THE INFLUENCE OF SOME CULTURE CONDITIONS

ON GROWTH OF PLANT TISSUES <u>IN VITRO</u>

by

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ABSTRACT.

The response of various plant tissues to different conditions of culture was compared. The tissues used were cambium-containing discs from carrot roots, undifferentiated carrot callus, bacteria-free sunflower tumorous (crown-gall) tissue, and segments of sunflower stems. The culture conditions compared, in combination, were agar versus liquid medium, shaken versus non-shaken liquid medium, and continuous light versus continuous dark. The response of the tissues to White's basal nutrient medium with added coconut milk (15 %) and indoleacetic acid (0.1 mg/l) and Hildebrandt's improved sunflower medium was also compared under these different culture conditions.

Agitation of the liquid medium was accomplished through the use of a newly designed shaker, which consists basically of a horizontally oscillating bank of shelves. The tissues rested on the bottom of culture flasks (medicine bottles) on these shelves and were alternately exposed to medium and air as the liquid medium washed back and forth. Any horizontally oscillating platform could replace this shaker and almost any type and size of culture flask could be used. Probably any type of plant tissue could be cultured under these shaking conditions. It is not necessary that the tissues adhere to the walls of the culture vessels as in other agitation

methods so far used in plant tissue culture.

Growth (weight increase) of all tissues in shaken liquid medium (in both light and dark) was markedly superior (two to six times greater average weight in 42 days) to that of tissues on agar and in non-shaken liquid medium. The superiority of growth in shaken liquid medium is probably due to several factors; nutrients and gasses are supplied to the entire surface of the tissues, there is no drying and hardening of the tissue surfaces, resulting in a greatly increased surface area, harmful excretions can not collect at the tissue surface, and diffusion of nutrients is not hindered by adsorptive effects of agar particles.

To compare the growth of these cultures with those of other workers using agitation methods is difficult due to the different sources of plant material, different sizes of tissues cultured, and different periods of culture used. In general the stimulatory results of shaking obtained appear to be at least as good as those obtained by Caplin and Steward with the much more elaborate and limited 'auxophyton'. There was no sign of eventual growth stoppage as obtained by White, using roller tubes.

Light consistently stimulated tissues grown in liquid medium, particularly those in shaken liquid medium. The effect was especially marked on carrot callus and tumorous sunflower tissues grown in Hildebrandt's medium. It is suggested that light may play a role in the synthesis of growth factors supplied by coconut milk. Light had no significant effect on the growth of tissues on agar medium, indicating that the primary limiting factor in the growth of such tissues may be the rate of diffusion of nutrients from the agar.

Carrot tissues showed better overall growth in the enriched White's

medium while the sunflower tumorous tissue grew better in Hildebrandt's medium. The effect on carrot was probably primarily through indoleacetic acid and coconut milk. The response of sunflower tissue is difficult to evaluate at present.

All carrot tissues developed chlorophyll throughout all of the experiments if cultured in light, while tumorous sunflower tissue remained white until placed in Hildebrandt's medium, when it turned light green. The significance of these differences is not known.

One experiment showed that carrot discs derived from different carrots grew at significantly different average rates, indicating that discs to be compared should be derived from the same root. The plane in which the discs were cut did not seem to influence subsequent growth. 'Intra root' variation in disc growth necessitates replication.

ACKNOWLEDGEMENTS.

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INTRODUCTION AND REVIEW OF LITERATURE.

I. General background.

The culture of isolated plant parts (organs and tissues) in
controlled nutrient media has been a major problem in experimental
botany since the turn of the century. In the beginning, the
establishment of growth _in vitro_ was simply an end in itself but the
emphasis has slowly shifted and today, tissue culture has become
primarily a means for controlled experiment with living processes.
Despite, however, notable successes in culturing plant tissues and
significant advances in our knowledge which have come about through
the use of the technique as an experimental tool, tissue culture
still has many imperfections and weaknesses which prevent full
exploitation of its many advantages. To appreciate both the present-
day advantages and weaknesses of plant tissue culture it is desirable
to briefly consider its development and present status.

Plant issue culture was first attempted by Haberlandt in 1902.
He used relatively highly differentiated cells (e.g. leaf parenchyma)
and very simple mineral nutrients; the experiment was unsuccessful
but provided stimulus to other workers who took up the problem. It was
not, however, until 1922 that partial success in growing plant tissues
artificially was obtained. In this year Kotte and Robbins independently

cultivated excised tomato roots for limited periods of time. Subsequently techniques were slowly improved and in 1934, White established cultures of excised tomato roots which were capable of unlimited growth in culture.

White (1943), considers the in vitro cultivation of excised roots to be organ culture rather than true tissue culture. He defines tissue culture in the strict sense as unlimited artificial growth of undifferentiated tissue. Cultures fulfilling this definition were obtained in 1939 when Gautheret, Nobecourt, and White independently established cultures of undifferentiated plant callus which were apparently capable of unlimited sub-culture.

Since these original researches the field of plant tissue culture has been extensively and diversely developed. To outline the more recent advances in the field it will be useful to consider separately the types of plant tissues which have been cultured and the general media and methods used.

II. Types of Plant Tissue Cultures.

Plant tissue cultures can be conveniently divided, according to the origin of the plant parts used for cultivation, into the following classes:

A. Embryo culture

B. Organ culture

C. Callus culture

D. Culture of tumorous tissues

E. Cultures of other origin

While the first two groups, embryo and organ cultures, do not fulfill the conditions of White's definition of true tissue culture (i.e. the tissue should be undifferentiated and capable of unlimited growth), they are included in this discussion because the techniques used with them are basically the same as those used in the culture of undifferentiated tissue. It should be further remarked that all the cells in undifferentiated callus cultures are not the same. Crown gall callus tissue of sunflower and hybrid tobacco callus tissue contain at least three different kinds of cells: parenchyma cells (large hyper-trophic cells), meristematic cells (small hyperplastic cells), and thick-walled scalariform cells (wound tracheids)(White, 1939; Caplin, 1947; Struckmeyer et al., 1949). Thus, White's use of the term 'undifferentiated' in his definition of true tissue culture applies at the histological and morhpological levels but not at the cytological level.

A. Embryo Culture.

Attempts to grow plant embryos on artificial media started early in the 20th century (e.g. Hanning, 1904; Stingl, 1907). However, it was not until 1922 that Knudson succeeded in germinating orchid seeds on artificial media under aseptic conditions. The orchid seed is considered an embryo rather than a seed as it consists of undifferenti-ated cells and can germinate in nature only in symbiosis with fungi. As media Knudson used mineral salts solutions, glucose and fructose as a carbohydrate source, and various plant extracts. He found extracts of Bacillus radicicola to be particularly beneficial to the development

of seedlings. Later Tukey (1933, 1934, 1938, and 1944) successfully cultured embryos of sweet cherry, peach, and other stone fruits.

Considerable improvement in the technique of embryo culture was brought about by the introduction of coconut milk into the nutrient media. This substance was used for the first time by van Overbeek et al. (1941) for the cultivation of Datura embryos. As a result it became possible to grow much younger embryos than had been grown before (van Overbeek et al., 1941, 1942, and 1944), and to obtain hybrids from incompatible crosses of Datura (Blakeslee and Satina, 1944).

Following this work embryo culture became widely used as a method of overcoming embryo abortion in crosses between incompatible varieties and species. Brink et al. (1944) obtained a hybrid between Hordeum jubatum and Secale cereale; the hybrid embryo does not mature in the seed but it was possible to grow it on an artificial medium. Similarly, Cooper and Brink (1945) were able to produce hybrids between diploid and tetraploid races of Lycopersicum pimpinellifolium through the use of embryo culture. As well as being a means for over-coming genetic incompatibility, embryo culture can also speed up breeding programs through the elimination of dormant period of seeds. The use of embryo culture in plant-breeding was discussed by Skirm (1942) and by Tukey (1944).

Weeping crab-apple embryos have been cultured by Nickell (1951), and potato embryos by Haynes (1954). Hybrid embryos of many other plants have been successfully cultured, among them: tobacco, cotton, lily, iris, violet, ginkgo, pine, apple, pear, plum, rose, and olive. A general review of embryo culture has been given by Rappaport (1954).

B. Organ Culture.

This category includes cultures of isolated roots, stem apices, leaves, flowers, and fruits. Roots have been most widely cultured of these organs, primarily because their isolation, culture, and measurement is relatively easy. Tomato roots were used by White (1934) for establishment of the first potentially unlimited plant tissue culture. Since then, tomato roots have been used for experimental studies by many other authors, among them by Robbins and Bartley (1937), Robbins and Schmidt (1939), Dormer and Street (1949), and Street (1953). Many other plant species have been used for root culture. Bonner and Addicott (1937) cultured pea roots while Bonner and Derivian (1939) cultured, besides pea and tomato roots, roots of radish and flax. Bonner (1940) established the minimal nutritional requirements for optimal growth of excised roots of alfalfa, clover, cotton, Datura, carrot, and sunflower. Almestrand (1949) cultured excised roots of barley and oats.

The culture of stem tips of Stellaria media was attempted by White (1933). Subsequently stem tips of Tropaeolum majus and of Lupinus albus were successfully grown by Ball (1946), shoot tips of Psilotum nudum by Marsden and Wetmore (1953), and apices of Adianthum pedatum by Wetmore (1954).

Leaves of tobacco were grown on artificial media by Dawson (1938) and flowers and fruits by Nitsch (1949). Pucher et al. (1937) have made extensive use of excised leaf cultures (e.g. tobacco and rhubarb) in the study of organic acid metabolism in plants.

The latter cultures, i.e. leaf, flower, and fruit cultures, are

only temporary and end with the full development of the cultured organ.
Cultures of roots and stem apices, which contain active meristems, can
be continued indefinitely.

C. Callus Culture.

The term callus culture is generally used to describe the culture
of undifferentiated tissue derived from cambium or other meristematic or
potenti-.ally meristematic tissue. Gautheret's (1939) first tissue
culture capable of indefinite growth was derived from the cambium of
carrot roots while White's (1939) came from procambium of Nicotiana
stems. Since these original experiments, callus cultures have been
obtained from cambium of many plant species, e.g. Ulmus campestris
(Gautheret, 1940), Vitis vinifera (Morel, 1944), Rosa sp. (Nobecourt and
Koefler, 1945), Helianthus annuus and Vinca rosea (deRopp, 1947),
Scorzonera sp. (Gautheret, 1948), Salix caprea (Gautheret, 1950), and
ferns (Morel and Wetmore, 1951).

To the group of callus cultures derived from meristems other
than cambium belong cultures of undifferentiated tissue proliferated
from embryos cultured on artificial media. Such cultures were isolated
from pro-embryos of Datura by van Overbeek et al. (1941, 1942), and
from seedlings of Pinus cultured by Loewenberg and Skoog (1952). Curtis
(1947) was able to produce undifferentiated tissue cultures from embryos
of orchids by adding barbiturates (e.g. 10 ppm phenyl ethyl barbituric
acid) to the culture media. Though derived from embryos, all of these
cultures are true tissue cultures in the sense of White's strict
definition, as the growth is undifferentiated and can be continued

imdefinitely.

