medium plus 6 other growth factors one at a time (Table 9). Growth was good in all cultures, but none were significantly better than the usual Murashige medium.

Figure 11 Effect of 2,4-D Concentration on Growth of Bean No. 8



10% Inoculum, initial weight 1.0 mg/ml, grown 30 days on the reciprocal shaker in the dark, 100 equals an increase of 15.6 mg/ml.

Table 6 Effe. . of Auxin on Growth of Bean No. 8

	Productivity (mg/ml/day)	
Auxin mg/L	Full M* + glycine	Minimal** + thiamine
None	0.17	0.02
2,4-D, 0.05	0.64	0.41
2.4-D, 0.10	0.61	0.53
2,4-D, 1.0	0.07	0.01
NAA, 0.1	0.47	0.28
NAA, 1.0	0.57	0.12
IAA, 0.1	0.35	0.03
IAA, 1.0	0.40	0.03
GA, 0.1	0.05	0
GA, 1.0	0.05	0

10% Inoculum, grown 20 days in the dark on the reciprocal shaker.

* Full M - all growth factors present (except auxin) Glycine 2 mg/L.
** Minimal - growth factors omitted except thiamine present at 0.10 mg/L.

IAA - Indoleacetic acid - sterilized separately by filtration.
GA - Gibberellic acid.

Table 7 Effect of Some Growth Factors on Bean No. 8

Transfer* No.	1	2	3	4	5	
Days Growth	25	24	19	21	29	Average 23
			Productivity mg/ml/day			
Full Murashige + glycine	0.22	0.63	0.59	0.39	0.37	0.44
Nicotinic Acid Omitted	0.31	0.41	0.58	0.26	0.51	0.42
Pyridoxine Omitted	0.34	0.47	0.59	0.40	0.46	0.45
Thiamine Omitted	0.12	0.04	0.01	dead		
Glycine Omitted	0.27	0.47	0.86	0.37	0.38	0.47
Kinetin Omitted	0.45	0.45	0.47	0.57	0.46	0.48
Inositol Omitted	0.20	0.36	0.60	0.50	0.49	0.43
**Minimal	0.10	0.01	0.01	dead		
Phytone 1.0 g/L added	0.11	0.04	0.04	dead		
Tryptone 1.0 g/L added	0.09	0.03	0.03	dead		

* Successive transfers 2,4-D 0.05 mg/L, 10% inoculum, grown in the dark in the reciprocal shaker.

** Minimal, omit Nicotinic Acid, Pyridoxine, Thiamine, Glycine, Kinetin, Inositol.

Table 8 Effect of Thiamine Concentration on Growth of Bean No. 8

Thiamine added mg/L	Productivity (mg/ml/day) Full M + glycine	Minimal**
0	0.67	0.09
0.1	0.66	0.50
0.2	0.64	0.59
0.4	0.69	0.59
0.5	----	0.56
1.0	0.76	0.47
2.0	0.72	0.53
4.0	0.65	0.50
0 *	0.79	0.56

* Yeast extract 1 g/L added.

** Minimal - omit Kinetin, Inositol, Glycine, Thiamine, Pyridoxine, Nicotinic Acid.

2,4-D 0.05 mg/L present in all media. 10% Inoculum, grown on the reciprocal shaker in the dark.

Full M - Thiamine 0.1 mg/L, glycine 2.0 mg/L.

Table 9 Effect of Single Growth Factors on the Growth of Bean No. 8 in the Presence of Thiamine and 2,4-D

Transfer* No.	1	2	
Days Growth	12	14	Average 16
		Productivity (mg/ml/day)	
Full Murashige + glycine	1.08	1.15	1.12
Minimal** + thiamine	1.02	0.79	0.90
+ 0.1 mg/L thiamine	1.14	0.60	0.87
+ 100 mg/L inositol	1.03	0.72	0.88
+ 0.32 mg/L kinetin	0.64	0.84	0.74
+ 1.0 mg/L Paraminobenzoic acid	0.93	0.68	0.80
+ 1.0 mg/L folic acid	0.85	1.10	0.98
+ 2.0 mg/L glycine	1.0	1.23	1.12
+ 0.5 mg/L pyridoxine	0.90	0.80	0.85
+ 0.5 mg/L nicotinic acid	0.98		0.98
+ 2.5 mg/L calcium pantothenate	0.96	0.90	0.93
+ 0.1 mg/L biotin	0.91	0.90	0.90
+ 1.0 g/L phytone	0.63	0.74	0.68

* Successive transfers.

** Minimal - Kinetin, Inositol, Glycine, Pyridoxine, Nicotinic Acid omitted 10% Inoculum, 2,4-D 0.05 mg/L, grown in the dark on the reciprocal shaker. Murashige (Table 1), Thiamine at 0.1 mg/L, glycine 2.0 mg/L.

In the second transfer, growth on the minimal medium declined, except in cultures with folic acid or glycine added (Table 9). Bean No. 12 behaved similarly. Thiamine is required, but the other growth factors had little effect (Table 10).

With NAA as auxin, bean no. 8 showed reduced growth, and growth was further reduced if thiamine was omitted. Several other growth factors increased growth with NAA but not to equal that on 2,4-D (Table 11).

Our conclusion from these studies is that bean cultures require thiamine and an auxin as organic growth factors. Other growth factors are not specifically required, but may stimulate growth. 2,4-D is the best auxin for bean cultures.

B. Growth Factors for Lettuce.

Lettuce cultures grow well with NAA as auxin, 0.10 mg/L is the optimum concentration (Fig. 12, Fig. 13). Indoleacetic acid and gibberellin are also suitable auxins, but 2,4-D is toxic (Table 12). When 2,4-D is the auxin, lettuce will not grow if thiamine is the only growth factor. Kinetin must also be supplied (Table 13).

A series of experiments was carried out to show the interactions of auxins and other growth factors in greater detail. Murashige medium was prepared with all 6 growth factors omitted. Growth factors were added singly and in various combinations at the usual concentrations. Cultures were inoculated originally from full M medium + Phytone (6% v/v) to give an initial dry weight of 0.5 mg/ml. After 7 days, cultures were transferred (10% v/v) to fresh medium of the same composition; this was repeated about every 2 weeks for a total of six transfers (Table 14, 15, 16). All cultures were grown in programmed light.

Table 10 Effect of Growth Factors on Bean No. 12

Transfer No.:	1	2
Days Grown :	24	33
	Dry wt mg/ml	
Full Murashige	8.0	11.6
Omit Thiamine	8.5	1.2
Omit Kinetin	8.4	12.3
Omit Inositol	8.8	12.6
Omit Pyridoxine	7.8	12.3
Omit Nicotinic Acid	7.9	12.7
Omit Thiamine, Kinetin	7.0	1.6
Omit Inositol, Pyridoxine, Nicotinic Acid	7.6	12.3
Omit Kinetin, Inositol, Nicotinic Acid, Pyridoxine	7.6	13.2
Omit Thiamine, Kinetin, Inositol, Nicotinic Acid	7.7	1.6

2,4-D 0.05 mg/L, 10% inoculum, grown in the dark on the rotary shaker, Thiamine at 0.5 mg/L, glycine omitted in all cultures.

