THE SINGLE CELL SUSPENSION CULTURE OF THE LICORICE
PLANT, GLYCYRRHIZA GLABRA

by

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ABSTRACT

The cells of the licorice plant, Glycyrrhiza glabra, were cultured as a "single cell" suspension. Their growth behaviour, yield and metabolic products were studied.

The suspension cultures of the licorice plant were established from the friable calluses obtained from the radicle, cotyledon and hypocotyl of the germinated seeds. The single cells, regardless of their origin showed little difference in cell size and morphology. After an apparent adjustment to the medium, the cells required 11-13 days of incubation to reach the maximum cell yield of 1.2 gm/100 ml medium, dry weight.

During the growth period, the pH of the growth medium decreased from pH 5.6 to pH 4.7 in the first few days and then increased to about pH 6. A level of 10% coconut milk in PRL-4-CM medium was found to support good cell growth; the lower the coconut milk level, the longer the growth period required to reach the maximum cell yield. It was also found that 0.5% yeast extract could be used to replace the coconut milk in the PRL-4-CCM medium.

The metabolites detected and examined in the licorice single cell suspension culture included a volatile apple aroma, a polysaccharide pectin-like material, steroids and triterpenoids.

The analyses of the licorice cell volatile apple aroma found under anaerobic conditions indicated the presence

of ethanol and some related esters.

The monosaccharides found in the pectin-like polysaccharide hydrolysate were glucose, fructose, galactose, arabinose, xylose, galacturonic acid and glucuronic acid. The pectin-like material in the cell preparations reached a maximum yield of 1.1 mg/ml after one month of growth.

Glycyrrhizinic acid, the common licorice constituent found in the root, could not be detected in the suspension cultures. However, several other related compounds which gave typical steroid and triterpenoid reactions were found.

Sorbitol and fructose were found to be the two major sugars which accumulated in free form in the licorice cell medium.

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INTRODUCTION

In 1902, Haberlandt formulated the theory of culturing the isolated vegetative cells of higher plants in-vitro as a method of studying the problems of cell differentiation and cellular interrelationships. Although he failed to establish a growing tissue culture, his concepts were pursued by several of his students and other investigators. From the time that Kotte^{69,70} and Robbins^{95,96} first initiated studies on the growth of excised root tips in sterile culture solutions in 1922, remarkable culture success has been reached. Callus and cell suspension culture from many different plant origin have been successfully grown. The induced differentiation of callus and single isolated cells into vascular elements, stem, leaf and roots and ultimately into whole plants^{24,33,61,107,113,116,126} has been shown to be possible.

As it has been demonstrated that whole plants can be generated from isolated single cells, it can be assumed that each cell possesses the full genetic potential of the whole plant. Similarly it may also be assumed that, any compound synthesized by plants is a potential by-product of cells grown in solution.

For many years, plant cell culture was considered too exacting and uncertain for general use. More recently, however, techniques have been developed employing relatively simple media which produced good cell yields. During the past

decade much of the research has been directed toward the growth of plant cells in suspension culture. The culture of plant cells in liquid media analogous to the culture of microorganisms is a convenient method for the production of cells of higher plants. Plant cell culture is now approaching the point where one can look upon the cultivation of isolated plant cells as one would the cultivation of bacteria, asking the same type of questions and employing the same techniques. Thus one may think of these cells as a new class of microorganisms.

In fact, suspension cell cultures derived from plants which synthesize particular secondary metabolites such as alkaloids, steroids and terpenoids, have in many instances been shown to synthesize those same compounds. The amino acid composition of the soluble proteins of wheat coleoptile and soybean hypocotyl resembled that of soluble proteins from cultured cells of these plants. All these observations have stimulated an increasing interest in plant tissue culture.

The development of techniques which allow the cultivation of plant cells on a large scale and on a routine basis such as the carboy system^{119,120,130} and the continuous system^{19,48,77,122} have been described for the submerged cultivation of a number of cell lines. From an industrial viewpoint any plant metabolite of interest to industry is a potential product of plant cell culture.

In the studies to be presented, single cell suspension cultures of the commercial licorice plant, Glycyrrhiza

glabra were studied. The growth conditions, the yield and the potential of producing various products were investigated.

LITERATURE REVIEW

I. Plant tissue culture

Many attempts were made following Haberlandt's studies during the years of the first two decades of twentieth century to culture single cells of the palisade tissue of leaves, of medullary parenchyma of epidermis and of various plant hairs. However the experimental results of these investigations were discouraging and in no case could the isolated cells be induced to generate tissue masses by cell division.

Kotte^{69,70} and Robbins^{95,96} reported independently in 1922 the experiments on the growth of root tips in sterile liquid medium. In 1932, White commenced his work on excised root culture, using seedling root tips of Triticum as experimental material. In attempts to obtain continuous growth of his excised wheat roots, White systematically varied such cultural factors as light, temperature, aeration, volume, pH and the inorganic composition of the medium^{133,134}. Under favourable conditions, the roots grew rapidly in culture for about 14 days and then rapidly declined to zero. This cessation of growth was found to be hastened by attempts to subculture the root apices.

In 1934, White¹³⁵, using a similar culture medium but with sucrose instead of dextrose as the source of carbon, succeeded in repeatedly subculturing excised tomato roots without diminution of the growth rate. In addition, the author found that by subculturing the lateral root tips which develop-

ed during culture, a clone of excised roots in continuous culture was established from a single radicle. By 1936¹³⁸, these roots had passed through 160 passages, each of 7 days duration, and the mean linear growth rate of the roots had continued at approximately 5 mm per day.