Several callus cultures have been developed from potentially meristematic tissues. Caplin and Steward (1948, 1949, and 1952) obtained callus cultures from secondary phloem of carrot. Cultures have also been derived from storage tissue of potato tuber (Steward and Caplin, 1951), and from similar tissues of sweet potato and Jerusalem artichoke (Wetmore, 1954).

It should be noted that while cambium produces callus 'spontaneously' it is necessary to treat potentially meristematic tissues with growth-promoting hormones such as indoleacetic acid (Gautheret, 1946) or coconut milk (Caplin and Steward, 1948) before they will start cell proliferation. However, once removed from contact with organized plant tissues, all callus cultures normally require an exogenous source of growth-hormone (see below).

C. Cultures of tumorous tissue.

The majority of culture work with tumorous tissue has been carried out on tissue derived from tumors originally induced by bacteria of the genus Agrobactërium (commonly called crown gall bacteria). Tissue from such tumors is similar to normal undifferentiated callus in genral cellular makeup and growth habit but it requires somewhat different techniques of isolation and is capable of growing on media without growth-hormone. The major problem has been to obtain in culture, tumorous tissue free of the inciting bacteria.

White and Braun (1942) first isolated and cultured bacteria-free crown gall tissue from secondary tumors on sunflower. Similar cultures

from primary galls on sunflower were later obtained by deRopp (1947).
Heat treatment was used by Braun (1947) to kill the bacteria in crown
gall tissue on Vinca rosea, allowing subsequent culture of bacteria-free
tissue. Bacteria-free crown gall cultures have been obtained from many
other plants, e.g. Scorzonera sp. and Helianthus tuberosus (Gautheret,
1947, and 1948), and Vitis vinifera and Antirrhinum majus (Morel, 1948).
The problem of crown gall and the results achieved in culturing of
tumorous tissue have been reviewed by many authors, among them by
Gautheret (1950), White (1951), and deRopp (1951).

Tumors on plants can be also initiated by other agents than
Agrobacterium sp. Tumors on roots of Rumex acetosa induced by wound
virus were cultured by Nickell and Brakke (1954). Genetic incompatibility
causes the appearance of spontaneous tumors in certain crosses of
Nicotiana species (Kostoff, 1930). This Nicotiana hybrid tumor tissue
has been cultured very extensively by White (1939), Caplin (1947),
Hildebrandt et al. (1945, and 1946), and Hildebrandt and Riker (1947).

E. Cultures of other origin.

Tulecke (1953) succeeded in culturing undifferentiated tissue
derived from pollen of Ginkgo biloba. The endosperm of maize has been
cultured by Strauss and La Rue (1954). Cultured endosporm appears to be
a good source of material for observation of mitoses in vivo (Bajer and
Mole-Bajer, 1954). Northcraft (1951) used ammonium oxalate in a liquid
medium to dissolve the middle lamella of cells in carrot callus cultures.
In doing so he claims to have obtained cultures of carrot callus tissue
derived from single cells.

Jablonski and Skoog (1954) have cultured pith tissue from tobacco. Through the use of indoleacetic acid and coconut milk or water extracts of vascular tissue they obtained continuous cell division and proliferation of the pith cells.

III. Tissue Culture Media.

The nutrient media used for plant tissue culture consist basically of mineral salts, carbohydrate, amino acids, vitamins, and growth-promoting hormones (Gautheret, 1942; White, 1943, and 1954).

The mineral salts generally used are basically the same as those used for cultivation of intact plants in water cultures. Gautheret (1935) used modified Knop's solution and White (1934, 1943, 1954) used a modification of the solution of Uspenski and Uspenskaja. White's original mineral solution consisted of the following salts: $MgSO_4$, $Ca(NO_3)_2$, Na_2SO_4, KNO_3, KCl, NaH_2PO_4, $Fe_2(SO_4)_3$, $MnSO_4$, $ZnSO_4$, H_3BO_3, and KI. This solution supports growth of most tissues and has been extensively used in plant tissue culture. More recently some changes which produce increased tissue growth have been proposed. Boll and Street (1951) suggested the addition of the trace elements molybdenum and copper. Hildebrandt et al. (1946) replaced ferric sulphate with the more stable ferric tartrate. Hildebrandt et al. (1946) also extensively investigated the optimal concentration requirements for the mineral salts and developed improved media for sunflower and tobacco tissues.

Different sugars in varied concentrations have been tried by many workers in attempts to find the best source of carbohydrate for tissue culture. The more important among these studies were those by

by Knudson (1924) with cultures of orchid embryos, White (1940) with

tomato roots, Bonner (1940) with excised roots of several plant species,

Hildebrandt et al. (1945) with tobacco and sunflower tissue cultures,

Dormer and Street (1949), Street and Lowe (1950), Street and McGregor

(1952) with culture of excised tomato roots, and Rappaport (1954) with

the culture of plant embryos. In most of these investigations sucrose

proved to be superior to any other carbohydrate; dextrose and levulose

also gave excellent results. Mannose, maltose, cellobiose, galactose,

and raffinose were satisfactory for some species but poor for other

species. Generally used now is either 2 % sucrose (White's and Hilde-

brandt's media), or 3-5 % dextrose (Gautheret's medium).

As a source of organic nitrogen White's medium contains glycine

and Gautheret's medium cysteine. Addicott and Bonner (1938) used a

mixture of seven different amino acids for culture of pea roots but

these were later found to be unessential for optimal growth. Various

organic and inorganic nitrogenous compounds were tested by Riker and

Gutsche (1948). These authors found that glycine was not essential for

continuous growth of sunflower gall tissue and recommended further

experiment with nitrate, urea, alanine, aspartic acid, and glutamic acid.

These substances are claimed to be not only a superior source of nitrogen,

but also to have a separte stimulatory effect on growth of tissue.

The vitamin requirements of different organs and tissues in

culture have been studied widely. The necessity of vitamin B_1 (thiamine)

for the growth of the majority of plant tissues in vitro has been

established by the studies of White (1937, and 1940), Robbins and Bartley

(1937), Robbins (1939), Bonner (1937, 1938, and 1940), and Bonner and

Devirian (1939). Similarly, the tissue culture requirements for vitamin B_6 (pyridoxine) and nicotinic acid have been studied by Robbins and Schmidt (1939), White (1940), and Bonner (1938, and 1940); most tissues (and organs) require these vitamins for sustained growth. Thiamine, pyridoxine, and nicotinic acid are now included in all basic tissue culture media although certain cultures will grow in the absence of one or the other of them, e.g. roots of alfalfa do not require pyridoxine for optimal growth (Bonner, 1940). In addition to these vitamins Gautheret (1950) uses biotin and Ca-pantothenate for the culture of certain tissues, e.g. Salix caprea.

The influence of growth-hormones has been studied extensively in cultured normal callus and in tumorous tissues. The work of Gautheret (1937, and 1939), Duhamet (1939), and others lead to the conclusion that indoleacetic acid or some other growth-hormone (e.g.naphtalene-acetic acid or indolebutyric acid) is indispensable for the growth in vitro of normal plant callus tissue. An exception to this statement occurs in the case of 'habituated' callus tissue, first obtained by Gautheret (1948a). Habituated tissue is normal callus tissue which in the course of continuous culture has lost the need for an exogenous supply of growth-hormone. The mechanism of this metabolic change is not understood. Bacteria-free tumorous tissue behaves the same as habituated callus tissue in this respect and grows optimally without supplied growth-hormone (Gautheret, 1947).

Tissue cultures have been widely used in attempts to elucidate the action of growth-hormones in tissue. The effects of indoleacetic acid on water absorption by potato tuber tissue have been studied

by Commoner et al. (1942), the histological effects of growth-hormones on crown gall tissue by Struckmeyer et al. (1949), the effect on growth and respiration of artichoke tissue by Hackett and Thimann (1952), and the influence on meristematic activities of tomato roots by Street (1953). Skoog and Tsui (1948) and Skoog (1951, and 1954) studied the effects of growth-hormones on growth, differentiation, and organ formation in callus culture, and Jablonski and Skoog (1953) examined the effect of growth-hormones on cell enlargement and cell division in isolated tobacco pith tissue.

The growth-promoting effect of coconut milk (liquid endosperm) on plant tissue cultures was first noticed by van Overbeek et al. (1941) in culturing of _Datura_ embryos. Coconut milk increases the rate of growth of plant tissue cultures considerably and is superior to other plant extracts such as tomato juice (Nitsch, 1951). The effect of different concentrations of coconut milk has been thoroughly studied by Caplin and Steward (1948, 1949, and 1952), Duhamet (1951a, b, and c), and Cutter and Wilson (1954). The optimal concentration lies between 10 and 20 % for the majority of plant tissues. There is obviously a different content of the growth-promoting substance in milk obtained from different nuts.

The growth-promoting effect of coconut milk on plant tissue cultures is similar to the effect of embryo extract on animal tissue cultures. Recently Miller and Skoog (in preparation) have isolated from coconut milk a factor of unknown structure which promotes cell division in isolated pith tissue and accelerates growth of tobacco callus. It is to be hoped that all active agents in coconut milk will eventually be

chemically characterized as the use of coconut milk in plant tissue culture at present introduces unknown factors which hamper controlled experimentation and interpretation of results.

The influence of environmental conditions on plant tissue cultures has been studied sufficiently to establish optimal temperatures, ion concentration, osmotic pressure, and pH of media (White, 1932, and 1943; Hildebrandt et al., 1945, and 1946). Optimal values of these factors vary with different kinds of tissues cultured. For example the optimal temperature for growth of tobacco callus tissue was found to be $26-32^\circ C$ and for sunflower callus tissue $24-28^\circ C$. The optimal pH for the former tissue is 5.0-5.9, and for the latter tissue 5.5-5.9 (Hildebrandt et al., 1945). While some authors have found that light has no influence on rate of growth of cultured tissues (Hildebrandt et al., 1945), Caplin and Steward (1952) reported a slight growth-promoting influence of light on the callus proliferation of cultured carrot discs. Bunning and Welte (1953) obtained significant differences in the rate of growth of carrot-disc cambium under different periods of illumination.

IV. Tissue Culture Technique.

For successful cultivation of plant tissue it is necessary to supply it with nutrient medium and air. The standard technique is to use liquid media for root cultures and semi-solid or solid media (with 0.5-2 % agar) for other kinds of tissue cultures. The roots float on the surface of the liquid medium and are thus aerated. Tissues on agar media obtain nutrients by diffusion from the agar at points of contact while the greater part of the tissue is directly exposed to the air.