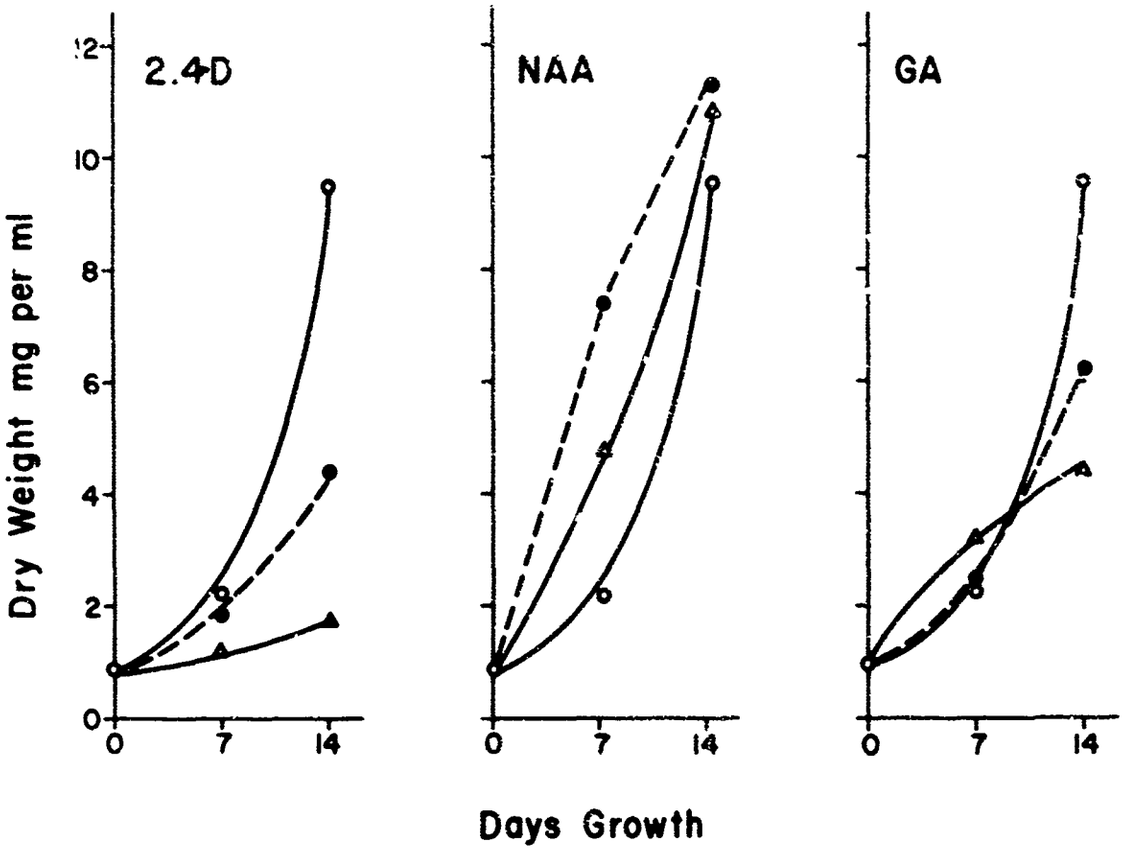
Table 11 Effect of Auxin and Other Growth Factors on Bean No. 8

	Productivity (mg/ml/day)	
	2,4-D (0.05 mg/L)	NAA (0.10 mg/L)
*Murashige Full + glycine	1.?	0.5
**Minimal	0.3	0.3
Kinetin added	0.4	0.3
Thiamine added	1.2	0.5
Thiamine and Kinetin added	1.2	0.7
Thiamine and Inositol added	1.5	0.8
Thiamine and Pyridoxine added	1.2	0.4
Thiamine and Biotin added	1.2	1.0
Thiamine and Calcium Pantothenate added	1.4	0.4
Thiamine and Paraminobenzoic acid added	1.1	0.9

10% Inoculum, cultures grown 17 days in the dark in the reciprocal shaker.

* Murashige (Table 1), Thiamine 0.1 mg/L.
** Minimal - omit Thiamine, Kinetin, Inositol, Nicotinic Acid, Pyridoxine, and Glycine.

Figure 12 Effect of Auxin on Lettuce No. 13



GA = Gibberellic Acid

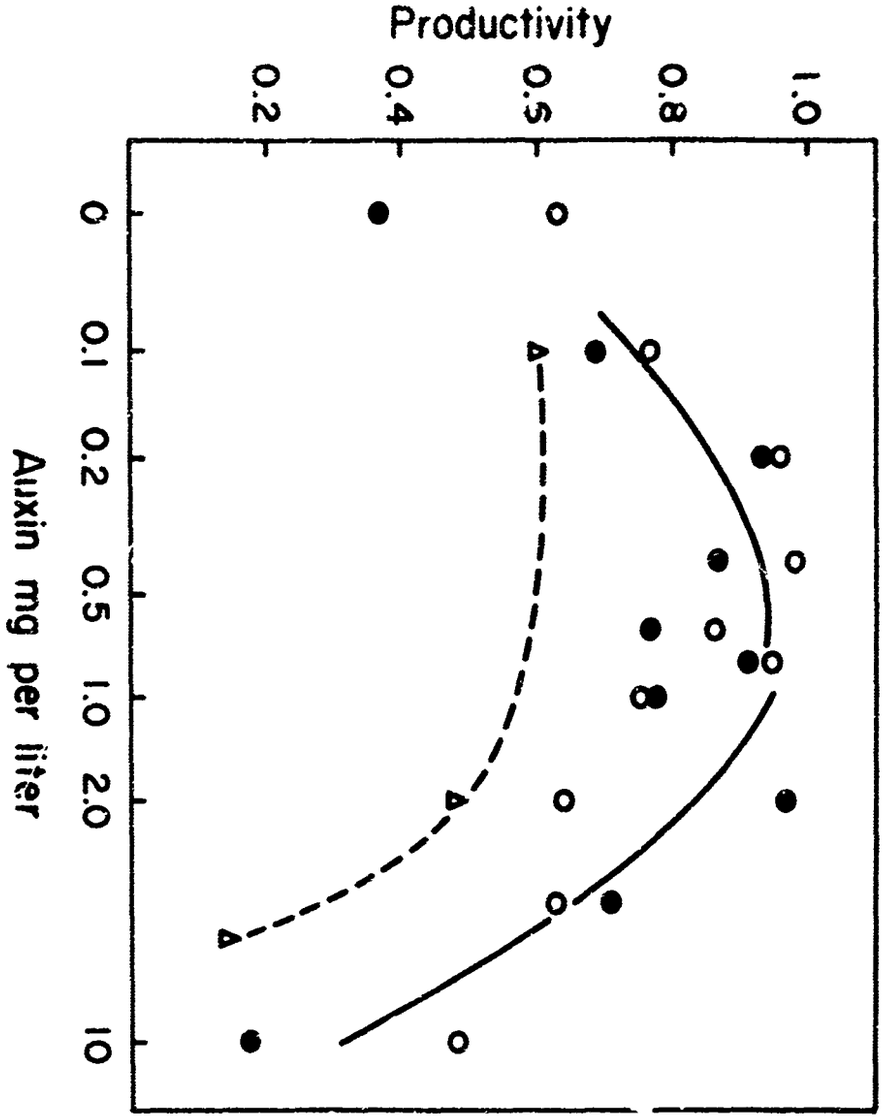
○ ——— ○ No auxin

● - - - - - ● 0.1 mg/L

△ ——— △ 1.0 mg/L

Grown in programmed light in the rotary shaker.

Figure 13 Effect of Auxin Concentration on Lettuce No. 13



Grown on the reciprocal shaker in programmed light.

NAA ○ First Transfer ● Second Transfer

2,4-D △- - - - - - -△

Productivity mg/ml/day

Table 12 Effect of Auxin on Growth of Lettuce No. 13

Auxin mg/L	Productivity	
	Full	Minimal + Thiamine
None	0.58	0.29
2,4-D 0.05	0.47	0.16
2,4-D 0.1	0.30	0.05
2,4-D 1.0	0.06	0.03
NAA 0.1	0.68	0.24
NAA 1.0	0.58	0.14
IAA 0.1	0.66	0.35
IAA 1.0	0.67	0.34
GA 0.1	0.74	0.45
GA 1.0	0.75	0.52

10% Inoculum, grown 20 days in the dark on the reciprocal shaker.

Full - all growth factors present (except auxin).
Minimal - 5 growth factors omitted. Thiamine present at 0.10 mg/L.
IAA - Indoleacetic acid - sterilized separately by filtration.
GA - Gibberellic acid.

Table 13 Effect of Single Growth Factors on the Growth of Lettuce No. 13 in the Presence of Thiamine

Transfer* No.	1	2
Days Growth	19	14
	Productivity (mg/ml/day)	
Murashige	0.57	0.49
Minimal + Thiamine	0.06	0.04
+ 0.1 mg/L thiamine	0.01	0.02
+ 100 mg/L inositol	0.05	
+ 0.32 mg/L kinetin	0.56	0.44
+ 1.0 mg/L paraminobenzoic acid	0.06	0
+ 1.0 mg/L folic acid	0.06	0
+ 2.0 mg/L glycine	0.06	
+ 0.5 mg/L pyridoxine	0.06	0.01
+ 0.5 mg/L nicotinic acid	0.04	0
+ 2.5 mg/L calcium pantothenate	0.08	0.03
+ 0.1 mg/L biotin	0.07	0.01
+ 1.0 g/L Phytone	0.14	0.04

* Successive transfers.