At about the same time, reports by Ganthert indicated that pieces of cambium removed under aseptic conditions from Salix capraea, Populus nigra and other trees and placed on a solidified medium containing Knop's solution, dextrose and cystein hydrochloride would continue to proliferate for some months, giving rise to algal-like outgrowth^{42,43}. Influenced by contemporary work with root cultures, Ganthert added thiamine and indole acetic acid to his medium and reported greatly enhanced growth in culture of the Salix capraea cambium^{44,45}. At the same time, Nobecourt reported some success in culturing explants of cambium from carrots and potatoes using a medium containing Knop's solution supplemented with Berthelet's mixture of accessory salts^{85,86,87}. Ganthert re-examined the behaviour of carrot explants on Nobecourt's medium modified by incorporating into the medium thiamine, cystein and a low concentration of indole acetic acid. The carrot explants gave rise to an "undifferentiated" mass of tissue capable of repeated subculture⁴⁵. A few days earlier, White had independently reported the continuous cultivation of a similar undifferentiated callus derived from the procambial tissue of young stems of a hybrid Nicotiana (N. glauca X N. langsdorffii) cultured in the medium he had developed for exci-

sed tomato roots containing 0.5% agar¹³⁹. These callus cultures of Ganther and White were the forerunners of similar cultures derived from the tissues of many dicotyledonous plants.

In 1954, Muir, Hildebrandt and Riker reported the growth of liquid cultures containing single cells and small clumps of cells of Tagetes erecta and of Nicotiana tabacum⁸⁰. These cultures arose when fragments of callus cultures of these species were transferred to a liquid medium agitated on a reciprocal shaker. Similarly, in 1955, Steward and Shantz reported that they were studying the growth of carrot phloem explants in liquid medium in specially constructed rotating culture vessels, the supernatant fluid became turbid owing to the development and growth of free floating cells¹¹¹. In 1956, Reinert obtained evidence for the occurrence of cell divisions in a similar suspension of single cells and cell clumps from Picea glauca⁹⁴. In the same year, Nickell reported that he had continuously subcultured for four years a suspension rich in single cells of hypocotyl of Phaseolus vulgaris⁸².

In 1957, Torrey and Shigemura in their paper titled "Growth and controlled morphogenesis in pea root callus tissue grown in liquid media"¹¹⁷ stated that under certain nutritional conditions the solid and woody callus became friable, and when transferred to the fresh medium in a flask, mild agitation produced a dense suspension of isolated viable cells. The reason for the observed morphogenesis was supposed to be due to an appropriate balance between auxin and yeast extract. Also

papers from Bergmann¹⁴, Torrey and Reinert¹¹⁸, Lamport and Northcote⁷¹, Earle and Torrey³² mentioned the release of cells and tissue fragments from friable callus masses and that the maintainance of a good degree of cell separation may often be promoted either by the presence in the liquid medium of a high auxin concentration, a balance between auxin and kinetin, or a defficiency of certain vitamins.

Suspension cultures have also been successfully established by introducing tissue fragments into the liquid medium obtained from sterile roots by mechanical disruption with a sterile homogenizer²⁰. In such cases the tissue fragments first gave rise to small callus masses from which free cells and small cell aggregates were later released. Especially designed culture tubes, shakers and methods of agitation have been recommended for single cell preparation during the past years^{19,41,72,80,112,119,120}. Recently it has become evident that the rate of cell growth and cell composition measured in terms of free cells and cell aggregates (the latter were always present) of a suspension culture were not primarily dependent upon agitation of the cultures but upon the origin of the plant tissue and the composition of nutrient medium.

According to Lamport⁷², cell suspensions can be defined as "cultures consisting of single cells and small clumps which can be pipetted accurately". Thus we may think of these cells as a new class of microorganism, and apply the microbiological techniques to higher plants via the cell suspension

technique.

A plant culture suspension composed entirely of free growing cells has not, as yet been achieved. However in recent years a number of plant cell strains have been developed from plants with great success. e. g. soybean^{37,38}, waxbean³⁷, mung bean³⁷, horse radish petiole³⁷, red kidney bean¹²⁹, rose^{37,141}, sugar cane⁸³, wheat barley³⁹, bush bean, spearmint¹³⁰, tobacco¹⁰⁹, Ammi visnagi⁶⁶, maple and ginkgo⁷².

In recent years, the induced differentiation from undifferentiated callus and single cells has been extensively studied. Differentiation has been demonstrated in callus tissues of tobacco¹⁰⁷, endive and parsley^{126,127}, carrot^{61,113}, asparagus¹⁴⁴, potato¹¹⁶, geranium^{24,92}, convolvulus³³ and chrysanthemum⁵⁵. In addition, it has been shown that whole plants can be generated from single carrot and tobacco cells grown in suspension culture^{60,114}. It, therefore, has been assumed that each cell possesses the full genetic potential of the whole plant and that a compound synthesized by adult plants would be a potential by-product of cells grown in isolation. The application of microbiological techniques to the culture of cell suspensions of higher plants is potentially useful in studying many aspects of plant metabolism, and in particular primary and secondary metabolites from the plant cell suspension.

A number of papers have been published recently which describe the relationships between plant cell cultures and respective the plant organ from which the cells

were derived. It has been found that the cultured cells differed biochemically from the parent plant presumably because the cultured cells were primarily meristematic, undifferentiated cells, while the intact tissue from a plant organ were differentiated cells. The difference between cultured cells and cells of plant organ was reflected in the chemical composition of their proteins. Tulecke et al.¹²¹ observed a notable difference between the protein amino acid composition of Ginkgo pollen and callus cultures derived from pollen. A comparison between the protein amino acids from stems and leaves of the rose and from the callus culture of rose stem also indicated a difference in the proteins in these tissues¹³¹, as might be expected. However, very recently, Gamborg and Finlayson⁴⁰ analysed the amino acids of the soluble and total protein from plant cells grown in suspension culture, the cell cultures originated from twelve different plant species, and the explants were taken from different organs of the plants. The latter authors found relatively small differences in the amino acid composition between cells originating from different organs of the same species, and between the same cultures grown in different media under the same environmental conditions. However some variation was found in the essential amino acid composition particularly in lysine, arginine and methionine. The latter amino acids were found to be proportionally higher in concentration in the cell proteins than those reported for seed proteins.