Liquid media have not been genrally used for cultures other than roots because the tissue does not float on the medium and consequently suffers from the lack of air. White (1939) tried growing callus tissue in liquid medium and found that growth was considerably slower than on agar and that histological differentiation occured and roots appeared. He presumed both effedts to be a result of the relatively anaerobic conditions present beneath the surface of the liquid medium where the tissue lay.

It was discovered by the workers with animal tissue culture that alternating submersion of tissue in liquid medium and exposure to sterile air in the culture vessel would provide for increased rate of growth. Using this principle Gey and Gey (1936) developed the roller tube method. Cultures are grown on the walls of tubes filled with small amount (1-2 ml) of liquid medium and the tubes are revolved along their longitudinal axis on a special drum. The cultures are thus alternately washed by the medium and exposed, for longer periods of time, to the air in the tube. The method was subsequently modified by Shaw et al. (1940). These workers substituted for the culture tubes, roller bottles with a hole in one side closed by a cover glass. This cover glass permitted direct microscopic observations on the tissue culture cells in vivo. Other modifications, such as introduction of perforated cellophane to hold the culture, were devised later.

Such techniques are not easily applied directly to plant tissue cultures as the plant cultures grow as relatively massive, solid lumps of tissue and do not readily adhere to the walls of the tubes (as do animal cultures). However, the possibility of accelerating growth of plant tissue cultures through the use of agitated liquid medium

attracted some workers. deRopp (1946) constructed an apparatus consisting basically of a U-tube with liquid medium in one arm and the tissue culture in the other arm. The tissue rests on some suitable support, such as quartz sand, and the medium is supplied to it by tilting the apparatus on a rack. After submerging the tissue in the liquid medium the apparatus is returned to the original position and the tissue exposed to the air in the tube.

Another method was devised by Caplin and Steward (1949) and used for extensive studies carried out by these authors (Caplin and Steward, 1952; Steward and Caplin, 1952a, b; Steward et al., 1952). These workers use a new type of culture tube which is closed and rounded at both ends, with a narrower open tube attached, at right angles, in the middle of the larger tube. The tissue sticks to the wall of one of the rounded ends of the main tube. The tube is attached to a circular disc and revolved at the speed 1 r.p.m., causing the liquid medium to flow slowly from one end of the tube to the other, thereby alternately immersing the tissue in the medium and exposing it to the air in the tube. The smaller side-neck tube (plugged with cotton) provides for the entrance to the tube and air exchange. The apparatus was originally named revolving klinostat and renamed auxophyton in 1952.

White (1953) used the roller tube method of Gey and Gey (1936) for plant tissue cultures.

All of these attempts to utilize agitating methods in plant tissue culture have had success in accelerating growth. However, in general they are somewhat cumbersome and require costly glassware and rotating devices. In 1952 the late Dr.L.C.Coleman began experiments to develop an agitating method for growth acceleration that would be, at the same

time, simple, reasonably inexpensive, and readily adaptable to large-scale experiments with all types of plant tissue cultures. The present author joined Dr. Coleman early in his studies along these lines.

This report describes the methods eventually developed and the results of experiments designed to measure the comparative growth response of several tissues to these and other methods of tissue culture. The study had, in effect, a two-fold aim; to empirically develop conditions for optimal growth of plant tissue cultures and, using these methods, to gain some insight into the metabolism of various cultured tissues.

MATERIALS AND METHODS.

I. Plant Material.

In the present experiments these different plant materials were
used:

A. Fresh carrot discs

B. Undifferentiated tissue of carrot

C. Bacteria-free sunflower tumor tissue

D. Stem sections of sunflower

A. Fresh carrot discs.

Carrot discs were cut from carrot roots of unknown variety bought
in local stores. The roots were washed in 50 % alcohol, sterilized for
20 min. in a 0.1 % aqueous solution of mercuric chloride, and subsequently
rinsed in sterile water. In a sterile transfer chamber discs for culture
were cut from the middle third of the sterilized roots.

In preliminary experiments discs with their diameters parallel
and at right angles to the longitudinal axis of the root (longitudinal
and transverse discs respectively) were used. To obtain longitudinal
discs, radial cylinders were bored out across the roots with a cannula
or a cork borer and then cut into discs with a multibladed cutter
similar to that used by Caplin and Steward (1949). Only discs containing
cambium were used for cultivation. Transverse discs were obtained by
cutting a transverse slice, 1 mm thick, across the root with a two-bladed
cutter. (The blades in this cutter are offset about 3 mm so that when

the upper blade has cut completely across the carrot, and thus cut off
the unusable portion, the lower blade has not completely severed the
slice. Leaving the slice partially attached to the carrot root in this
manner provides support for the slice which makes the cutting of sterile
discs from it easy). Discs were cut from the transverse slice with a
cannula or a borer so that the cambium was running completely across
their diameters.

Discs of various diameters were tested in preliminary experiments.
Discs cut with a 1.8 mm cannula weighed 2 mg, with a 4 mm borer 18 mg,
and with a 6 mm borer 42 mg each. Discs 6 mm in diameter were eventually
found to give the most uniform growth and were used in the main experi-
ments reported here.

B. Undifferentiated tissue of carrot.

Undifferentiated carrot tissue used in these experiments was
derived from callus proliferated by the cambium of fresh carrot discs
in culture. Fresh transverse carrot discs weighing 18 mg (4 mm in
diameter, see above) were cultured in agitated liquid White's basal
medium with added indoleacetic acid and coconut milk (see below) for
14 weeks. They were transferred to fresh media every two weeks during
this period. At the end of this time irregularly shaped masses of undif-
ferentiated tissue, yellowish green with reddish spots, and covered with
mamillary outgrowth had formed. These masses of undifferentiated callus
tissue were cut with a scalpel into small pieces, approximately 80 mg
each, and sub-cultured. The culture was maintained and expanded by
continuous sub-culture, and the sub-cultured pieces of callus were used

in the experiments on tissue culture conditions reported below.

C. Bacteria-free sunflower tumor tissue.

Bacteria-free sunflower tumor tissue was isolated from a small secondary tumor which developed on the main vein of a leaf of a sunflower plant eight weeks after the plant was inoculated with Agrobacterium tumefaciens. The secondary tumor was washed in 50 % alcohol, sterilized for a few minutes in a 0.1 % aqueous solution of mercuric chloride, and opened aseptically. Small pieces of tissue were cut out with a scalpel, cultured in agitated liquid White's basal medium (see below) for 8 weeks, and then sub-cultured. Stock sub-cultures of the tissue were transferred every two weeks and used ... for experiments.

D. Stem sections of sunflower.

In one of the preliminary experiments segments of sunflower stem were used. When young sunflower plants were about 5 inches tall the leaves were removed and the upper parts of the stems were cut off. The excised stem pieces were washed in alcohol, sterilized for 10 min. in 0.1 % mercuric chloride, and rinsed in sterile water. Finally, the epidermis of each stem piece was peeled off and the first internode cut into 4 mm long segments which were used directly in the experiment.

II. Handling of Material.

All transfers of tissue cultures were carried out in a transfer chamber; the instruments used were dipped in alcohol and flamed.

The amount of bacterial and fungal contamination was at first about 10-15 % but this was lowered to about 5 % when a special small transfer chamber was used. (This part of the experiments was carried out in the Dominion Laboratory of Plant Pathology in Saanichton, B.C. Because of the great amount of plant pathological work that was being done in the laboratory it was difficult to keep the cultures free from contamination during transfers and weighing). After the laboratory was moved to Vancouver a transfer chamber that could be steam-sterilized was used and almost no contamination was subsequently encountered.

Increase in wet weight was used as a measure of growth of the tissue cultures. Tissue pieces from liquid media were surface-dried with sterile filter paper in sterile Petri plates, transferred into tared sterile Petri plates, weighed, and returned to fresh media. Tissue pieces from agar media were freed of adhering agar and rinsed in sterile double distilled water before surface-drying and weighing. Contaminated cultures were usually discovered within two days after transfer; they were immediately surface-dried, weighed, and discarded. The weight was subtracted from the original total weight of tissue in the treatment.

III. Growth Conditions.

A. Glassware.

Medicine bottles of 170 ml content were used for cultures grown in liquid media and vials 22x94 mm for cultures grown on agar media. Bottles and vials were closed with cotton plugs wrapped in cheese cloth which had been previously boiled in distilled water. All glassware used in the preparation of media and for culturing was acid cleaned in a

saturated solution of potassium dichromate in concentrated sulphuric

acid, and subsequently rinsed ten times in hot running water, twice in

distilled water, and twice in double distilled water.

B. Media.

Two basal media were used in the experiments: White's standard

medium (1943, and 1954*) and Hildebrandt's improved sunflower tissue

medium (Hildebrandt et al., 1946). Except in preliminary experiments

(see results section) White's medium was supplemented with 15 % coconut

milk and 0.1 mg/l indoleacetic acid, and Hildebrandt's medium with

0.01 mg/l indoleacetic acid.

Composition of media is given on next page.

Double distilled water (first distillation in a metal still,

second in a Pyrex-glass still) was used throughout the experiments.

CP grade reagents were used exclusively. Stock mineral salt solutions

were kept at room temperature while stock solutions of vitamins and of

indoleacetic acid were stored in a freezer. Fresh stock solutions of

all chemicals were prepared every six weeks.

Coconut milk was obtained from mature coconuts. Each nut was

opened and the milk poured into a stender and examined for quality.

Disintegrating milk (cloudy and odoured) was discarded. The good quality

milk was mixed, filtered, distributed in 150 ml aliquots, and stored in

a deep-freezer. Preliminary experiments showed that there was no difference

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*The concentration of vitamins in White's 1954 book (p.74) is given, by
a mistake, ten times stronger than it should be.

Composition of Media:

Nutrient ingredient	White's basal medium	Hildebrandt's improved sunflower t. medium
Na_2SO_4	200.00 mg	100.00 mg
$Ca(NO_3)_2 \cdot 4 H_2O$	200.00 "	800.00 "
$MgSO_4 \cdot 7 H_2O$	360.00 "	720.00 "
KNO_3	80.00 "	160.oo "
KCl	65.00 "	130.00 "
$NaH_2PO_4 \cdot H_2O$	16.50 "	132.00 "
$Fe_2(C_4H_4O_6)_3$	---	5.00 "
$Fe_2(SO_4)_3 \cdot 6 H_2O$	2.50 "	---
$MnSO_4 \cdot 4 H_2O$	4.50 "	4.50 "
$ZnSO_4 \cdot 7 H_2O$	1.50 "	3.00 "
H_3BO_3	1.50 "	3.00 "
KI	0.75 "	0.375"
glycine	3.00 "	12.00 "
nicotinic acid	0.50 "	---
thiamine	0.10 "	0.10 "
pyridoxine	0.10 "	0.80 "
sucrose	20.00 g	20.00 g
water	1000.00 ml	1000.00 ml

in the growth of cultures on media with autoclaved coconut milk and those
on media with filter sterilized coconut milk. In experiments reported
here coconut milk was added to the media before autoclaving.