Murashige medium (Table 1) 2,4-D 0.05 mg/L, Thiamine 0.1 mg/L.
Minimal - omit 5 growth factors - thiamine 0.1 mg/L and 2,4-D 0.05 mg/L present.

Table 14 Effect of Growth Factors on Lettuce No. 13 Grown Without Auxin

Transfer No.	1	2	3	4	5	6	
Days Growth	7	13	15	13	14	9	Average 12
			Productivity (mg/ml/day)				
Full	1.0	0.8	0.3	0.06	0.03	--	dead
- Thiamine	1.0	0.07	0.2	--	--	--	dead
- Kinetin	1.0	1.2	0.8	0.05	0.01	--	dead
- Thiamine and Kinetin	0.7	0.03	0.03	--	--	--	dead
Minimal	1.0	0.02	0.03	--	--	--	dead
+ Thiamine	1.0	1.3	0.7	0.1	0.3	0.1	0.6
+ Kinetin	0.9	0.08	0.05	--	--	--	dead
+ Thiamine and Kinetin	0.5	0.8	0.8	0.2	0.3	0.05	0.5

Full includes Thiamine (0.5 mg/L), Kinetin, Inositol, Pyridoxine, Nicotinic Acid.

Minimal - above growth factors omitted.

Auxin omitted in all cultures.

Table 15 Effect of Growth Factors on Lettuce No. 13 Grown with Naphthaleneacetic Acid

Transfer No.	1	2	3	4	5	6	
Days Growth	7	13	15	13	14	9	Average 12
				Productivity (mg/ml/day)			
Full	0.8	1.1	1.0	1.0	0.4	1.5	1.0
- Thiamine	0.4	0.1	0.07	--	--		dead
- Kinetin	1.0	1.0	0.8	0.3	0.3	0.1	0.6
- Thiamine and Kinetin	0.9	0.06	0.05	--	--		dead
Minimal	0.9	0.06	0.07	--	--		dead
+ Thiamine	0.7	1.0	0.8	0.3	0.7	0.8	0.7
+ Kinetin	1.0	0.03	0.07	--	--		dead
+ Thiamine and Kinetin	1.2	1.0	0.5	cont.	0.6*	1.0	0.8

Full includes Thiamine (0.5 mg/L), Kinetin, Inositol, Pyridoxine, Nicotinic Acid.

Minimal - above growth factors omitted.

NAA, 0.1 mg/L in all cultures.

* New culture from no hormone (Minimal + Thiamine and Kinetin, Table 14).

Table 16 Effect of Growth Factors on Lettuce No. 13 Grown with 2,4-Dichlorophenoxyacetic Acid

Transfer No.		1	2	3	4	5	6	
Days Growth		7	13	15	13	14	9	Average 12
	Phytone*	Productivity (mg/ml/day)						
Full	+	1.2	0.8	0.5	0.04	0	--	0.5
Full	-	0.7	0.7	0.7	0.02	0.01	--	0.4
- Thiamine	+	1.2	0.7	0.5	--	--	--	0.8
- Thiamine	-	0.5	0.07	0.03	--	--	--	dead
- Kinetin	+	1.4	0.8	0.6	0.8	0.7	--	0.9
- Kinetin	-	0.5	0.4	0.5	0.7	0.7	0.3	0.6
- Thiamine and Kinetin	+	1.2	0.2	0.3	--	--	--	0.6
- Thiamine and Kinetin	-	0.4	0.03	0.01	--	--	--	dead
Minimal								
+ Thiamine	+	--	--	--	0.3a	0	--	0.2
+ Thiamine	-	--	--	--	0.04a	0	0.4	0.1
+ Thiamine and Kinetin	+	--	--	--	0.3b	0.7	--	0.5
+ Thiamine and Kinetin	-	--	--	--	0.4b	0.7	0.7	0.6

a New culture from no hormone (Minimal + Thiamine)
b Thiamine and Kinetin, Table 14.

2,4-D 0.05 mg/L in all cultures.

* Phytone 1.0 g/L, if added.

Full includes Thiamine (0.5 mg/L,, Kinetin, Inositol, Pyridoxine, Nicotinic Acid.
Minimal - above growth factors omitted.

With no auxin (Table 14) but all other growth factors, growth was good for three transfers, but then declined. The decline was earlier and steeper in the four series with thiamine omitted. Minimal media with thiamine, kinetin, or both supported better growth at first than the full Murashige medium. However, these cultures also declined after the third transfer.

With NAA as auxin (Table 15) the full growth factor cultures maintained excellent growth for six transfers. This was the best medium. Cultures without thiamine declined steeply by the second transfer. Minimal media with thiamine or thiamine and kinetin were maintained but were not equal to the full nutrient cultures.

With 2,4-D as auxin (Table 16) the full growth factor cultures declined after the third transfer (as with no auxin). Phytone was favorable, but did not prevent this decline. In the absence of thiamine the culture died by the third transfer unless Phytone (which contains some thiamine) was present. Cultures with kinetin omitted maintained a good growth rate for five or six transfers. Limited data suggest (in agreement with Table 13) that on minimal medium growth is poor if thiamine alone is added, but good when thiamine and kinetin are both present.

Finally with NAA (0.10 mg/L) as the auxin we tried all possible combinations of the five growth factors: Thiamine 0.5 mg/L, Kinetin 0.32 mg/L, Inositol 100.0 mg/L, Pyridoxine 0.5 mg/L, Nicotinic acid 0.5 mg/L. First, to the full medium we added all combinations of the five growth factors in excess (Table 17). In a single transfer (10% v/v from NAA medium) growth was excellent in all cultures, indicating that none of the factors in the basic medium were present at much below or much above favorable concentrations.

Table 17 Effect of Excess Growth Factors on Lettuce No. 13

Add to Medium	Thiamine + Kinetin	Thiamine	Kinetin	--	Average
		Productivity (mg/ml/day)			
I P N	1.1	1.1	1.0	1.2	1.1
P N	1.1	0.9	1.0	1.2	1.1
I N	1.1	1.0	1.2	1.1	1.1
I P	1.1	0.9	1.1	1.1	1.1
Nicotinic Acid	1.0	1.0	1.2	1.2	1.1
Pyridoxine	1.0	1.0	1.2	1.3	1.1
Inositol	1.0	0.9	1.2	1.3	1.1
--	1.0	1.1	1.3	1.2	1.2
Average	1.1	1.0	1.2	1.2	1.1
		Final dry weight (mg/ml)			
I P N	14.9	14.8	14.7	16.7	
P N	14.9	12.4	14.4	16.1	
I N	15.9	13.9	16.4	16.0	
I P	15.4	12.5	15.2	16.0	
Nicotinic Acid	14.4	14.3	16.1	16.7	
Pyridoxine	13.7	13.8	16.2	17.4	
Inositol	14.3	13.3	16.9	17.4	
--	14.6	14.9	17.7	17.3	

Full medium plus excess growth factors as noted. Thiamine 0.5 mg/L, Kinetin 0.32 mg/L, I = Inositol 100 mg/L, P = Pyridoxine 0.5 mg/L, N = Nicotinic Acid 0.5 mg/L. NAA 0.10 mg/ml present in all cultures. 10% inoculum, single transfer, giving an initial weight of 1.2 mg/ml, grown 13 days.

Next, we omitted the five growth factors from the basic medium, and added them separately in all possible combinations. Cultures were inoculated (10% v/v) from NAA medium to give an initial weight of 1.7 mg/ml, incubated in the reciprocal shaker in programmed light and transferred (10% v/v) after about 2 weeks' growth to fresh media of the same composition for a total of six transfers.

All cultures with thiamine and kinetin (Table 18a) maintained good growth for the six transfers regardless of the presence or absence of inositol, pyridoxine or nicotinic acid. Cultures with thiamine but no kinetin (Table 18b) grew well for four transfers, but declined in the fifth when kinetin would be reduced at least to 1 part in 3×10^{11}. Again the presence or absence of inositol, pyridoxine, and nicotinic acid had no effect. Cultures with kinetin but no thiamine (Table 18c) and cultures without thiamine or kinetin (Table 18d) declined in the third transfer when thiamine was reduced to 1 part in 2×10^{9}. In these cultures the absence of inositol, pyridoxine, and nicotinic acid was possibly slightly favorable.