In addition, a number of investigations claim that

suspension cell cultures derived from plants which synthesize particular secondary metabolites, such as alkaloids, steroids and terpenoids have been shown to synthesize these same compounds in culture.

In 1957, French and Gibson³⁶ first reported the effect of glutamic acid on the biosynthesis of alkaloids (hyoscyamine and hyoscyne) in the three-week old cultures of Datura tatula L. In 1964, Speake et al.¹⁰⁹ reported that nicotine could be successfully isolated and identified from several lines of callus tissue culture of Nicotiana tabacum. In 1965, Carew²² reported that the callus of Alstonia constricta has the capability to biosynthesize reserpine. In 1967, Kaul and Staba⁶⁴ demonstrated the biosynthesis of visnagin from A. visnaga cell suspension with the yield at 0.31%.

In 1965, Williams and Goodwin¹⁴¹ examined the terpenoids of tissue cultures of Paul's scarlet rose, they found the carotenoids present in tissue cultures are zeaxanthin, violaxanthin, auroxanthin and neoxanthin. β -carotene, lutein and neoxanthin were the main pigments in stem and leaf from the intact rose. The total carotenoids in stem and leaf from the intact plant was four and thirty times greater, respectively, than the same tissue grown in culture. The triterpenoids isolated and identified from tissue culture and from stem and leaf were identical. β -sitosterol was the main triterpenoid component, with traces of γ -sitosterol, lanosterol, β -amyrin and squalene. In 1968, Heble et al.⁵² isolated diosgenin and

β -sitosterol from Solanum xanthocarpum tissue culture with a reported yield higher than that found in the plant berries.

Antibiotic substances have been shown to be produced by many plant tissue culture, e. g. lettuce, cauliflower, aspen and avocado^{47,65,75}.

A number of enzymes such as glucanases³⁹, different dehydrogenases and transaminases^{26,29,30,37,58} have also been demonstrated to be present in the filtrates of plant tissue cultures.

In conclusion, although the development of plant tissue culture is of relatively recent origin, the technique promises great potential. For example, in plant biology, tissue culture has been used to study differentiation, cellular interrelationships, metabolic pathways and genetic control. In the microbial industry, plant cultures have been suggested as a source of food, food additives, medicines and industrial materials.

II. Licorice

Several review articles concerning licorice have been written within the last fifty years^{1,11,25,73,78,84,104}. In order to obtain a better insight on the importance of single cell suspension cultures of licorice cells, it is necessary to outline the history, pharmacology, and important chemical components of the licorice plant.

Licorice is a leguminous shrub that may reach a

height of several feet, occurring chiefly in subtropical regions where it can be found growing wild on river banks. The roots of the plant are used commercially for the production of licorice extract. The licorice root is collected in the fall, leaving parts of the root stump to assure the propagation of a new crop. The main licorice-growing countries are found in the subtropical Eurasian zone which includes Spain, the south of France, Italy, Greece, Syria, Iran, Iraq, Russia (Volga and Black sea area) and China. Most commercial licorice plants belong to the species Glycyrrhiza glabra L.

The products of the licorice root, *Glycyrrhiza glabra*, have been treasured for over 40 centuries. Their value has been stated in many of man's earliest medical records. As early as 500 B.C. in documents of the Greek physician Hippocrates and in the essay of the Greek philosopher Thaophrastus, licorice was mentioned as sweet Scythian root. In the first Chinese herbal "Shen nung Pen tsa'o King", licorice is classified amongst the 120 "drugs of the first class" which are supposed to exert godly influence on the body and to lengthen life.

Licorice is also mentioned in the earliest medical record of the Emperors of Rome, Greek, Egypt in the list of drugs as a panacea, a cosmetic and a part of the elixier.

In ancient time, licorice was used as a remedy for human ailments, particularly as medication for dry coughs, inflammation of the throat and other respiratory infections.

Today licorice is used in various potions and medi-

caments, a number of which are included in the pharmacopoeias of most countries. Sometimes licorice extract is added to correct the bitter acrid taste of other drugs; in other cases, however licorice is considered useful against a variety of ailments, mainly affections or inflammations of respiratory tract, including throat, bronchia and chest. Bergefors¹³ found ground licorice root to be an excellent pill base.

Investigations for the physiological and pharmacological properties of licorice have been carried out, however, with the complex juice, it is seldom possible to attribute some effect to a specific component of the extract.

No ill effects of oral administration of reasonable quantities have been recorded. The metabolism of the characteristic sweet-tasting glycosides in licorice, glycyrrhizinic acid, was investigated by Van Katwijk and Huis in't Veld^{61, 62, 63}, and Hudson et al.⁵⁶ According to the latter authors, glycyrrhizinic acid is not metabolized to identifiable steroid substances. The decrease of arterial blood pressure by glycyrrhizinic acid upon intravenous injection (Clere et al.,⁸⁴) is coupled with a reduction of the surface tension. In 1922, Kofler⁸⁴ found a hemolytic effect of glycyrrhizinic acid in concentration as low as 0.05%, and Busacca⁸⁴ confirmed the hemolytic activity, which was considered to be in accordance with the saponin nature of the compound. However results obtained with the licorice extract or with insufficiently purified glycyrrhizinic acid preparations are to be judged carefully, as other physiologically active constituents are

present in the root extract.