The pH of the media was always adjusted with 0.1 N NaOH to
approximately 5.8 before autoclaving and in some experiments checked
after autoclaving and also after a 3-week culture period. No significant
changes in pH occurred during autoclaving and only slight rises (less than
0.5 pH units) resulted after 3-week culture of tissue.

Both solid and liquid media were used in the experiments. For
solid media 1 % agar (Difco) was added to White's medium and 0.5 % to
Hildebrandt's medium. Media were autoclaved for 20 min. at 15 lb. per
square inch. Ten ml of medium per culture (both liquid and solid) were
used throughout the experiments.

C. Agitation.

The major experiments here reported are concerned with measuring
the growth response of tissues cultured in agitated liquid media.
Agitation of the liquid media was obtained by placing the culture bottles
on a mechanical shaker originally conceived by Dr.L.C.Coleman, and
constructed by Mr.C.J.Lines of the Dominion Laboratory of Plant Patho-
logy, Saanichton, B.C. This shaker basically consists of a rigid bank of
five wooden shelves, 26x76 inches each, spaced 12 inches above each other.
The bank of shelves is hung on a movable supporting frame and is oscil-
lated horizontally $\frac{1}{4}$ of an inch, 82 times per minute. Oscillation is
provided by a cam-shaft driven by a $\frac{1}{4}$ HP electric motor. Culture bottles
(medicine bottles) were laid on the shelves with their long axis parallel

to the direction of oscillation. The oscillation caused the medium to
wash back and forth on the bottom of the bottle, thereby alternately
exposing the tissue inside to medium and air.

D. Light conditions.

The growth response of tissue cultures to conditions of continuous
light and dark were also studied. Continuous lighting of the shaken
cultures was accomplished by four 40 W fluorescent lights suspended on
the underside of each shelf of the shaker. These lights provided an
illumination intensity of 400 foot-candles to the upper surface of the
shelf immediately below. To obtain dark conditions on the shaker one
shelf was covered with thick cardboard and the lights above extinguished.
Cultures which received no shaking were kept on separate stationary
shelves with the same light and dark conditions as those on the shaker.

E. Temperature and Humidity.

Culture was carried out in an insulated room with thermostatically
controlled air-conditioning. The cooling effect of the air-conditioner
and heating action of the lights were balanced so that the temperature
within the culture bottles remained at $25 \pm 1°$ C. Lights beneath the shelves
were used to maintain this temperature in the cultures grown in dark.
The humidity in the room was maintained at approximately 80 % through
evaporation of water from large trays.

EXPERIMENTAL RESULTS.

A preliminary experiment was performed to test the influence of coconut milk and indoleacetic acid (IAA) on the development of callus tissue from cultured carrot discs. Fresh carrot discs weighing 18 mg were cultured for three weeks in White's basal medium alone and in combination with IAA (0.1 mg/l) and coconut milk (15 %). The cultures were grown in shaken liquid medium under continuous light. The results of this experiment are summarized in Table I.

Indoleacetic acid had no significant effect on growth, either in the basal medium alone or in combination with coconut milk. On the other hand, coconut milk gave a highly significant stimulation of growth with or without IAA. The average weights of tissues cultured in media with coconut milk were approximately double those of tissues in the media without coconut milk.

A second preliminary experiment was designed to compare the growth rate of fresh carrot discs derived from different carrot roots and from different planes within the same carrot root. Discs in both transverse and longitudinal planes (see Materials and Methods) were cut from four different carrot roots and cultured for three weeks, under light, in shaken liquid White's basal medium with added IAA (0.1 mg/l) and coconut milk (15 %). The results are summarized graphically in Figure 1 and are statistically analyzed in Table II.

The average growth of carrot discs derived from different carrots showed considerable variation, the extremes being highly significant (explants from carrot 1 grew approximately twice as fast as those from

TABLE I. Results of experiment designed to test the effect of indole-
acetic acid and coconut milk on the growth of fresh carrot discs.

A. Mean wet weights of carrot discs after three weeks of culture.
Original weight of each disc was 18 mg. Wh - White's basal
medium, Wh IAA - Wh plus 0.1 mg/l indoleacetic acid, Wh CM -
Wh plus 15 % coconut milk, and Wh IAA CM - Wh plus 0.1 mg/l
indoleacetic acid and 15 % coconut milk.

Medium	Mean weight, in mg, of five carrot discs
Wh	45.0
Wh IAA	38.6
Wh CM	86.8
Wh IAA CM	87.2

B. Analysis of variance.

Source	D.F.	S.S.	M.S.	F
Total	19	16,960		
Between media	3	10,318	3,439	8.3**
Within media	16	6,642	415	

L.S.D. - 5 % level - 27.3 mg

1 % level - 37.6 mg

carrot 4). Only slight mean differences occurred between discs from
different planes of the same root. The standard deviations and coefficients
of variability show that there is noticeable variation in growth of discs
derived from the same root. The degree of this 'intra-root' growth variation
seems to change from carrot to carrot (range of C.V. 14.03 - 30.36) but is
essentially independent on the plane in which discs are taken (mean C.V.:
transverse discs, 22.94; longitudinal discs, 20.57).

TABLE II. Analysis of variance of results (shown in Figure 1) from
experiment on growth of cultured carrot discs derived
from longitudinal and transverse planes of four
different carrot roots.

Source	D.F.	S.S.	M.S.	F
Total	78	57,698,74		
Between carrots	7	35,136.48	5,019.49	15.79**
(Between planes	1	35.68	35.68	0.11)
Within carrots	71	22,562.26	317.77	

Table III summarizes the results of a preliminary experiment
designed to compare the effects of shaken liquid and agar based (solid)
media on the proliferation of callus from sunflower stem sections.
White's basal medium with added IAA and coconut milk was used in liquid
and solid (1 % agar) condition. All cultures were kept on the shaker
(which, of course, has no effect on the agar medium), under continuous
light, for 10 days.

Figure 1. Mean wet weight in mg, Standard Deviation (S.D.), and
          Coefficient of Variability (C.V.) of cultured longitudinal
          and transverse carrot discs derived from four different
          carrot roots. Each disc weighed 18 mg originally and was
          cultured for 21 days in shaken liquid White's basal medium
          plus o.1 mg/l IAA and 15 % coconut milk. Means are
          calculated from 5 - 16 discs.
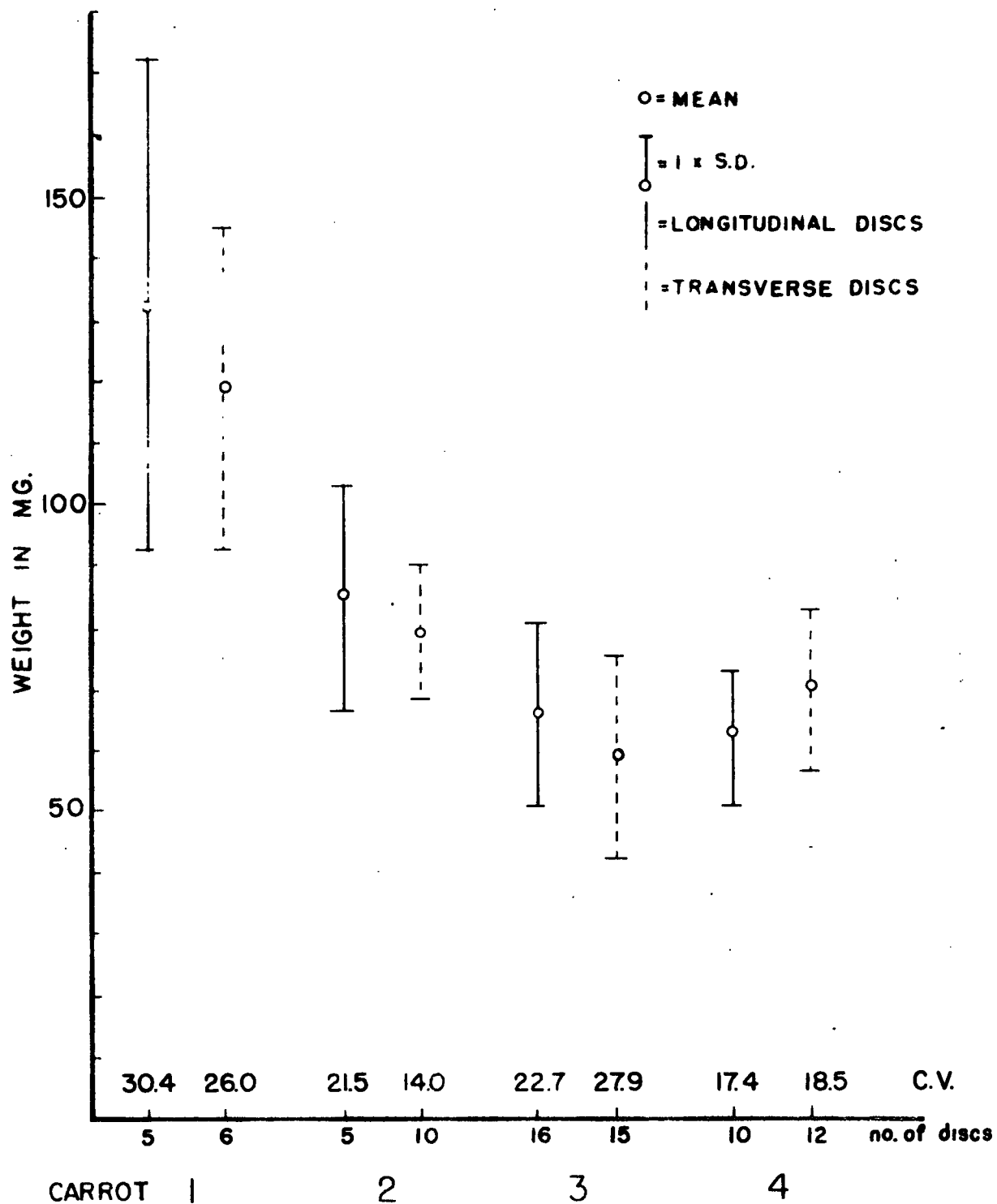
TABLE III. Results of experiment designed to compare the effect of
shaken liquid and agar based media on the proliferation
of callus by sunflower stem segments.

A. Mean wet weights of stem segments after 10 days culture
on White's basal medium plus 0.1 mg/l IAA and 15 %
coconut milk. Average weight of the stem segments
was originally 85 mg.

Type of medium	Mean wet weight, in mg, of 5 stem segments
Agar (5 %) based	364.2
Shaken liquid	590.0

B. Analysis of variance:

Source	D.F.	S.S.	M.S.	F
Total	9	171,906		
Between media	1	127,464	127,464	22.9**
Within media	8	44,442	5,555	

L.S.D. - 1 % level - 158.0 mg

The average weight of the stem sections (4 mm in length) at the beginning of the experiment was 85 mg. As can be seen from Table III the shaken liquid media produced an average growth almost double that given by the agar media, the difference being statistically highly significant.