Our conclusions from these studies are that the lettuce cells have low but definite requirements for an auxin and thiamine. NAA is the most favorable auxin, and with it lettuce cells require some kinetin, as well as thiamine. On 2,4-D medium kinetin seems to be unfavorable when inositol, pyrodoxine, and nicotinic acid are present, but required when they are absent. The nature of the auxin thus affects the response of lettuce cells to other growth factors.

C. Growth Factors for Carrot.

Carrot cells will grow for one or two transfers with no auxin, but growth is better if an auxin is present. 2,4-D, NAA, IAA, or gibberellic acid are all suitable (Table 19). Thiamine is required, with an optimum

Table 18 Effect of Growth Factors on Lettuce No. 13
a. All + Thiamine + Kinetin + NAA

Transfer No.	1	2	3	4	5	6	
Days Grown	13	15	15	14	13	18	Average 15
Add to Medium				Productivity (mg/ml/day)			
I P N	0.9	0.8	0.8	0.9	1.0	0.6	0.8
P N	0.9	0.8	0.7	0.9	0.8	0.5	0.8
I N	1.1	0.8	0.7	1.0	1.0	0.6	0.9
I P	1.0	0.8	0.8	1.0	0.9	0.7	0.9
Nicotinic Acid	0.9	0.9	0.7	0.9	0.9	0.6	0.8
Pyridoxine	0.9	0.8	0.7	1.0	0.8	0.6	0.8
Inositol	1.0	0.9	1.7	1.0	0.7	0.5	0.8
--	0.9	0.8	0.9	0.8	0.8	0.7	0.8
Average	1.0	0.8	0.8	0.9	0.9	0.6	0.8
				Final dry weight (mg/ml)			
I P N	13.4	13.1	13.0	13.8	15.0	11.8	
P N	12.8	13.2	12.2	14.2	11.5	10.0	
I N	16.1	13.5	12.0	14.4	14.4	11.0	
I P	14.6	13.4	13.5	14.8	12.4	12.1	
Nicotinic Acid	12.8	15.4	13.3	14.4	13.1	10.1	
Pyridoxine	12.5	13.6	11.1	14.5	11.5	11.4	
Inositol	14.0	14.4	13.0	14.8	10.9	9.3	
--	12.8	13.8	14.1	12.2	12.1	13.4	

10% inoculum to give an initial weight of 1.7 mg/ml.

I = Inositol
P = Pyridoxine
N = Nicotinic Acid

Table 18 Effect of Growth Factors on Lettuce No. 13
b. All + Thiamine - Kinetin + NAA

Transfer No.	1	2	3	4	5	6	
Days Grown	13	15	15	14	13	18	Average 15
Add to medium				Productivity (mg/ml/day)			
I P N	1.1	0.7	0.7	0.7	0.07	0.01	0.4
P N	1.1	0.8	0.7	0.8	0.05	0	0.4
I N	1.2	0.8	0.7	0.8	0.01	0	0.4
I P	1.2	0.7	0.7	0.8	0.7	0.07	0.5
Nicotinic acid	1.0	0.7	0.6	0.9	0.1	0	0.4
Pyridoxine	1.0	0.8	0.6	0.8	0.1	0	0.4
Inositol	1.0	0.8	0.6	0.9	0.2	0.04	0.4
--	1.2	0.8	0.6	0.7	0.6	0.03	0.5
Average	1.1	0.8	0.7	0.8	0.2	0.02	
				Final dry weight (mg/ml)			
I P N	15.4	12.1	12.0	10.4	1.8	0.3	
P N	16.5	12.6	11.4	12.9	1.9	0.3	
I N	17.7	12.6	11.1	11.8	1.6	0.3	
I P	17.2	12.6	11.8	11.8	10.2	2.3	
Nicotinic acid	14.4	12.9	10.7	13.1	3.2	0.3	
Pyridoxine	15.1	14.2	10.5	12.8	2.9	0.3	
Inositol	15.0	12.7	10.5	13.4	3.9	1.0	
--	17.5	13.7	10.6	11.4	9.3	1.4	

Table 18 Effect of Growth Factors on Lettuce No. 13
c. All - Thiamine + Kinetin + NAA

Transfer No.	1	2	3	4	
Days Grown	13	15	15	14	Average 14
Add to Medium			Productivity (mg/ml/day)		
I P N	0.7	1.0	0.1	0.06	0.5
P N	1.0	0.8	0.2	0.08	0.5
I N	1.1	0.8	0.2	0.1	0.6
I P	0.9	0.8	0.4	0.2	0.6
Nicotinic acid	1.4	0.9	0.3	0.2	0.7
Pyridoxine	1.3	0.7	0.2	0.1	0.6
Inositol	0.8	0.7	0.3	0.2	0.5
--	1.1	0.9	0.2	0.1	0.6
Average	1.0	0.8	0.2	0.1	
			Final dry weight (mg/ml)		
I P N	11.3	15.3	3.2	2.2	
P N	14.6	13.5	4.4	1.6	
I N	16.4	13.7	4.1	2.1	
I P	13.1	13.1	6.4	2.7	
Nicotinic acid	19.8	15.3	5.4	2.7	
Pyridoxine	18.1	11.7	4.4	2.2	
Inositol	12.0	11.5	6.0	2.9	
--	16.1	14.5	3.8	2.3	

Table 18 Effect of Growth Factors on Lettuce
d. All - Thiamine - Kinetin + NAA

Transfer No.	1	2	3	4	
Days Grown	13	15	15	14	Average 14
Add to Medium			Productivity (mg/ml/day)		
I P N	0.8	0.7	0.1	0.02	0.4
P N	1.5	0.7	0.05	0.01	0.6
I N	1.3	0.7	0.08	0.02	0.5
I P	1.4	0.8	0.1	0.02	0.6
Nicotinic acid	1.2	0.8	0.04	0.03	0.5
Pyridoxine	1.1	0.8	0.08	0.01	0.5
Inositol	1.1	0.8	0.1	0.02	0.5
--	1.3	0.6	0.09	0.01	0.5
Average	1.2	0.7	0.08	0.02	
			Final dry weight (mg/ml)		
I P N	11.8	12.0	2.9	0.6	
P N	21.0	12.1	2.0	0.4	
I N	18.7	12.1	2.6	0.5	
I P	20.0	13.8	3.0	0.6	
Nicotinic acid	17.2	13.4	2.0	0.6	
Pyridoxine	16.3	13.5	2.5	0.5	
Inositol	16.1	13.7	2.9	0.6	
--	19.0	11.2	2.6	0.4	

Table 19 Effect of Auxin on Growth of Carrot No. 18

	Productivity	
Auxin, mg/L	Full*	Minimal** + Thiamine
None	0.39	0.45
2,4-D,0.05	0.41	0.45
2,4-D,0.10	0.50	0.33
2,4-D,1.0	0.42	0.29
NAA, 0.1	0.47	0.54
NAA, 1.0	0.56	0.49
IAA, 0.1	0.54	0.65
IAA, 1.0	0.59	0.45
GA, 0.1	0.54	0.37
GA, 1.0	0.48	0.36

10% inoculum, grown 20 days in the dark in the reciprocal shaker.

* Full - all growth factors present (except auxin).
** Minimal + Thiamine - 5 growth factors omitted. Thiamine present at 0.10 mg/L.

IAA - Indole acetic Acid - Sterilized separately by filtration.
GA - Gibberellic Acid.

concentration of about 0.5 mg/L (Table 20).

A detailed study of the interaction of five growth factors and auxin on carrot growth was made. All possible combinations of thiamine 0.5 mg/L, kinetin 0.32 mg/L, inositol 100.0 mg/L, pyridoxine 0.5 mg/L, and nicotinic acid 0.5 mg/L were added to basic media from which all of these had been omitted.