A stimulus in the medicinal status of licorice extract started in Holland. In 1946, Revers reported that the extract had a therapeutic effect on stomach ulcers. Verheyen (1948) suggested that the favorable influence of licorice extract on the gastric ulcer might be related to the spasmolytic (cramp abolishing) action of the succus, which was subsequently demonstrated in rats. The therapeutic effect of licorice on stomach ulcers was further confirmed by Schulze and Franke (1951), Standner (1952), Lange (1952), Boller (1952), Irle (1952), Schulze et al. (1954) although Revers (1948) discovered that in about 20% of the cases in which stomach ulcer was treated with liberal doses of pure licorice, a side effect occurred resulting in edema of the face and extremities, usually accompanied by headaches and sometimes by dizziness. The mechanism by which licorice cures ulcers is yet unknown. According to Czok and Kreienberg (1954) the drug could regulate acid formation in the stomach, or at least is considered to be in some way interrelated with acid formation (Kreienberg and Harth, 1955).

Studying the cause of edema as a result of overdose of licorice extract, Borst et al. (1950) made the most interesting discovery that licorice influenced the water and electrolyte (salt) metabolism of the organism. Daily administration of 20 to 45 grams of licorice extract to hospitalized patients resulted in retention of water, sodium and chloride, whereas potassium output was much increased. This group of investiga-

tors came to the conclusion⁷⁹ that the oral administration of succus liquiritiae is in almost all respects similar to that of large doses of intravenously administered deoxycortone. Groen et al. and Pelser et al. (1953)⁹¹ considered that glycyrrhizinic acid was the responsible component for the deoxycorticosterone-like activity since the ammonium salt of glycyrrhizinic acid proved to be active.

Card et al. (1953)²¹ showed that glycyrrhetinic acid, the aglycon of glycyrrhizinic acid had the same effect on the water and salt metabolism as deoxycortone, but the acid, unlike the hormone, did not prolonged the survival time of adrenalectomized rats. Hassan et al. (1954)⁵¹ confirmed the electrolyte regulating activity of licorice.

Several derivatives of glycyrrhetinic acid and some compounds with analogous structure were also tested clinically; oleanolic acid and ursolic acid proved inactive.

The use of licorice extract for the sole purpose of flavoring confectionery products started during the eighteenth century when an English chemist, George Dunhill, blended the extract with sugar, molasses, flour and other ingredients to produce so called Poute-fract Cakes. This gradually led to the manufacture of other licorice confections which up to the present day have been very popular in Holland, England, France and other European countries where the consumption of these confections is many times that in the United States. In some type of chewing gum, licorice extract is incorporated to ensure a flexible texture; the antioxidant properties of lico-

rice are said to improve keeping quality and freshness of the product. In certain chocolate candies, licorice is also added to stabilize the fat dispersion, therefore preventing bloom formation.

Many licorice confectionery lines, especially in Holland, combine the flavoring properties of licorice with its soothing qualities. In cough and throat lozenges, pastilles, and gums, licorice is definitely recognized as a valuable health ingredient. Between delicious licorice sweet and pharmaceutical preparations containing licorice, there is a wide field of licorice products having energy-yielding, flavoring, and medicinal qualities.

The tobacco industry, especially in the United States, is by far the largest user of licorice, incorporating the juice into cigarettes, cigars, smoking mixtures, and even snuff tobacco. In tobacco, licorice serves several purposes: it imparts a sweet taste and characteristic flavor and at the same time enhance the mildness of a tobacco mixture, it also acts as a moisture controlling agent.

Another interesting use of licorice extract is in beer, where by virtue of its surface-active properties the foaming qualities of the extract increase the head-forming character of the beverage.

Like many plants, the licorice root or *Radix liquiritiae* (the name of the drug form) contains glycosides, compounds built up from an aglycon joined to a sugar residue. In most cases the sugar-free moiety, the aglycon, has a

complicated structure containing one or more hydroxylic groups. In the licorice root, glycyrrhizinic acid, the characteristic sweet-tasting compound is one of the most studied of the aglycons. The amount of this glycoside in the root may vary markedly, 7-10% is usually accepted as a reliable average figure.

The sugar moiety of glycyrrhizinic acid according to Lythgoe and Trippett (1950)⁷⁴ is:

It consists of 2-glucopyruronic acid residues joined by a β -1':2-link.

The aglycon of glycyrrhizinic acid, glycyrrhetic acid, according to Ruzicka and Jeger (1942)¹⁰² is:

or called: $\Delta^{12,13}$ -2-oxy-11-oxo-oleanene 30 acid.

A second glycoside found in licorice root which has been examined closely, is liquiretin. According to Shinoda and Ueeda (1934) this is a true glucoside, the aglycon being linked to one molecule of glucose. Pure liquiretin consists of colorless crystals of melting point 212°C . The aglycon, liquiretigenin, (m.p. 207°C) has been identified by Shinoda and Ueeda¹⁰⁶ as 4',7 dihydroxyflavanone. The structure of the glucoside, 7-hydroxy-4'-glucosidoxy-flavanone is as follows:

Isoliquirtin (Puri and Seshadri, 1954) and Rhamno-liquiritin (Van Hulle, 1968)¹²⁵ also can be obtained from licorice root. The aglycon of isoliquiritin, isoliquiritigenin, shows a remarkable spasmolytic action.

The qualitative detection of licorice extract in pharmaceutical preparations by means of ultraviolet light, according to Steiner (1946) is apparently based upon the strong fluorescent capacity of these flavanone.

A neutral saponin related to the glycosides was reported in licorice root by Kobert in 1915⁶⁸. This glycoside occurs on isolation as an ether-insoluble resin consisting of glucuronic acid and hemolytic sapogenin. Houseman and Swift⁵⁵ isolated from Chinese licorice root (1929) a compound of

gross formula $C_{20}H_{21}O_9$, probably related to lapachol.