Following these preliminary experiments a series of major experiments designed to compare the growth response of a number of tissues to different culture conditions was carried out. The culture conditions compared were: agar (i.e. solid medium with an agar base) medium (A) versus liquid medium (Lq); shaken liquid medium (Sh) versus stationary or non-shaken liquid medium (NSh); and continuous light (L) versus continuous dark (D). These conditions were combined so as to give the following six basic culture conditions (treatments) which the tissues were subjected to:

1. Agar medium, continuous light (A L)

2. Agar medium, continuous dark (A D)

3. Liquid medium, non-shaken, continuous light (Lq NSh L)

4. Liquid medium, non-shaken, continuous dark (Lq NSh D)

5. Liquid medium, shaken, continuous light (Lq Sh L)

6. Liquid medium, shaken continuous dark (Lq Sh D)

Figure 2 shows, graphically, the results obtained with undifferentiated carrot callus tissue grown under these conditions for six weeks (with weighing after both three and six weeks of culturing). White's basal medium with 0.1 mg/l IAA and 15 % coconut milk was used throughout the experiment.

Figure 2. Bar graph showing mean weights of undifferentiated carrot callus tissues, as percentage of weight at the start of the experiment, cultured under different conditions. A - agar medium, Lq - liquid medium, Sh - shaken, NSh - non-shaken, L - continuous light, D - continuous dark. Values at three weeks are means from three replicates, and at six weeks means from two replicates. Each replicates represents six individual pieces of callus tissue cultured separately and weighed jointly. White's basal medium plus 0.1 mg/1 IAA and 15 % coconut milk was used in this experiment.

Figure 3. Bar graph showing mean weights of tumorous sunflower tissues, as percentage of weight at the start of the experiment, cultured under different conditions. Abbreviations used for culture conditions and medium are the same as in Figure 2. All values are means of two replicates. Each replicate represents six individual pieces of tissue cultured separately and weighed jointly.
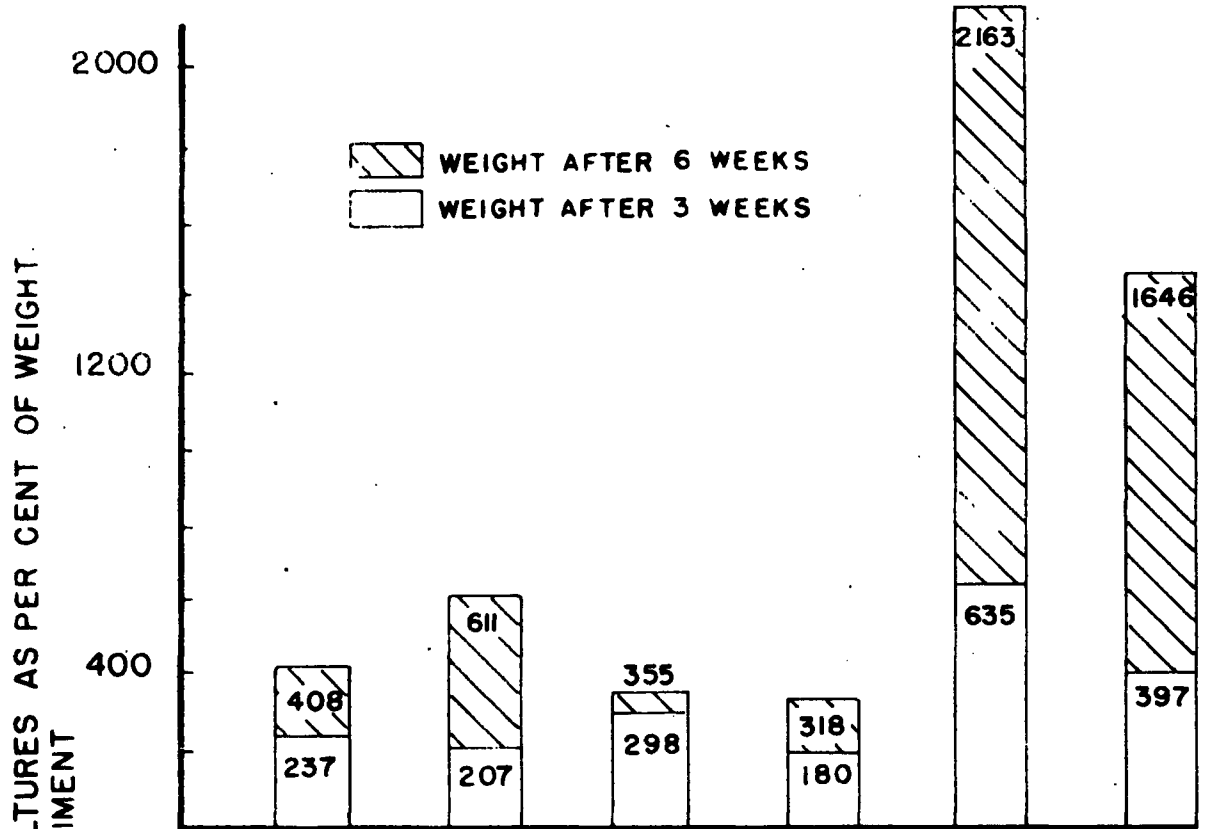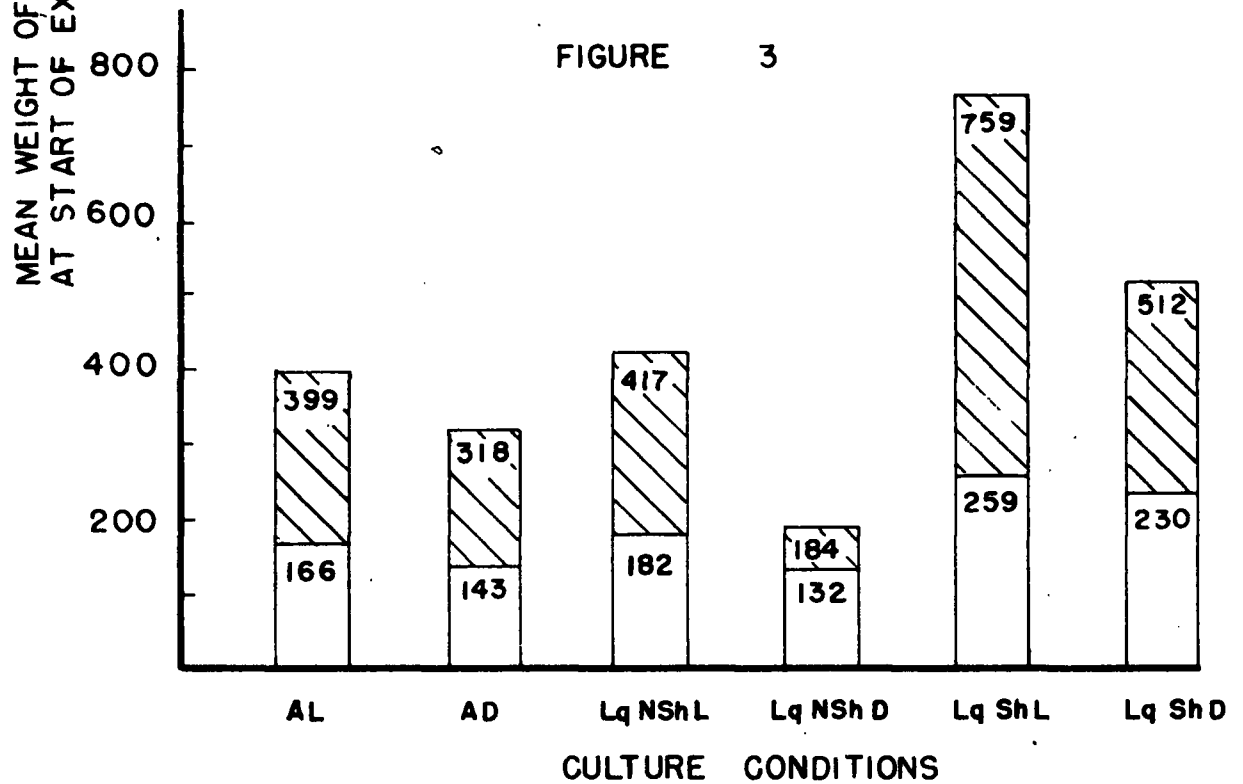
FIGURE 2

FIGURE 3

MEAN WEIGHT OF CULTURES AS PER CENT OF WEIGHT AT START OF EXPERIMENT

WEIGHT AFTER 6 WEEKS
WEIGHT AFTER 3 WEEKS

CULTURE CONDITIONS

AL   AD   Lq NSh L   Lq NSh D   Lq Sh L   Lq Sh D

The most striking effect was that given by the shaken liquid medium. In both light and dark it caused a very marked stimulation of growth over agar and non-shaken liquid media. Agar medium, however, was slightly superior to the non-shaken liquid medium. Light appears to stimulate growth in the liquid medium (particularly in the shaken liquid medium) but to slightly retard growth on agar medium.

TABLE IV. Analysis of variance on weights of undifferentiated carrot callus tissue cultured under different conditions for three weeks (Figure 2).

Source	D.F.	S.S.	M.S.	F
Total	17	468,518.95		
Replications	2	8,907.11	4,453.55	1.69
Treatments:				
A. Agar vs liquid m.	1	96,513.77	96,513.77	36.62**
B. Shaking vs non-sh.	1	229,633.33	229,633.33	87.14**
C. Light vs dark	1	74,626.72	74,626.72	28.31**
Interactions:				
AxC	1	21,805.44	21,805.44	8.27*
BxC	1	10,680.33	10.680.33	4.05
Error	10	26,352.23	2,635.22	

A statistical analysis of the results obtained at three weeks is given in Table IV. Highly significant differences were obtained between agar and liquid media, shaken and non-shaken liquid media, and light and dark. Furthermore, there was a significant interaction between the state

of the medium (solid or liquid) and the light-dark condition. All the differences obtained at three weeks are merely more marked at six weeks (Figure 2).

At the end of the six-week growth period the cultures grown in dark were pale yellowish while those grown in light were green and covered with brownish-red spots. The cultures grown in liquid medium in dark were much more friable and usually broke into several pieces before the end of the culture period (especially those shaken). Occasionally, roots developed on the cultures in shaken liquid medium, particularly on those in the dark. No roots developed on cultures grown on agar or in non-shaken liquid medium. Cultures grown on agar medium were harder and more compact than those in liquid medium. There tended to be more variation in shape, size, and appearance of cultures grown on agar or in liquid non-shaken media than in those grown in liquid/shaken medium.

A similar experiment was carried out to test the effect of the same culture conditions as in previous experiment, on the growth of bacteria-free tumorous tissue of sunflower. Again the medium used throughout was White's basal medium plus 0.1 mg/l IAA and 15 % coconut milk. The results of this experiment are shown graphically in Figure 3 and an analysis of variance is given in Table V.