In the first experiment (Table 21) cultures were transferred from M (10% v/v) to media with 0.05 mg 2,4-D/L to give an initial weight of 1.0 mg/ml. Cultures were transferred (10% v/v) at intervals of about two weeks to media of the same composition except that 0.10 mg/L of NAA was used as the auxin. All cultures were grown in shake flasks on a reciprocal shaker in programmed light. Cultures with thiamine and kinetin (Table 21a) all grew, all remained healthy and undifferentiated, although growth rate declined after the second transfer. The presence of inositol, pyridoxine, and nicotinic acid was somewhat favorable after the second transfer. Cultures with thiamine, but no kinetin (Table 21b) showed a decreased growth rate even in the first transfer, but continued to grow slowly for the four transfers. All of these cultures differentiated leaves and roots by the second transfer and from then on grew as semi-differentiated cultures. Cultures with kinetin but no thiamine (Table 21c) declined rapidly in growth and turned brown and died by the third transfer. There was no differentiation. Cultures with neither thiamine nor kinetin (Table 21d) declined very steeply in growth even by the first transfer, but did not die and continued to grow slowly for the four transfers. All of these cultures differentiated, although because of the slow growth rate it

Table 20 Effect of Thiamine Concentration on Growth of Carrot No. 2

Medium	Thiamine Added mg/L	Productivity mg/ml/day
Full M*	0	0.49
	0	0.07
	0.1	0.32
Minimal**	0.5	0.46
	1.0	0.47
	2.0	0.31

2,4-D 0.05 mg/L in all cultures.

* Full M medium includes thiamine at 0.1 mg/L.
** Minimal - omit all growth factors except auxin.

Culture grown 21 days in the dark on the reciprocal shaker.

Table 21 Effect of Growth Factors on Carrot No. 18 on NAA medium
a. All + Thiamine + Kinetin

Transfer No.	1	2	3	4
Days Grown	11	16	13	14
Medium	M	NAA	NAA	NAA
Add to Medium		Productivity (mg/ml/day)		
I P N	0.9	0.9	0.3	0.5
P N	0.9	0.9	0.2	0.2
I N	0.7	0.9	0.2	0.2
I P	0.9	0.9	0.2	0.2
Nicotinic acid	1.0	0.8	0.02	0.06
Pyridoxine	1.0	0.9	0.2	0.3
Inositol	0.7	0.8	0.2	0.1
--	0.8	1.1	0.1	0.1
		Final wt (mg/ml)		
I P N	11.1	16.1	4.9	7.6
P N	10.6	15.1	4.3	3.0
I N	9.0	14.8	4.0	3.7
I P	11.1	15.4	3.9	3.8
Nicotinic acid	11.9	14.5	2.4	1.0
Pyridoxine	12.2	16.0	3.8	4.2
Inositol	8.3	13.3	4.1	2.3
--	10.1	18.3	3.4	1.5

10% inoculum to give an initial weight of 1 mg/ml.

First transfer NAA 0.1 mg/L.
Later transfers 2,4-D 0.05 mg/L.

N = Nicotinic acid
P - Pyridoxine
I = Inositol

Table 21 Effect of Growth Factors on Carrot No. 15 on NAA medium
b. All + Thiamine - Kinetin

Transfer No.	1	2	3	4
Days Grown	11	16	23	14
Medium	M	NAA	NAA	NAA
Add to Medium		Productivity (mg/ml/day)		
I P N	0.5	0.4	0.1	0.2
P N	0.4	0.6	0.04	0.1
I N	0.5	0.6	0.05	0.1
I P	0.4	0.6	0.01	0.1
Nicotinic acid	0.2	0.3	0.1	0.09
Pyridoxine	0.3	0.5	0.01	0.1
Inositol	0.3	0.5	0.05	0.1
—	0.4	0.4	0.05	0.2
		Final wt (mg/ml)		
I P N	5.9	6.7	2.2	2.5
P N	5.1	10.2	1.7	2.1
I N	6.3	11.2	1.4	1.7
I P	5.3	9.7	1.5	2.1
Nicotinic acid	3.3	4.9	1.9	1.5
Pyridoxine	3.8	8.1	1.8	2.9
Inositol	4.5	8.4	1.6	1.9
—	5.3	7.4	1.7	3.2

All cultures differentiated by the second transfer.

Table 20 Effect of Growth Factors on Carrot No. 18 on NAA Medium c. All - Thiamine + Kinetin

Transfer No.	1	2	3	4
Days Grown	11	16	13	14
Medium	M	NAA	NAA	NAA
Add to Medium		Productivity (mg/ml/day)		
I P N	0.7	0.2	0.05	0.04
P N	0.6	0.09	0.04	0
I N	0.8	0.2	0.03	0.03
I P	0.8	0.2	0.03	0.02
Nicotinic acid	0.4	0.3	0	0.01
Pyridoxine	0.5	0.06	0.01	0.01
Inositol	0.6	0.1	0.07	0.02
--	0.6	0.07	0.03	0.01
		Final wt (mg/ml)		
I P N	9.0	4.0	1.2	0.8
P N	7.7	2.5	0.8	0.2
I N	9.3	3.7	0.8	0.5
I P	9.5	4.0	0.8	0.3
Nicotinic acid	5.6	5.2	0.5	0.1
Pyridoxine	6.5	1.7	0.3	0.2
Inositol	7.4	2.4	1.1	0.3
--	7.5	1.8	0.6	0.2

Table 21. Effect of Growth Factors on Carrot No. 18 on NAA Medium d. All - Thiamine - Kinetin

Transfer No.	1	2	3	4
Days Grown	11	16	13	14
Medium	M	NAA	NAA	NAA
Add to Medium		Productivity (mg/ml/day)		
I P N	0.2	0.1	0.05	.07
P N	0.5	0.4	0	0.1
I N	0.3	0.2	0.1	0.1
I P	0.3	0.3	0.09	0.09
Nicotinic acid	0.6	0.2	0.08	0.01
Pyridoxine	0.4	0.3	0.08	0.01
Inositol	0.4	0.2	0.1	0.1
--	0.3	0.2	0.07	0.2
		Final wt (mg/ml)		
I P N	2.7	2.1	1.0	1.1
P N	6.4	7.3	1.2	2.0
I N	4.6	3.0	1.8	2.0
I P	4.5	4.3	1.7	1.3
Nicotinic acid	7.5	3.6	1.4	1.1
Pyridoxine	5.7	4.5	1.7	0.4
Inositol	5.2	3.9	2.1	2.1
--	4.5	3.4	1.3	2.5

All cultures differentiated by the second transfer.

Table 21 Effect of Growth Factors on Carrot No. 18 on NAA Medium (4 Transfers)
e. Average Productivity Values

	Average Productivity Values (mg/ml/day)				
Growth Factors	+ Thiamine + Kinetin	- Thiamine + Kinetin	+ Thiamine - Kinetin	- Thiamine - Kinetin	Average
+ I + P + N	0.65	0.25	0.23	0.10	0.32
- I + P + N	0.53	0.19	0.28	0.27	0.32
+ I - P + N	0.49	0.25	0.32	0.19	0.31
+ I + P - N	0.56	0.25	0.28	0.19	0.32
- I - P + N	0.48	0.18	0.18	0.23	0.27
- I + P - N	0.60	0.15	0.21	0.19	0.29
+ I - P - N	0.44	0.19	0.25	0.21	0.27
- I - P - N	0.53	0.18	0.27	0.18	0.29
Average	0.54	0.21	0.26	0.20	0.30

was less evident than in the cultures with thiamine but no kinetin. An average of all Productivity values for the four transfers (54 days of growth) (Table 21e) suggests that thiamine and kinetin are required for good growth, inositol, pyridoxine ard nicotinic acid have little effect. It appears that kinetin supresses differentiation. In sc doing, it must repress the synthesis of many compounds. Thiamine is required for carrot cells, but probably some can be synthesized by the cells if kinetin is omitted from the medium.

A second experiment was run similarly,using 0.05 mg/L 2,4-D as auxin for the whole experiment (Table 22). The first transfer was from M medium but instead of pipetting a cell suspension for the initial transfer we used a small slotted spoon, transferring only cells and no medium to give 0.2 mg/ml as an initial weight. Subsequent transfers were made at about 2-week intervals using cell suspensions (10% v/v). This led to a more rapid response to nutrient differences. Cultures with thiamine and kinetin (Table 22a) grew rather slowly in the first transfer but recovered and grew vigorously for five subsequent transfers. There was no effect of the presence or absence of inositol, pyridoxine or nicotinic acid. The remaining cultures all grew very poorly and were discontinued after the third transfer (Table 22b, c, d). No differentiation occurred in any of these cultures. With 2,4-D as an auxin, carrot cells require both thiamine and kinetin.