Glabric acid, a triterpenoid constituent in licorice root, was first reported in 1956 by Beaton and Spring¹² and the structure was determined in 1968 by Elgamal and Fayez³⁵. Liquoric acid, another triterpenoid constituent of glycyrrhiza was reported by Elgamal, Fayez and Snatzk in 1965³⁴.

Recently, the Japanese investigators, Shibata and Saitoh identified some additional new constituents in the licorice root, these constituents are reported as licoricidin¹⁰⁵ glycyrol, 5-o-methyl glycyrol and isoglycyrol¹⁰³. Licoricidin can be represented as 3',6-diisopentenyl-2',4'5-trihydroxy-7-methoxyisoflavan. The glycyrols have the structures as:

(glycyrol, $R=H$)
(5-o-methylglycyrol, $R=CH_3$)

(isoglycyrol)

Other triterpene compounds meristotropic acid, macedonic acid and enathic acid have been found also in the roots and rhizomes of G. glabra, G. korshinskyi and G. uralensis as reported by a Russian worker⁶⁷.

The purpose of this study is to apply tissue culture techniques, via single cell suspension culture to the study

the growth of the licorice and the potential of producing glycyrrhizinic acid or other cell byproducts of academic and industrial interest.

MATERIALS AND METHODS

I. Plant origin, cultural conditions and preparation of single cell suspension.

A. Origin of the plant

The plant used throughout the studies was Glycyrrhiza glabra (licorice). The seeds of the plant were supplied by a number of botanical and seed suppliers throughout the world.

B. Medium

The main basal medium used throughout these studies was a modified PRL-4-C medium of Gamborg³⁷. This basal medium, the composition of which is listed below, was found suitable for all cultures.

i. PRL-4-C medium

Component	mg/liter	mM
NaH ₂ PO ₄ H ₂ O	90	0.65
Na ₂ HPO ₄	30	0.21
KCl	300	4.0
(NH ₄) ₂ SO ₄	200	1.5
MgSO ₄ ·7H ₂ O	250	1.0
KNO ₃	1000	10.0
CaCl ₂ ·2H ₂ O	150	1.0
KI	0.75	
iron-EDTA*	5.0 ml	
micronutrients**	1.0 ml	
vitamins***	10.0 ml	
sucrose	20.0 gm	
N-Z amino type A	2.0 gm	
2,4-D or NAA	2.0 mg	

*Stock solution. Dissolve in 100 ml of water:
278 mg $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ and 372 mg Na_2EDTA . Kept frozen.

**Stock solution. Dissolve in 100 ml of water:
1000 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 300 mg H_3BO_3 , 300 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$,
25 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 25 mg CuSO_4 , 25 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$.
Kept frozen.

***Stock solution. Dissolve in 100 ml of water:
10 mg of nicotinic acid, 100 mg thiamine HCl, 10 mg
pyridoxine HCl, and 1000 mg myo-inositol. Kept
frozen.

In the studies, N-Z amino type A was replaced by
casein hydrolysate, and the quantity was decreased to 0.50 gm
per liter.

ii. PRL-4-C-CM

PRL-4-C medium containing 10% coconut milk.

The coconut milk was obtained from mature coconuts
from the local market. The milk was drained, filtered through
Whatman No.1 paper and stored frozen in plastic bottles.

iii. PRL-4-DM

This is a chemically defined medium, containing
the same mineral salts, sucrose, and growth regulators as the
general basal medium, but the vitamin solution was more complex
and the protein hydrolysate was replaced by a mixture of amino
acids.

Table 1. Concentrations of vitamins and amino acids used in the chemically defined medium (PRL-4-DM)

Vitamin	Amount per liter(μ g)	Amino acids	Amount per liter(mg)
Biotin	0.25	L-asparagine	40
Riboflavin	15	L-glutamine	60
Folic acid	15	glycine	20
p-aminobenzoic acid	200	L-tryptophan	40
Choline	200	L-phenylalanine	20
Ascorbic acid	400	L-arginine	40
Nicotinic acid	500	L-methionine	30
Thiamine HCl	500		
Pyridoxine HCl	500		
Calcium pantothenate	400		
myo-inositol	100 mg		

iv. PRL-4-1%CM-GM

This medium was PRL-4-C containing 1% coconut milk and different growth factors as described in the text.

v. PRL-4-YM

This medium was PRL-4-C containing various amount of yeast extract as described in the text.

vi. PRL-4-CHM

This medium was PRL-4-C containing various amount of casein hydrolysate as described in the text.

C. Callus formation

Seeds of Glycyrrhiza glabra were sterilized by 0.5%

NaOCl solution and 70% ethyl alcohol and then placed aseptically on the surface of a sterile 1% agar-water medium in the sterile petri dishes. The seeds were germinated in the dark at 25 C.

The radicle, cotyledons or hypocotyl of the germinated seeds were sectioned and four to six of these sections were transferred to the surface of 10 ml of PRL-4-CCM agar medium located in a 100 ml milk dilution bottle. The cultures were incubated in the dark at 25 C. Callus tissue formed within two weeks to a month.

D. Preparation of single cell suspension.

When the calluses were big enough to transfer (about 4 weeks), several were transferred to 100 ml of PRL-4-CCM liquid medium located in a 250 ml erlenmeyer flask. The culture was incubated on a rotatory shaker (Gyrotory shaker) at a speed of 160 revolutions per minute at 28 C. The cells were grown in the dark with short periodic exposure to indirect overhead fluorescent light during observation.

The cells were transferred to new medium at regular time intervals in order to maintain culture vigour.

II. Measurement of the cell growth

The growth of the cells was measured by following cell dry weight. The cells from duplicate or triplicate cultures were collected by filtration through Whatman No.1 paper, dried to a constant weight in a Virtis freeze drier.