The results essentially parallel those obtained with the carrot callus tissue although the overall growth throughout the experiment was lower. Relative growth was markedly superior in the shaken liquid medium, particularly under the light condition. Growth in the non-shaken liquid and agar media was essentially the same in the light, but growth in the non-shaken liquid medium in the dark was markedly inferior to that on

agar in the dark. The effect of light was generally more marked in this experiment, and in contrast to the carrot callus tissue, the tumorous tissue grown on agar was stimulated by light. The stimulatory effect of light on the shaken liquid medium cultures was particularly noticeable by 6 weeks. The trends in growth established at three weeks were again only extended and more marked by 6 weeks.

TABLE V. Analysis of variance on weights of tumorous sunflower tissue cultured under different conditions for six weeks (Figure 3).

Source	D.F.	S.S.	M.S.	F
Total	11	423,259,67		
Replications	1	14,560.33	14,560.33	2.36
Treatments				
A. Agar vs Liquid m.	1	32,047.04	32,047.04	5.19
B. Shaking vs Non-sh.	1	224,115.12	224,115.12	36.33**
C. Light vs Dark	1	104,907.00	104,907.00	17.00**
Interactions:				
AxC	1	16,695.37	16,695.37	2.70
BxC	1	91.12	91.12	0.01
Error	5	30,843.67	6,168.73	

The analysis of variance of the data obtained at six weeks (Table V) shows that there were highly significant differences between cultures grown in shaken and non-shaken liquid medium, and between cultures grown in light and dark. The difference between agar and liquid media only approaches significance and is somewhat misleading; non-shaken and shaken liquid

media tend to nullify each other as they are respectively inferior and superior to agar medium. No significant interactions were obtained.

A series of major experiments was carried out to test the effect of the same growth conditions as before on tissues cultured in Hildebrandt's improved sunflower medium. The tissues used were fresh carrot discs, undifferentiated carrot callus, and tumorous sunflower tissue. Indole-acetic acid (0.01 mg/l) was added to the media for carrot discs and carrot callus, but not for the sunflower tissue. No coconut milk was used so that the media were completely chemically defined.

Figures 4, 5, and 6 summarize graphically the results obtained with carrot discs, carrot callus, and tumorous sunflower tissue, respect-ively. All carrot callus and sunflower tissues in the same treatment (culture condition) were weighed together, giving no statistical repli-cation. With the darrot discs, six individual discs in each treatment were weighed separately, allowing a statistical analysis, which is given in Table VI.

In the main these experiments paralleled those with White's medium. However, the stimulatory effect of light on growth in shaken liquid medium was much more marked with the carrot callus and tumorous sunflower tissues than in the previous experiments. This effect was also much more marked in the above mentioned experiments than in the experiment with the carrot discs. Tissues cultured in non-shaken liquid medium in the dark showed the poorest growth in all experiments. The growth on agar media, in both light and dark, and in non-shaken liquid medium in the light was essen-tially the same for all tissues.

TABLE VI. Analysis of variance on weights of fresh carrot discs

          cultured under different conditions for six weeks

          (Figure 4). Six discs were cultured in each treatment

          and these were weighed separately.

Source	D.F.	S.S.	M.S.	F
Total	35	306,084.98		
Treatments:				
A. Agar vs Liquid	1	16,866.72	16,866.72	5.77*
B. Shaking vs Non-Sh.	1	126,150.00	126,150.00	43.21**
C. Light vs Dark	1	25,122.25	25,122.25	8.60**
Interactions:				
AxC	1	41,472.00	41,472.00	14.20**
BxC	1	8,893.50	8,893.50	3.04
Error	30	87,580.50	2,919.35	

It should be noted that the total growth of the undifferentiated
carrot tissues in Hildebrandt's medium was markedly inferior to that in
White's medium with coconut milk. Tumorous sunflower tissue, on the other
hand, grew better in Hildebrandt's medium. It is interesting that all
of the tissues showed a marked decline in growth during the second
three weeks of culture, except when cultured in shaken liquid medium in
light. Such declines were only obtained in non-shaken liquid medium in

the experiments using White's medium.

The results obtained by analysis of variance (Table VI) resemble very closely results obtained in previous experiments. The difference between agar and liquid media is significant, the differences between shaken and non-shaken liquid media, between light and dark, and the interaction between agar-liquid media and light-dark conditions are highly significant.

After 6-week cultivation the carrot discs grown in light developed greenish strips in the cambial region while the regions distal to cambium were brownish-red. Discs grown in dark were uniformly pale orange. The appearance of the undifferentiated carrot callus tissues in this experiment was generally the same as described for the experiments with White's medium (see above). No roots were developed on any tissue grown in Hildebrandt's medium. The tumorous sunflower tissue grown in light, however, turned green instead of remaining whitish colourless as it did in White's medium.

Figure 4. Bar graph showing mean weights of fresh carrot discs in mg after
three and six weeks in culture. Each treatment consisted of
6 discs cultured and weighed separately. The original weight
of a disc was 42 mg. Medium: Hildebrandt's improved sunflower
medium plus 0.ol mg/l IAA. Abbreviations used for culture
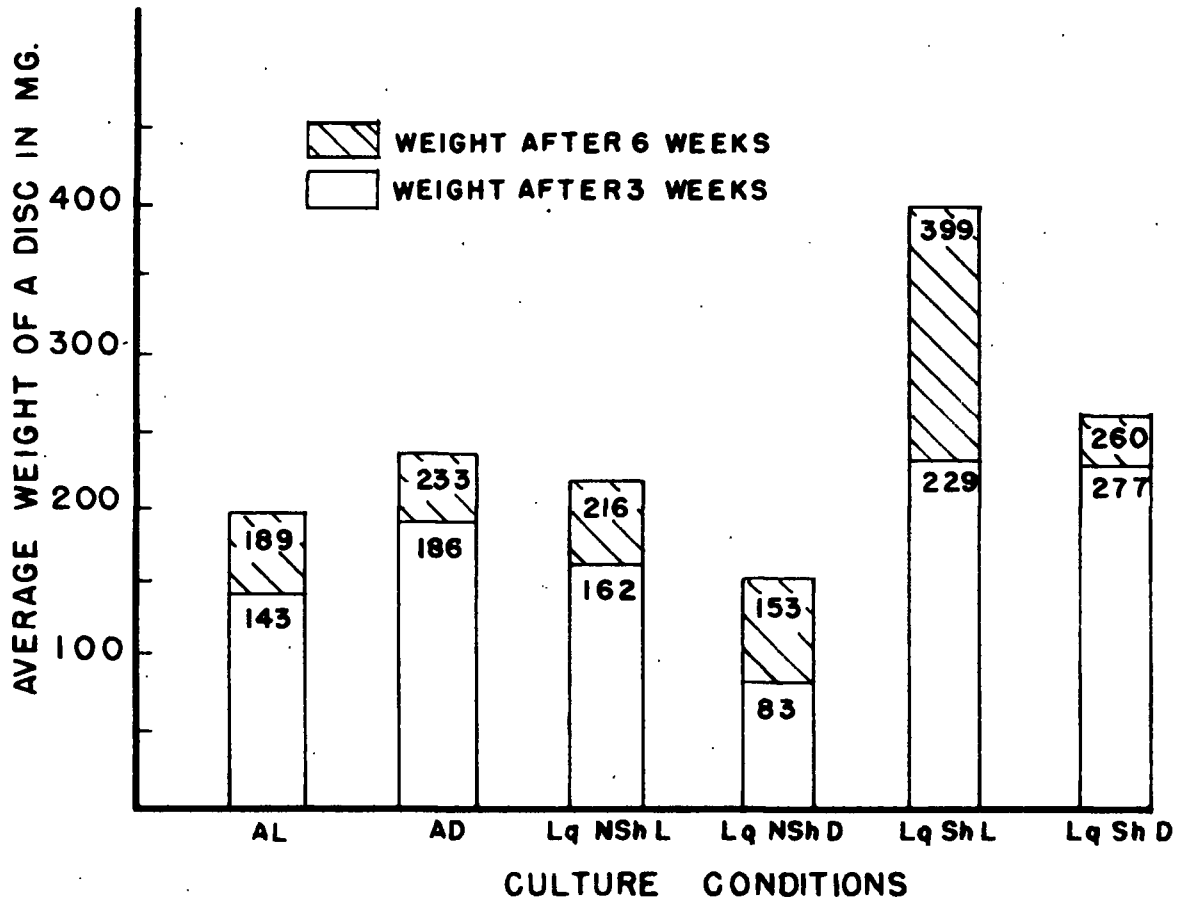conditions are the same as in Figure 2.

FIGURE 4

Figure 5. Bar graph showing mean weights of undifferentiated carrot
callus tissues, as percentage of weight at the start of the
experiment, cultured under different conditions. Values are
means of 1 replicate consisting of six individual pieces
cultured separately and weighed together. Medium: Hildebrandt's
improved sunflower medium plus 0.01 mg/l IAA. Abbreviations
used for treatments are the same as in Figure 2.