Our conclusion from these studies is that carrot cells grow well with several auxins. Thiamine and kinetin are also required. On NAA medium differentiation occurs if kinetin is omitted. Differentiation does not occur if 2,4-D is the auxin.

Table 22 Effect of Growth Factors on Carrot No. 18 on 2,4-D Medium
a. All + Thiamine + Kinetin

Transfer No.	1	2	3	4	5
Days Grown	15	14	16	13	14
Add to Medium			Productivity (mg/ml/day)		
I P N	0.2	0.9	0.8	0.4	1.1
P N	0.2	0.9	0.8	0.5	0.9
I N	0.1	0.9	0.9	0.8	1.1
I P	0.4	1.0	0.8	0.4	1.0
Nicotinic acid	0.3	0.9	0.8	0.4	1.0
Pyridoxine	0.4	1.0	0.8	0.6	1.1
Inositol	0.3	0.9	0.8	0.5	1.1
--	0.2	0.7	0.9	0.9	1.1
			Final wt (mg/ml)		
I P N	3.8	11.8	14.6	6.9	15.8
P N	3.0	11.7	14.1	9.0	13.5
I N	2.2	12.3	14.9	11.6	16.6
I P	6.8	14.8	15.0	6.4	17.1
Nicotinic acid	3.9	12.5	14.6	7.2	16.6
Pyridoxine	5.8	13.9	14.6	9.5	16.0
Inositol	4.3	13.1	14.0	8.3	16.2
--	3.5	10.8	14.8	13.8	17.2

Small inoculum to start (no liquid), after that 10% v/v. 2,4-D 0.05 mg/L.

Table 22 Effect of Growth Factors on Carrot No. 18 on 2,4-D Medium
b. All + Thiamine - Kinetin

Transfer No.	1	2	3
Days Grown	15	14	16
Add to Medium		Productivity (mg/ml/day)	
I P N	0.04	0.03	0.03
P N	0.05	0.04	0.02
I N	0.07	0.07	0.01
I P	0.10	0.05	0.01
Nicotinic acid	0.07	0.02	0
Pyridoxine	0.05	0.06	0.01
Inositol	0.05	0.06	0.01
--	0.05	0.01	0.02
		Final wt (mg/ml)	
I P N	0.7	0.5	0.6
P N	0.9	0.7	0.5
I N	1.3	0.3	0.3
I P	1.6	0.9	0.2
Nicotinic acid	1.3	0.4	0.1
Pyridoxine	0.9	1.0	0.4
Inositol	0.9	0.9	0.2
--	0.9	0.3	0.5

Table 22 Effect of Growth Factors on Carrot No. 18 on 2,4-D Medium c. All - Thiamine + Kinetin

Transfer No.	1	2	3
Days Grown	15	14	16
Add to Medium		Productivity (mg/ml/day)	
I P N	0.02	0.06	0.01
P N	0.10	0.02	0
I N	0.09	0.03	0
I P	0.12	0.06	0
Nicotinic acid	0.17	0.04	0.02
Pyridoxine	0.18	0.05	0
Inositol	0.13	0.03	0
--	0.10	0.04	0.02
		Final wt (mg/ml)	
I P N	1.5	0.9	0.2
P N	1.7	0.5	0.1
I N	1.5	0.6	0.1
I P	2.0	1.2	0.2
Nicotinic acid	2.6	1.0	0.4
Pyridoxine	2.8	1.2	0.2
Inositol	2.1	0.8	0.2
--	1.6	0.8	0.3

Table 22 Effect of Growth Factors on Carrot No. 18 on 2,4-D Medium
d. All - Thiamine - Kinetin

Transfer No.	1	2	3
Days Grown	15	14	16
Add to Medium		Productivity (mg/ml/day)	
I P N	0.07	0.05	0.01
P N	0.04	0.03	0
I N	0.05	0.02	0.03
I P	0.12	0.04	0.04
Nicotinic acid	0.11	0	0
Pyridoxine	0.04	0.03	0
Inositol	0.05	0.02	0.01
--	0.10	0	0
		Final wt (mg/ml)	
I P N	1.3	0.8	0.2
P N	0.8	0.6	0.1
I N	0.9	0.4	0.6
I P	2.0	1.0	0.8
Nicotinic acid	1.9	0.3	0.1
Pyridoxine	0.8	0.5	0.1
Inositol	0.9	0.5	0.3
--	1.6	0.2	0.1

D. Differentiation in Plant Cell Cultures.

Variability in plant cell cultures has been noted by many workers and studies of differentiation have been an important area of plant cell research (3, 5, 7, 15). Fluctuations in growth rate were noted in our first report.

Chlorophyll development has been noted in plant cell cultures (1, 10, 17) but significant autotrophic growth of green morphologically undifferentiated cells has not been reported. We find that chlorophyll often develops in static cultures of carrot, lettuce and cucumber grown in light. Bean cultures do not develop chlorophyll on the regular sucrose medium, but do turn green when starch is substituted for sucrose. Possibly sucrose gives a feed-back inhibition of chlorophyll formation. The slow release of glucose from starch may be less inhibitory. Chlorophyll development occurs rarely in undifferentiated suspension cultures, and then only when the cells grow in large (ca. 1 mm or more in diameter) nuggets. Carrot cultures produce the highest chlorophyll levels in our experience.

Bean and pepper cultures have never shown any differentiation. Static cultures of cucumber showed differentiation only in the first few transfers. Static cultures of lettuce differentiated into plantlets for a year or so, but now seem to have lost this ability. Static cultures of carrot and marigold still differentiate, although they have been maintained in culture for over three years and have been transferred more than 25 times. The carrot (especially No. 18) readily differentiated into plantlets, especially on old plants. The marigold cultures produce abundant roots, but no shoots. None of these differentiations require coconut milk.

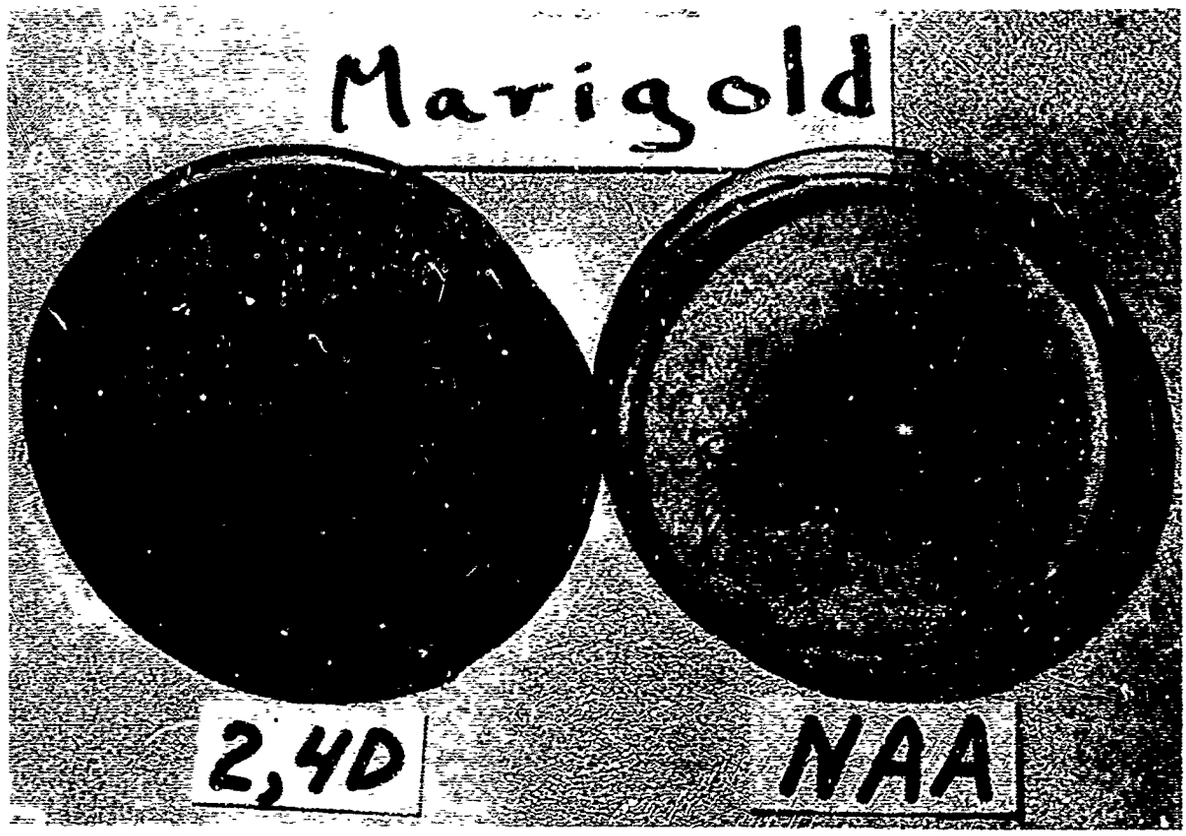
Differentiation is rare in suspension cultures. Marigold cultures however, frequently produce roots with NAA as auxin, but not with 2,4-D (Fig. 14). Bean cells tend to produce larger lumps in NAA medium than on 2,4-D, but do not show true differentiation (Fig. 15). Carrot suspension cultures sometimes differentiate spontaneously when transferred from 2,4-D to NAA medium (Fig. 16) but differentiation on NAA medium is nearly 100% when kinetin is omitted (as described in section C). Carrot cultures without auxin will also differentiate, particularly if kinetin is omitted (Fig. 17).