III. Quantitative measurement of the pectin-like material

The pectin-like material found in the growth medium was measured as follows:

The cold or hot culture medium with the cells (as described in text) was vacuum filtered through Whatman No. 1 filter paper. A constant volume of filtrate was placed into a $\frac{1}{2}$ " diameter cellophane dialysis tubing and dialyzed against running cold tap water for 16 hours. The dialyzed medium was measured for total high molecular weight carbohydrate by the phenol-sulphuric method³¹. The principle of this method is that phenol in the presence of sulphuric acid can be used for the colormetric micro-determination of sugars and their methyl derivatives, oligasaccharides, and polysaccharides. A known concentration of glucose solution was used as a reference standard.

IV. Isolation and hydrolysis of the pectin-like material

A. Isolation

The pectin-like material present in the growth medium was extracted as follows:

The cold or boiling medium (as described in text) was filtered through a whatman No.1 paper. Two volumes of 95% ethanol was added to the filtrate⁸⁹. The colloidal precipitate which formed was collected by centrifugation and washed twice with ethanol, once with acetone and once

with ether; after removal of the ether by air drying, the final precipitate was freeze dried.

B. Hydrolysis

The isolated pectin-like material was hydrolysed for 3 hours or 18 hours^{9,10,115} depending on the experiment in 3% (v/v) nitric acid containing 0.05% (w/v) urea. The hydrolysates were neutralized with NaOH solution.

V. Qualitative carbohydrate analysis; Paper chromatography

The sugars present in the hydrolysate and growth medium were analyzed by descending paper chromatography on No. 1 filter paper at room temperature.

A. Solvents

Solvent A: ethyl acetate-pyridine-water 8:2:1 (v/v)⁹

Solvent B: n-butanol-ethanol-water 5:1:4 (v/v)⁹

Solvent C: ethyl acetate-acetic acid-pyridine-water 5:1:5:3 (v/v) in a tank equilibrated with ethyl acetate-pyridine-water 40:11:6 by volume⁹.

Solvent D: ethyl acetate-acetic acid-formic acid-water 18:3:1:4 (v/v)⁹

Solvent E: the organic phase from a mixture of phenol, 100 ml of water and 1 ml of formic acid⁹.

Solvent F: n-propanol-ethyl acetate-water 7:1:2 by volume¹⁵.

Solvent G: 80% phenol¹⁵.

Solvent H: n-butanol-pyridine-water 6:4:3 (v/v)¹⁵

B. Location reagent

The following reagents were used for the detection of compounds resolved by paper chromatography.

i. For reducing sugars and reducing substances: silver nitrate acetone solution and sodium hydroxide alcohol solution dip followed by treatment with sodium thiosulfate to clear the background¹⁰².

ii. For all sugars: aniline phosphate solution dip followed by heating in a oven at 105°C for 10 minutes¹⁴⁵.

VI. Isolation of steroids

The dried plant cells were ground to fine powder and the steroids extracted with CHCl_3 for 24 hours in a soxhlet⁵³ apparatus. The extracts were concentrated under vacuum by using a water aspirator.

VII. Extraction and purification of glycyrrhizinic acid from licorice root stick or dried cells.

The procedure listed in figure 1 was designed for isolation of ammonium glycyrrhizinate^{17,76,90}.

Fig. 1 Procedure for isolation and purification of glycyrrhizinic acid from licorice root stick or dried plant cells

VIII. Hydrolysis of glycyrrhizinic acid

Glycyrrhizinic acid was hydrolyzed for 4 hrs with 6 N. sulphuric acid in a reflux apparatus^{17,76}. The product was extracted with chloroform or ether. The reaction can be expressed by equation:

IX. Reduction of glycyrrhetinic acid

An amount of LiAlH_4 equal to the glycyrrhetinic acid by weight was refluxed for six hours in glycyrrhetinic acid ether solution¹⁷. The refluxing apparatus was assembled with a dry-tube at the top to prevent the entrance of moisture. The product of the reaction was poured carefully onto ice, and shaken with an equal volume of ether. The ether solution was allowed to stand for a period of time and then the ether phase was removed from the water phase with the aid of a separatory funnel. The substance in the ether portion was the reduced glycyrrhetinic acid.

X. Qualitative steroids and triterpenoid analysis; thin layer chromatography (TLC)

A thin layer of silica gel G (E. Merck AG, Darmstadt, Germany) was prepared on glass plates as described by E. Stahl (1965)¹¹⁰.

Solvents used were:

- i. chloroform-methanol 2:1 by vol.
- ii. chloroform-95% ethanol 2:1 by vol.
- iii. isopropanol-methanol 2:1 by vol.
- iv. heptane-benzene-absolute ethanol 50:50:1 by vol.⁵⁷
- v. n-hexane-acetone 2:1 by vol.⁵³

Solvents i, ii, iii, were used mainly for steroids.

Solvents iii, iv, v, were used mainly for triterpenoids.

XI. Methods used for locating the steroids and triterpenoids

A. Observation under ultra-violet light

Fluorescent spots of different colour and absorbence could be distinguished under ultra-violet light. Standard samples of glycyrrhetic acid (K & K Laboratories Inc. Plainview, N. Y. Hollywood, Calif.) showed three absorbent spots under UV.

B. Spray with coloration reagent

- i. antimony trichloride, 20% in chloroform⁵⁷.
- ii. antimony pentachloride, 20% in chloroform^{53,57}.
- iii. acetic anhydride (10%) and sulphuric acid (10%) in absolute alcohol. Liebermann-Burchard reagent^{57,110}.

After spraying, the plates were heated at 120°C for 5 minutes.

XII. Measurement of oxygen consumption

Oxygen uptake of the cells was measured by a Gilson differential respirometer. Each Warburg flask contained 3.0 ml of a 7 days cell suspension (approx. 50 mg dry weight of cells). The center well contained a fluted filter paper saturated with 0.15 ml of 20% KOH.