Figure 6. Bar graph showing mean weights of tumorous sunflower tissues,
as percentage of weight at the beginning of the experiment,
cultured under different conditions. Values are means of 1
replicate consisting of six individual pieces of tissue
cultured separately and weighed jointly. Medium: Hildebrandt's
improved sunflower medium. Abbreviations used for culture con-
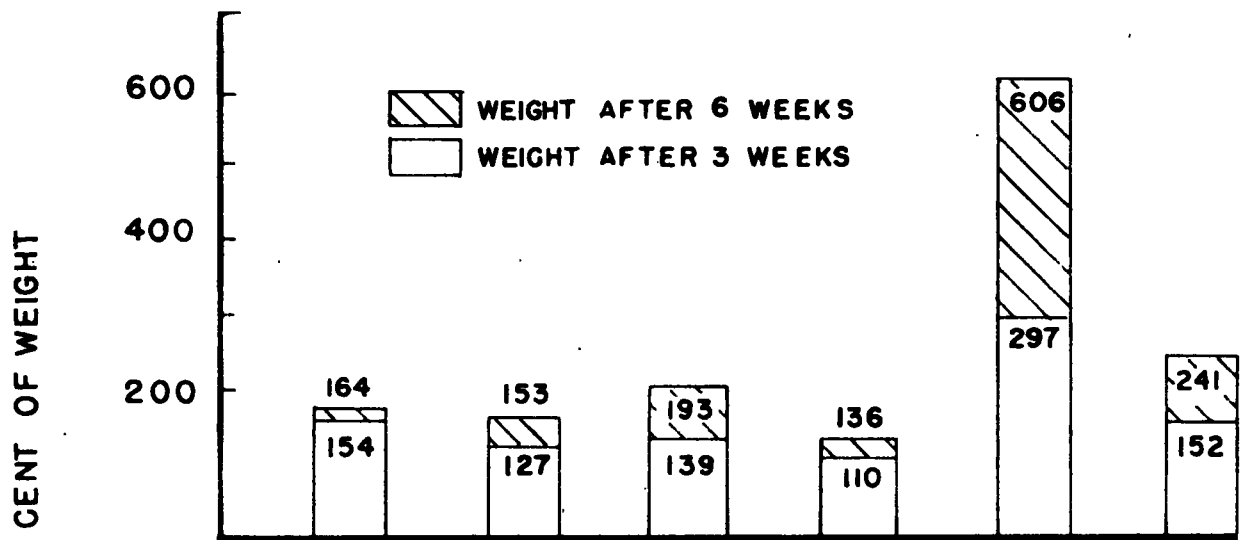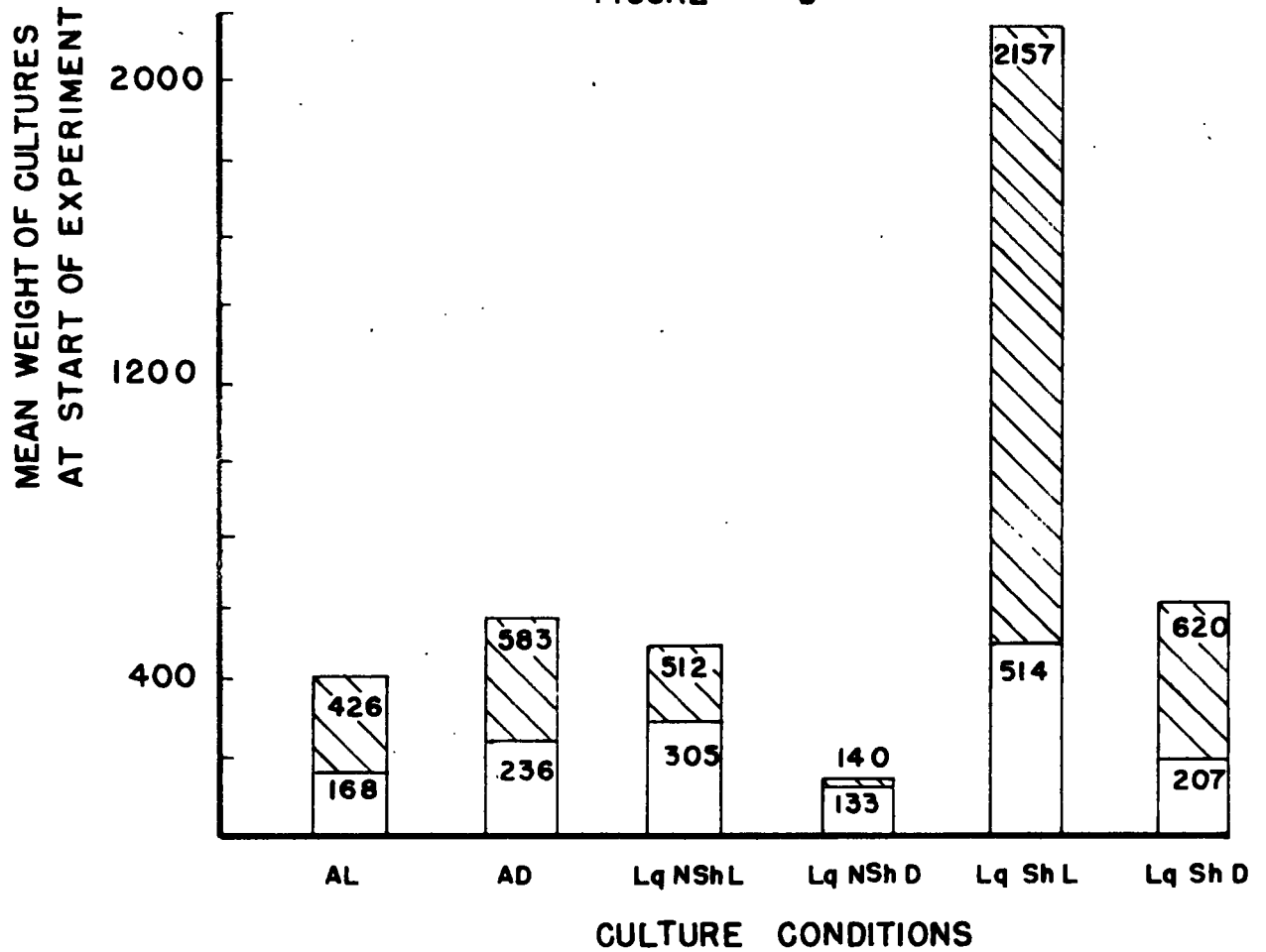ditions are the same as in Figure 2.

FIGURE 5

FIGURE 6

MEAN WEIGHT OF CULTURES AS PER CENT OF WEIGHT AT START OF EXPERIMENT

CULTURE CONDITIONS

WEIGHT AFTER 6 WEEKS
WEIGHT AFTER 3 WEEKS

DISCUSSION.

The results in genral corroborate and extend the findings of other workers. They demonstrate that growth of plant tissue cultures can be markedly stimulated by certain combinations of culture conditions. At the same time they raise some interesting questions concerning the metabolism of tissues grown in vitro.

The beneficial influence of coconut milk on growth of cultured carrot discs, as revealed by the first preliminary experiment (Table 1), was to be expected. This effect has been noted by several workers (van Overbeek, 1941; Caplin and Steward, 1948, 1949, and 1952; Duhamet, 1951a, b, and c) and is probably at least partly due to an as yet uncharacterized factor, 'kinetin', isolated from coconut milk recently by Skoog and Miller (in preparation). Since the carrot discs (and the callus derived from them) will grow in the absence of coconut milk it appears that tissues either can synthesize the active substance supplied by the coconut milk, or that this substance can be substituted to some extent by indoleacetic acid necessary for continuous growth of normal callus tissue. Apparently the growth-stimulating action of coconut milk is not a result of its content of growth-hormone for in the same experiment IAA was completely unable to stimulate growth. Besides, according to all studies known to the author coconut milk stimulated growth of cultured tissues much more than IAA or any other related substance. Because in the present experiment carrot discs could proliferate callus tissue also when grown on medium without IAA or coconut milk (Wh) it seems probable that the fresh tissue may have an endogenous content of

growth hormone sufficient to support initial callus proliferation.
Wiggans (1953) found that 10 mg/l IAA gave the best growth of carrot
discs while Caplin and Steward (1948) found 0.01 mg/l to be optimal.
This suggests that the endogenous supply of growth-hormone may be
quite variable.

The experiment comparing growth of discs from different carrots
and different planes in carrots has considerable practical significance
in the design of experiments. Since the average growth rate of discs de-
rived from different carrots was highly significantly different it
appears desirable that all discs to be compared in an experiment be
derived from the same carrot. This procedure was followed with the
experiments reported here. The plane from which the disc is cut seems
to have little effect on subsequent growth. Since transverse discs were
found easier to obtain in sterile condition they were used exclusively
in all other experiments.

There was also considerable variation in the growth of discs
derived from the same carrot root. Caplin and Steward (1952) found the
same to be true for discs cut from the secondary phloem of carrot roots.
They further noted that discs containing cambium were more variable in
growth than those with only phloem tissue. However, Wiggans (1953)
reported that discs containing cambium grew considerably faster than
those cut from phloem or xylem. The cambium containing discs are, there-
fore, excellent material for experiments in which high rates of growth
are desirable while phloem discs will give more uniform results. The
variation in growth between discs may be due to variations in the
original amount of cambium present. If this is the case, however, the
discs from the longitudinal plane should grow faster as they contain

more cambium than transverse discs. It seems that there are minor
inherent variations in the growth capacity of cells in different parts
of the root. In experiments using discs this 'intra root' variation
can only be controlled by adequate replication and statistical treatment.
In experiments in which carrot callus tissue derived by sub-culture from
discs is used it would seem advisable to have all culture pieces derived
originally from a single disc. This was the case with all callus cultures
used in the experiments described here.

The major result obtained from the experiments with different
culture conditions was the consistent and striking demonstration that
growth in shaken liquid medium was superior to that on agar medium,
(and, of course, superior as well to growth in liquid non-shaken medium).
This applied to all tissues tested in these experiments, i.e. fresh
carrot discs, carrot callus, tumorous sunflower tissue, and sunflower
stem segments, regardless either White's medium with IAA and coconut milk
or Hildebrandt's improved sunflower medium was used.

The reason for the growth-stimulating action of shaken liquid
medium is undoubtedly complex. Probably the major effect is due to
the action of the medium washing over the tissue. This allows nutrients
to enter the tissue at all surface points rather than just at the base,
as on agar media. The washing over the tissue culture prevents, at the
same time, the surface-drying which occurres in the parts of agar-cultured
tissues exposed continuously to air. The latter effect probably accounts
for the loose, flaccid surface of the callus growing in shaken liquid
medium, as compared with relatively smooth, harder surface of callus
grown on agar. The surface area of the cultures grown in shaken liquid

medium is thus considerably greater, permitting increased exchange of nutrients and gasses.

Secondary reasons for the superiority of the shaken liquid medium may be the prevention of accumulation of harmful excretions ('staling' products) at the tissue surface of cultures, and greater availability of the ions from nutrient solution because the movement of these substances is not hindered by adsorption on agar particles. Another reason for greater growth in shaken liquid medium may be better exchange of gasses between the outer atmosphere and the atmosphere of the culture bottle due to the movement of the bottle. The influence of improved aeration is corrobated indirectly by results obtained by White (1953); he compared growth of undisturbed culture with growth of culture that had been removed from the tube and weighed every three weeks (both cultures were grown on agar medium); the latter culture grew approximately twice as fast as the former, probably due to better aeration.

Caplin and Steward (1948, 1949, and 1952), and White (1953), reported a beneficial effect of agitated liquid media on growth of tissue cultures (using the auxophyton and roller tube techniques, respectively). To make a direct comparison between their results and the results reported here is very difficult for several reasons. Firstly, although all these authors used carrot tissue, their cultures were of quite different origin and differed, most probably, in the inherent capability to grow: Caplin and Steward' used discs cut from secondary phloem, White used habituated tissue isolated in 1937 by Gautheret, and the present author used callus tissue isolated some three months before the beginning of experiments (the experiment with carrot discs cannot be used for this comparison because they were grown in medium without coconut milk and the growth

was, consequently, much smaller). Secondly, the sizes and weights of tissue

pieces used for culturing were different. Caplin and Steward used 3 mg discs,

White rectangulars about 15 mg, and the present author more or less irregular

pieces about 70 mg each. Thirdly, the culture periods were different - Caplin

and Steward's 20 days, White's 24 days for cultures in agitated medium and

120 days for cultures on agar medium, present author's 42 days.

Even if the first objection is disregarded, the different weights

at the beginning of experiments and the different periods of culturing

make it impossible to calculute some ratio that could be reliably used

for comparison of growth between the cultures. Weight increase of culture

pieces is to some extent geometrical when they are small, but gradually

slows down as they enlarge (surface to volume ratio diminishes). White

(1953) simply assumes a straight-line growth and divides the total growth

by the time of culturing to give an average figure for growth per day.