DISCUSSION AND SUMMARY

Murashige medium is well designed for optimum growth of plant cell cultures with most nutrients present in excess. Considerable reductions and omissions are possible without reducing growth rate or productivity. No changes tried by us have resulted in significant increases in growth rates or Productivity.

Specific findings include: (1) Calcium is already at optimum level and could be reduced without lowering yield, (2) Doubling magnesium level and doubling or tripling phosphate levels may cause slight growth increases, (3) Sucrose is an optimum carbon source. Glucose or starch will also support growth of some cultures, but glycerol is not suitable, (4) Sucrose irradiated to 5 Mrad supports normal growth to our plant cell cultures. Growth is retarded on glucose irradiated at +20° to 0.5 Mrad, but glucose irradiated at -80° supports normal growth, (5) Nitrogen level in this medium can be considerably lowered without reducing growth. Plant cell cultures grow well on NH_4NO_3, KNO_3 or amino acids as the sole source of nitrogen. We have not succeeded in growing plant cells on urea or NH_4Cl. Urea at 500 mg or more per

Figure 14 Differentiation of Suspension Culture of Marigold No. 26 in Response to Naphthaleneacetic Acid

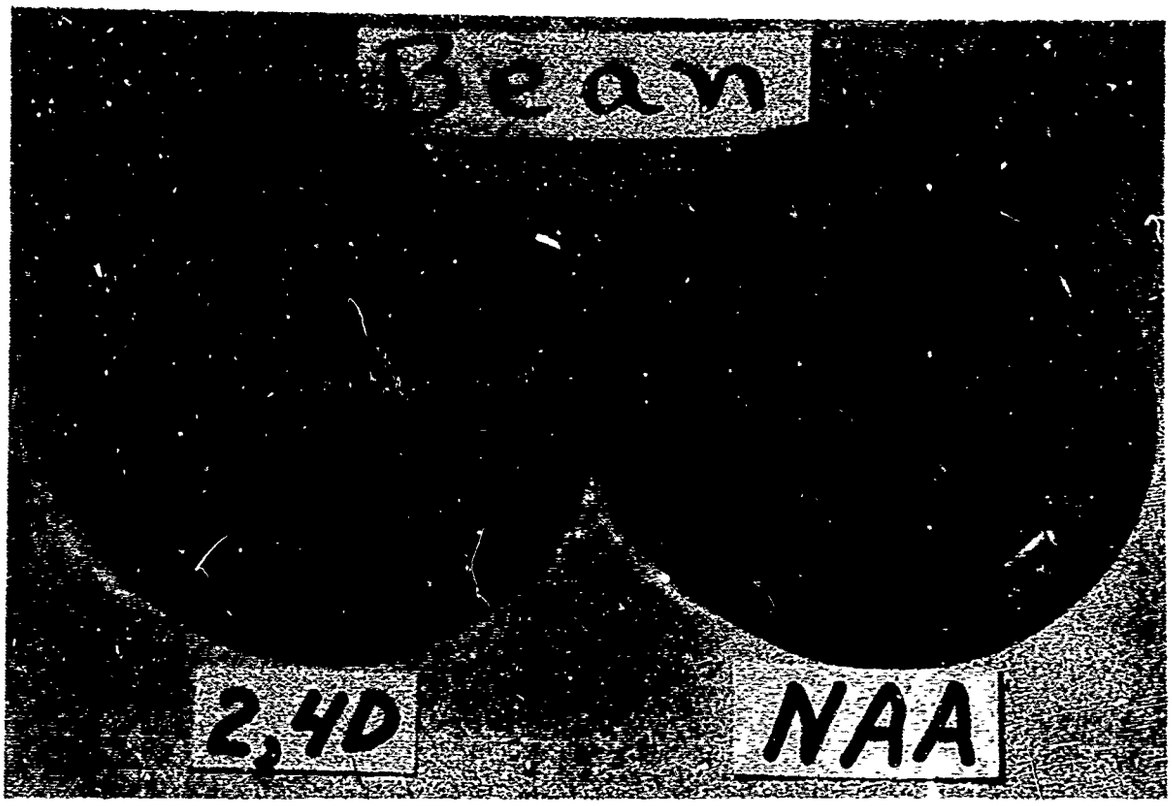


10% Inoculum from 2,4-D medium grown 28 days on rotary shaker in programmed light.

2,4-D - 0.05 mg/L (dry wt 5.2 mg/ml)

NAA - 0.10 mg/L (dry wt 4.3 mg/ml)

Figure 15 Growth of Bean No. 8 in Presence of Different Auxins

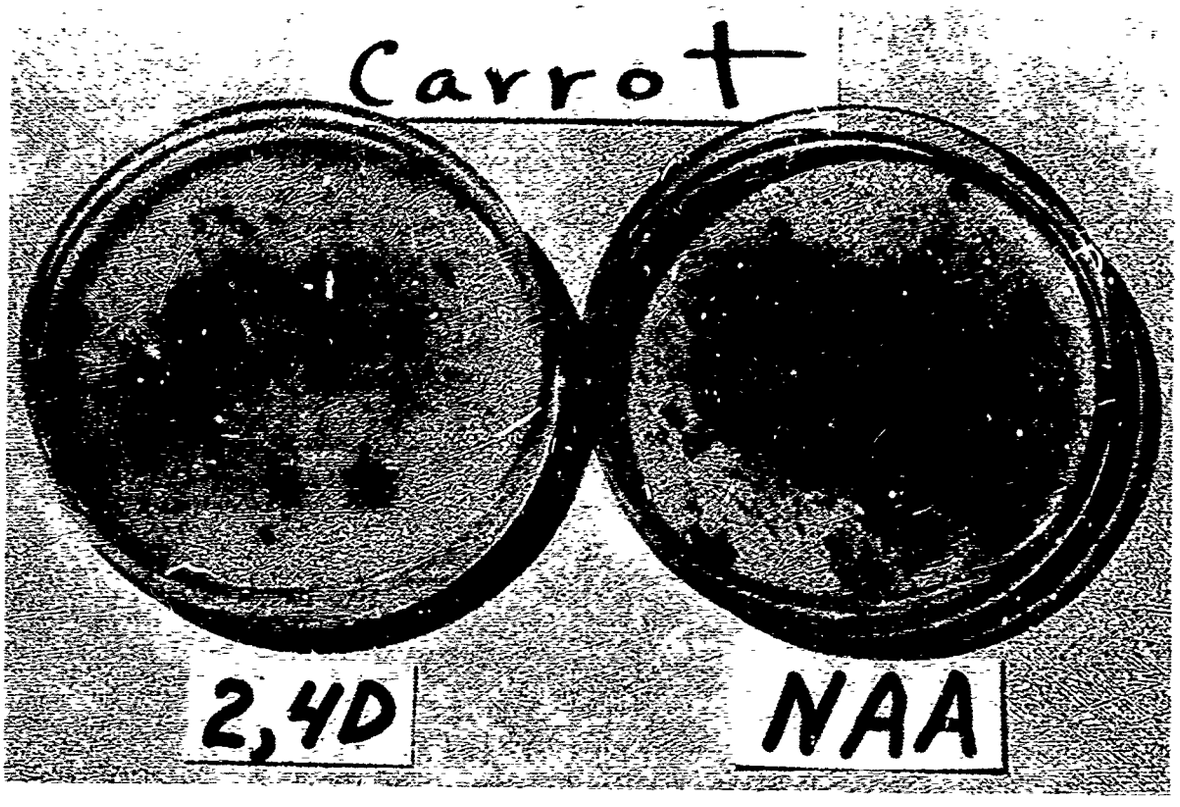


10% Inoculum from 2,4-D medium, grown 13 days on rotary shaker in programmed light.