XIII. Qualitative apple aroma analyses

A Becker research dual column gas chromatograph, type 3810, equipped with dual flame ionization detectors was used for the analyses of the apple aroma from the licorice cell growth media. The stainless steel columns contained chromosorb W coated with diethylene glycol succinate polyester; mesh size 80/100; 12 feet long; outside diameter 1/8".

XIV. Enzymatic determination of alcohol

The enzyme, alcohol dehydrogenase, was used to measure the amount of ethanol contained in the supernatant growth medium following the method of Vallee and Hoch^{123,124}. Cell supernatant (0.5 ml) was added to 0.3 ml of nicotinamide adenine dinucleotide (NAD) solution and 0.2 ml enzyme solution. The amount of ethanol contained in the growth medium was calculated by comparing the rate of increase in absorbancy at 340 m μ of the unknown solution with standard ethanol solution.

EXPERIMENTAL RESULTS

PART I. Growth experiments

I. Callus formation and the preparation of single cell suspensions

The glycyrrhizan seeds sprouted on the agar medium within a week; even though they were incubated in the dark, the plumules were a dark green colour and remained the same colour for more than one month.

The 1/16 inch sections of the hypocotyl, cotyledons and roots all formed calluses on PRL-4-CCM agar medium. The time needed for callus formation varied from ten days to month. The cotyledon sections took a longer time to convert to undifferentiated cell masses or callus than did the other tissues.

Some of the calluses formed from cotyledons and roots contained two different colours within the one callus, white at one end of the callus and brown at the other end. The white section of the tissue grew slower than the brown coloured area, as a result the whole callus gradually became predominantly brown in colour.

The calluses had different friabilities, some were smooth on the surface with little friability and others were coarse in appearance and were very friable.

When the friable calluses were of sufficient size, approximately 1/2 inch in diameter, to provide a large number

of fragments, they were transferred to PRL-4-CCM liquid medium containing 2 mg/l 2,4-D (2,4 dichlorophenoxy-acetic acid). The cultures were incubated in a rotatory shaker at 28°C in the dark.

The time required for the disintegration of the callus to form fragments consisting one to several cells varied from callus to callus. Generally speaking at the initiation the cultures grew and divided rather slowly. The cells of different origin (root, hypocotyl, cotyledons) did not display a difference in morphology. However at least three different types of cells could be recognized in the culture under microscope. One type of cell was coil-like in shape and a light yellow colour. The second type of cell occurred in a kidney-like shape and was also light yellow in colour, the third type of cell was a very large brown coloured cell.

Cell aggregates containing at least ten or more cells were found in the culture for the first several transfers. The percentage of single cells vs. clumps increased with every culture transfer, and divided at a faster rate from one transfer to another. Following a growth period which included about six transfers, the cells appeared to grow in an almost constant rate. The coil-like cells decreased in quantity either due to their apparent slow growth or to a change in their morphology during the growing period. The large yellowed cells could be found occasionally in all cultures.

Licorice cells reached the maximum total dry weight in about 11-13 days. Following inoculation into fresh medium,

the cells divided vigorously for three to seven days forming many small clumps and the small single cells. After this initial growth most cells enlarged and elongated showing differences in sizes and morphology. The active movement of small mitochondrial particles from the region of nucleus to the places around the cell wall could be seen clearly under 400x phase contrast microscope in healthy cells.

II. Growth curve

The growth of licorice cells in PRL-4-CCM medium is shown in table 2 and figure 10.

Table 2. Growth rate of licorice cells

Growth period (days)	Dry weight (grams)*	pH
0	0.0800	5.60
3	0.1600	5.21
5	0.2533	4.97
6	0.3942	4.80
7	0.6127	4.60
8	0.6590	4.55
10	0.8348	4.50
11	0.9699	4.70
12	0.9708	4.75
13	1.0004	4.70
14	1.0108	4.96
17	1.0133	4.88
18	1.0734	5.15
20	0.9621	5.60
24	0.8926	5.90
28	0.8482	6.10

* average of triplicate. grams per flask (100 ml medium).

Fig. 2 Callus formed from cotyledon section; begining stage.

Fig. 3 Callus formed from root section; begining stage.

Fig. 4 A callus, white at one end and brown at the other end with clear dividing line.

Fig. 5 Large seaweed -like cells (400X)

Fig. 6(a) Kidney-like cells (400X)

Fig. 6(a) Kidney-like cells (1200X)

Fig. 7(a) Coil-like cells (400X)

Fig. 7(b) Coil-like cell (1200X)

Fig. 8 Cells in different sizes and morphology (400X)

Fig. 9 Dried cells grown in PRL-4-CCM (with 2 mg/l 2,4-D) medium and in PRL-4-1%CM-GM medium

Figure 10. Dry weight and pH changes during the growth period of licorice cells

symbols: circles, dry weight; triangles, pH.

The growth medium of licorice cells indicated a strong reducing ability when tested with Fehling solution. Two major silver nitrate reducing compounds were observed following paper chromatography and will be discussed in a later section.

The protein content of the plant cells and the surrounding medium after ten days growth was not detectable by the biuret method. This would suggest that the protein level of the cells and the growth medium was below 0.1 mg/ml.

III. Effect of coconut milk level and growth factors on the growth of licorice cells

High concentrations of coconut milk have been found necessary for the growth of many plant calluses and suspension cultures. However, the addition of this chemically undefined material in such high levels limits the usefulness of cell suspension for metabolic studies. As a result an attempt was made to replace coconut milk with synthetic medium.

Defined synthetic medium (PRL-4-DM) was selected for the experiment. This medium was reported to support the growth of several plant cell cultures although the growth rate was considered to be considerably lower than that obtained with the complex media. The growth rate of licorice cells on PRL-4-DM medium was compared with PRL-4-CCM medium containing 2,4-D and coconut milk. Results are shown in

table 3. The synthetic medium PRL-4-DM with or without 2,4-D both could not support active sustained growth. Coconut milk was a required ingredient in the synthetic medium for licorice cell growth.