Caplin (1947), on the other hand, uses Blackman's (1919) compound interest

law to calculate growth as percentage increase per day. Both methods can be

extremely misleading and the results may be completely opposite according

to which method is used. The question of evaluation of growth in such a way

that the result of one experiment can be compared reliably with the result

of another experiment is very important but has been nearly completely

neglected by workers in plant tissue culture. The only serious consideration

of the problem was given by Caplin (1947).

In very general terms, the growth response to agitation obtained

here may be compared with that obtained by Caplin and Steward in the

following way: Caplin and Steward's cultures grew approximately twice as

fast in agitated medium as on agar medium, in 20 days, while similar

cultures used in this study grew nearly four times faster in agitated liquid

medium than on agar medium in 42 days if cultures grown in light and dark were counted together, and more than five times faster if only cultures grown in light are considered. Since White used different periods of culture for agitated and agar media it is impossible to make a numerical comparison. Initially his cultures in agitated medium grew faster than those on agar but eventually they completely stopped growth and were passed by the cultures on agar. No such reversal in growth trends was found in the experiments here described, even though the cultures greatly exceeded the size of White's cultures. The ratios expressing the response of other cultures used in this study to agitation are generally smaller than in the case of carrot callus tissue but, nevertheless, they are always highly significant (see analyses of variance).

Certainly, the agitation technique described here is simpler and more adaptable than those used previously (see Introduction). Any horizontally oscillating platform could be substituted for the shaker used, and almost any type and size of bottle or flask could be used as a culture vessel. Furthermore, it is not necessary that the tissue adhere to the surface of the culture vessel. This factor limited the size of Caplin and Steward's cultures to about 180 mg and White's to even less. On the other hand, cultures weighing more than 3000 mg have been successfully grown under the shaking conditions described here. Furthermore, organ cultures, which are massive and non-adhesive, can be grown under these conditions but can not be grown in the special tubes of the auxophyton or in roller tubes.

It must be added that one disadvantage of the here described technique (shared by all the techniques which use liquid media and cotton plugs) is that unless the surrounding humidity is kept high, rapid evaporation of the water from the medium can quickly produce detrimental changes in the concen-

tration of nutrients. It is recommended, even if the humidity is high, that tissues be transferred to fresh medium every two or three weeks.

The finding that growth on agar medium was as good or better than that in non-shaken liquid medium is not too surprising. Although there may have been some unknown growth-promoting substances contaminating the agar, the effect was most probably a result of aeration conditions. The tissues in the liquid medium sank and were partially submerged in the liquid. Such submersion prevents adequate aeration of the tissue, thereby retarding growth. White (1939) obtained a similar effect although in his case the difference between growth on agar and in liquid was more marked than in these experiments. He used longer times and the tissue in liquid medium was completely submerged.

The presence of continuous light had significant stimulatory effect on the growth of all tissues cultured in liquid medium, particularly those in shaken liquid medium. Steward et al. (1952) obtained increased growth of carrot discs cultured in rotated liquid medium although the difference due to light was not so significant as in the present study. Light and dark conditions had only small, insignificant effects on the growth of tissues on agar medium. This agrees with the finding by Hildebrandt et al. (1945) that light conditions had little effect on the growth of tobacco callus and sunflower tumorous tissues cultured on agar. These results in general support the interpretation that cultures grown on agar are primarily limited by the diffusion rate of nutrients from the agar, whereas in liquid culture this limitation is alleviated and light can have significant effect on the utilization of the nutrients. Since tissues grown in shaken liquid medium presumably receive a better supply of nutrients than those in non-shaken medium (see above), the effect of light would be expected, as was found, to be more marked in tissues grown in the former medium.

The actual physiological action of the light is difficult to ascertain. The stimulation of growth exercised by light is probably not primarily due to increased photosynthesis. Firstly, all the culture media contained an optimal concentration of sucrose; secondly, the sunflower tumorous tissue, used in one series of experiments, was stimulated by light (Figure 3 and Table V) despite the fact that it showed no green coloration (chlorophyll).

It is interesting to note that the most marked stimulation by light on tissues grown in shaken liquid medium was given with carrot callus and sunflower tumorous tissues cultured in Hildebrandt's medium without coconut milk (Figures 5 and 6). The stimulation was much less marked when these tissues grown in White's medium with added coconut milk (Figures 2 and 3). This might suggest that light plays a role (direct or indirect) in the synthesis, by the tissues, of a growth stimulating factor which is supplied by coconut milk. Consistant with this hypothesis is the fact that fresh carrot discs, which might contain an endogenous supply of such a factor, were not markedly stimulated by light when grown in Hildebrandt's medium.

An interesting difference in growth occurred between tissues cultured on White's medium with coconut milk and those on Hildebrandt's medium. All tissues on the former medium, except those in non-shaken liquid medium in the dark, showed approximately the same growth during the first and the second three-week culture periods. On the other hand, all tissues on Hildebrandt's medium, except those in shaken liquid medium in light, showed a marked decline in growth during the second three-week period. This might suggest that only under the conditions of shaken liquid medium in light can the tissues on a chemically defined medium synthesize an adequate supply of the growth-promoting substances supplied by coconut milk.

Another noticeable difference between the responses to the two

nutrient media was that the carrot callus tissue grew significantly better
on White's medium with added coconut milk (Figures 2 and 5) while the growth
of the sunflower tumorous tissue was markedly superior on Hildebrandt's
medium (Figures 3 and 6). Part of this effect is probably directly due to
the fact that the concentrations of nutrients in Hildebrandt's medium are
specifically designed for sunflower tumorous tissue and may not be optimal
for carrot tissue (or at least not so favourable as the concentrations in
White's medium). However, difference in growth-hormone metabolism between
the two tissues must also be taken into consideration. Carrot callus is a
normal tissue and needs an exogenous supply of growth-hormone (and/or
growth factor from coconut milk) for its growth (Gautheret 1942b, 1946,
1947a, and b) while sunflower crown gall tissue generates itself an excess
of growth-hormone and does not respond to growth-hormone in the medium
(deRopp, 1947). Therefore, enriched White's medium (containing 0.1 mg/l
IAA and 15 % coconut milk) was superior to Hildebrandt's medium (containing
only 0.01 mg/l IAA) for growth of normal carrot callus tissue, while
Hildebrandt's medium with improved concentration of nutrients was superior
to White's medium for growth of tumorous sunflower tissue. With the
sunflower tissue, it is surprising that by merely using more favourable con-
centrations of nutrients the growth can exceed that with coconut milk.
The change in colour of sunflower tumorous tissue, discussed in next para-
graph, may have been another factor contributing to the result.

A rather notable phenomenom, which may have been partially a cause
or result of the improved growth of the sunflower tumorous tissue in Hilde-
brandt's medium, was observed. During all of the experiments in which
White's enriched medium was used, the sunflower tumorous tissue remained
whitish and translucent. However, when the tissue was transferred to

Hildebrandt's medium for the later experiments the cultures grown in light suddenly turned light green. The cause or significance of this change is difficult to explain. It may be that the optimal nutrient concentration of Hildebrandt's medium overcame a limiting factor in chlorophyll synthesis or that the coconut milk contained inhibitors of chlorophyll synthesis (coconut milk did not, of course, prevent chlorophyll synthesis in other tissues). The continuous cultivation (for some five months) of the sunflower tissue in light might have been also an important factor influencing the metabolic capability of sunflower tumorous tissue to synthesize chlorophyll. Certainly there is a marked difference between normal carrot tissue and tumorous sunflower tissue to synthesize chlorophyll; carrot callus tissue developed chlorophyll in both media within about a week. Whether these differences are specific or are due to a more meristematic nature of the tumorous cells cannot be said at present.

SUMMARY.

The response of various plant tissues to different culture conditions was compared. The tissues used were cambium-containing discs from carrot roots, undifferentiated carrot callus, bacteria-free sunflower tumorous (crown-gall) tissue, and segments of sunflower stems. The culture conditions compared, in combination, were agar versus liquid medium, shaken versus non-shaken liquid medium, and continuous light versus continuous dark. The response of the tissues to White's basal nutrient medium with added coconut milk (15 %) and indoleacetic acid (0.1 mg/l) and Hildebrandt's improved sunflower medium was also compared under these different culture conditions.

Agitation of the liquid medium was accomplished through the use of a newly designed shaker, which consists basically of a horizontally oscillating bank of shelves. The tissues rested on the bottom of culture flasks (medicine bottles) on these shelves and were alternately exposed to medium and air as the liquid medium washed back and forth. Any horizontally oscillating platform could replace this shaker and almost any type and size of culture flask could be used. Probably any type of plant tissue could be cultured under these shaking conditions. It is not necessary that the tissues adhere to the walls of the culture vessels as in other agitation methods so far used in plant tissue culture.

Growth (weight increase) of all tissues in shaken liquid medium (in both light and dark) was markedly superior (two to six times greater average weight in 42 days) to that of tissues on agar and in non-shaken liquid medium. The superiority of growth in shaken liquid medium is probably due to several factors: nutrients and gasses are supplied to the entire surface of the tissue, there is no drying and hardening of the tissue

surfaces, resulting in a greatly increased surface area, harmful excretions cannot collect at the tissue surface, and diffusion of nutrients is not hindered by adsorption on agar particles.

To compare the growth of these cultures with those of other workers using agitation methods is difficult due to the different sources of plant material, different sizes of tissues cultured, and different periods of culture used. In general the stimulatory results of shaking obtained appear to be at least as good as those obtained by Caplin and Steward with the much more elaborate and limited 'auxophyton'. There was no sign of eventual growth stoppage as obtained by White, using roller tubes.

There were no significant differences in the growth of tissues on agar, in both light and dark, and in non-shaken liquid medium in light. Tissues in non-shaken liquid medium in the dark always showed the poorest growth. Tissues in non-shaken liquid medium received inferior aeration.

Light consistently stimulated tissues grown in liquid medium, particularly those in shaken liquid medium. The effect was especially marked on carrot callus and tumorous sunflower tissues grown in Hildebrandt's medium. It is suggested that light may play a role in the synthesis of growth factors supplied by coconut milk. Light had no significant effect on the growth of tissues on agar medium, indicating that the primary limiting factor in the growth of such tissues may be the rate of diffusion of nutrients from agar.

Carrot tissues showed better overall growth in the enriched White's medium while the sunflower tumorous tissue did better in Hildebrandt's medium. The effect on carrot was probably primarily through indoleacetic acid and coconut milk. The response of sunflower tissue is difficult to evaluate at present.

All carrot tissues developed chlorophyll throughout all of the experiments if cultured in light while tumorous sunflower tissue remained white until placed in Hildebrandt's medium, when it turned light green. The significance of these differences is not known.

One experiment showed that carrot discs derived from different carrots grew at significantly different average rates indicating that discs to be compared should be derived from the same carrot. The plane in which the discs were cut did not seem to influence subsequent growth. 'Intra root' variation in disc growth necessitates replication.

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