2,4-D - 0.05 mg/L (dry wt 15.7 mg/ml)

NAA - 0.10 mg/L (dry wt 5.0 mg/ml)

Figure 16 Differentiation of Carrot No. 18 in Response to Naphthaleneacetic Acid

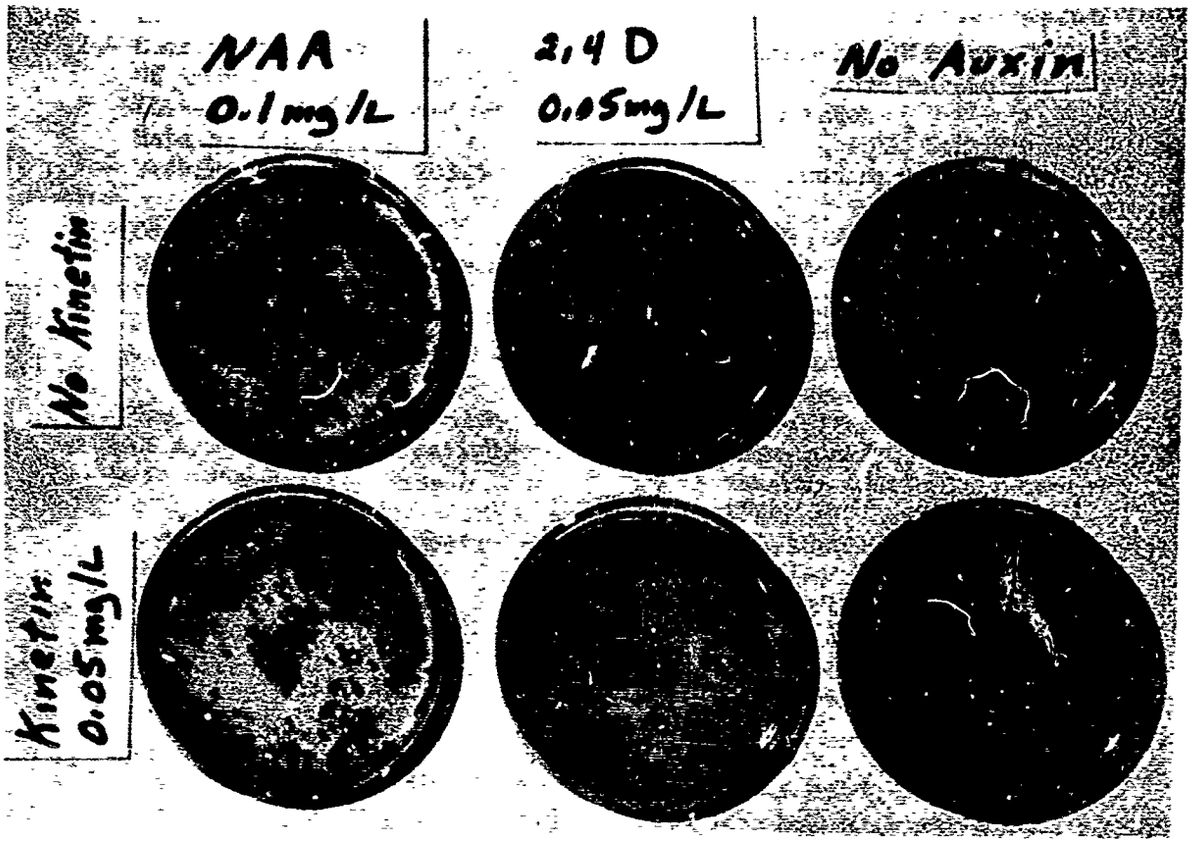


10% Inoculum from 2,4-D medium grown 28 days on the rotary shaker in programmed light.

2,4-D - 0.05 mg/L (dry wt 4.0 mg/ml)

NAA - 0.10 mg/L)dry wt 9.8 mg/ml)

Figure 17 Effects of Auxin and Kinetin on Differentiation of Carrot No. 18



Full medium except for auxin and kinetin added as noted:

Grown on rotary shaker in programmed light for 13 days.

	NAA 0.1	2,4-D 0.05	No Auxin
No Kinetin	5.4 mg/ml	8.4 mg/ml	7.0 mg/ml
Kinetin 0.05 mg/L	8.2	14.6	5.3

liter is toxic, (6) Bean cells require an auxin, preferably 2,4-dichlorophenoxyacetic acid, and thiamine as organic growth factors, (7) Lettuce cells require an auxin, preferably naphthaleneacetic acid, thiamine, and kinetin as organic growth factors, and (8) Carrot cells require an auxin, thiamine and kinetin to grow as undifferentiated cells. With naphthaleneacetic acid as auxin, omission of kinetin leads to differentiation of carrot cells, and this in turn appears to reduce the requirements for thiamine. Differentiation does not occur if 2,4-dichlorophenoxyacetic acid is the auxin. No complex additives such as coconut milk are required for this differentiation.

Our general conclusions are that plant cells can be maintained in suspension culture for several years by periodic transfer on a simple inexpensive defined medium similar to the media used for growing non-fastidious microorganisms. Although carbohydrate must be supplied, the cell yield is 50% or more, and the cost of sucrose, starch, or glucose is certainly less than the cost of supplying light to a photosynthetic culture under fermenter conditions. Growth rate of these cultures remains slow in comparison to other microbial systems. At present the use of plant cell cultures to produce food would seem to be practical only under very unusual circumstances. Plant cell cultures do not make a good candidate for a closed ecological system. Oxygen is consumed, not produced, carbohydrate must be supplied, and as yet plant cell cultures have not been grown using urea or ammonia as a nitrogen source.

Plant cell cultures, however, may have a potential to produce high-cost products such as drugs, enzymes or flavors. Our finding that differentiation can be induced in some suspension cultures on simple defined media is of

interest for such use. Differentiated cultures are much more likely to produce desirable secondary growth products. In cultures grown for food, differentiation could lead to more attractive textures and flavors. Further studies may still uncover approaches to further simplify the growth environment and, hence, make plant cell cultures more attractive as a food source. The genetic capacities of such cell lines provide a unique biological source that can be used for undifferentiated (mass culture) continuous fermentation, or for the production of differentiated tissues.

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13. ABSTRACT

A detailed study has been made of some nutrient requirements of several plant cell suspension cultures. These cultures require the usual inorganic salts including inorganic nitrate nitrogen. Hydrolyzed protein or amino acids will serve as the sole source of nitrogen for growth, but organic nitrogen is not required. Urea or ammonia nitrogen in the absence of nitrate will not support growth. Sucrose is an excellent carbon source. Glucose and starch will also support growth of some cultures. Sucrose sterilized by gamma irradiation supports normal growth of these cultures. Growth is retarded on glucose sterilized by gamma irradiation at ambient temperature, but growth is normal on glucose irradiated at -80°C. A few growth factors at low concentration are also required. An Auxin (2,4 dichlorphenoxyacetic acid or naphthaleneacetic acid) and thiamine are required by all cultures. Kinetin (6 furfurylaminopurine) is also required by some cultures. Maximum productivity on this simple medium is about 1.5 grams dry weight per liter of culture per day, equal to, but not significantly greater than productivities obtained on much more complex media.

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	ROLE	WT	ROLE	WT	ROLE	WT
Nutritional requirements	8					
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Cultures (Biology)	9,5					
Culture media	9,5		5			
Growth	8		7			
Inorganic salts			6			
Proteins			6			
Amino acids			6			
Urea			6			
Ammonia			5			
Sucrose			6			
Starches			6			
Glucose			6			
Auxins			6			
Thiamine			6			
Kinetin			6			