Among the flasks exposed to the same treatment, a variation in the colour of the cells and the medium was apparent. A deep brown colour consistently indicated unfavorable growth. In PRL-4-CCM medium the cells and the medium were a pale yellow colour which turned to a yellow-brown color as the cells approach the late stationary phase.

Table 3. Yields of licorice cells in PRL-4-DM medium

*	1st transfer	2nd transfer	3rd transfer
PRL-4-DM without 2,4-D	0.6832	0.2720	0.0351
PRL-4-DM with 2,4-D	0.7082	0.3482	0.2809
PRL-4-CCM	1.1064	1.0923	1.0983

*average of triplicate. grams per flask (100 ml medium).

In a subsequent experiment, 2,4-dichlorophenoxyacetic acid was found to be important for cell growth. Coconut medium without 2,4-D supported slow single cell growth, abnormal dark coloured cells and a tendency toward large solid clumps.

Table 4. Yield of licorice cells (gm/100 ml) in PRL-4-C medium with different level of coconut milk (%)

level of coconut milk	1st transfer				2nd transfer			
	12 days		20 days		11 days		18 days	
	dry wt.	pH	dry wt.	pH	dry wt.	pH	dry wt.	pH
0.5%	0.9406	4.54	1.0258	4.75	0.3368	4.60	0.7048	4.80
1.0%	0.9353	4.40	0.9340	4.60	0.3547	4.50	0.7376	4.70
2.0%	0.9074	4.38	1.0736	4.80	0.5003	4.40	0.8252	4.70
3.0%	0.9351	4.35	1.0565	4.80	0.5781	4.50	0.8659	4.70
5.0%	0.9863	4.45	1.1217	4.65	0.7142	4.50	0.9349	4.80
7.0%	0.9898	4.78	1.0891	4.85	1.0178	4.70	1.1320	5.00
10.0%	0.9463	5.14	1.1451	4.80	1.1516	4.80	1.2114	5.20
15.0%	1.0553	5.17	1.0452	4.95	1.2272	4.70	1.2565	5.50

*average of triplicate.

Because of the improved cell growth in the defined medium containing coconut milk, the optimum level of coconut milk present in the medium was determined. From the results shown in table 4 and figure 11, the lower the coconut milk level in the medium, the longer the growth period needed to reach the maximum cell yield. When the cells were harvested at 12 days after inoculation, the resulting cell yield was found to increase in a linear fashion as the coconut milk level increased from 0.5% to 7%. Only a slight difference in cell yield was shown in coconut milk between the 10% and 15% level. A longer cell growth period decreased the difference in cell dry weight observed between coconut milk levels.

Figure 11. Dry weight of licorice cells (gm/100 ml) in PRL-4-C medium with different coconut milk levels

solid line: cells harvested at 11 days incubation
dotted line: cells harvested at 18 days incubation

Generally two or three cell transfers are necessary to show the visible differences between plant cell treatments. The results of second transfer was taken for the graph in figure 11. The reason for the long delay in cell response following a specific treatment was due likely to the large amount of nutrients transferred with the cells and within the cells.

In order to observe the effects of various plant hormones on licorice cells growing on a medium containing limiting amount of coconut milk, licorice cells were transferred to 1% coconut milk in PRL-4-C medium with the addition of different plant growth factors. As observed in table 5, the hormone treatments did not promote increased cell yield. In fact a decrease in cell yield was observed for all treatments suggesting that these hormones at these concentration levels under the condition of the experiment in the presence of limiting concentration of coconut milk did not overcome the coconut milk nutrient deficiency. A great difference in colour and growth rate was noted between treatments and within a particular treatment. During third growth transfer, all the cells changed to deep brown colour and appeared to be in poor condition.

In conclusion, the addition of 10% coconut milk to PRL-4-C medium was found to be the optimum concentration required for cell growth rate and good cell yield. The addition of the hormones, kinetin, gibberellic acid, naphthalene acetic acid, indole acetic acid, and 2,4-dichlorophenoxyacetic acid at the levels recorded did not replace or augment the

growth activity contributed by the coconut milk.

Table 5. Yield of licorice cells (visible growth response)*
in different PRL-4-1%C-GM following 2-3 week
inoculation

PRL-4-1%C	1st transfer	2nd transfer	3rd transfer
0.5mg/l kinetin	++++	+++	+
2mg/l kinetin	+++	++	+
5mg/l kinetin	+	—	—
0.5mg/l G. A.	+++	++	+
2mg/l G. A.	+++	++	+
5mg/l G. A.	+++	++	+
0.5mg/l NAA	++++	+++	+
2mg/l NAA	++++	+++	+
5mg/l NAA	++++	+++	+
0.5mg/l IAA	++++	+++	+
2mg/l IAA	+++	++	+
5mg/l IAA	+	+	+
2mg/l 2,4-D**	0.9522	0.8196	0.4634

* results of triplicate

** weight (grams/100 ml medium)

IV. Yeast extract medium

Complex nutrients other than coconut milk such as yeast extract or casein hydrolysate were examined to their ability to replace the coconut milk in the PRL-4-CCM medium. According to the results of these experiments, as summarized in table 6, yeast extract served as a better nutrient than casein hydrolysate. Yeast extract at the 0.3% or the 0.5% levels in PRL-4-C medium gave satisfactory licorice cell growth through five or more transfers. It is of interest to note that when the cells from the stationary phase of growth were used for inoculation, a much longer lag growth phase was needed for the cell to recover their growth activity as the next day following inoculation the cells and the medium altered from yellow to brown. At higher concentration of yeast extract in the medium (0.8%) growth was inhibited; a longer lag phase, sometimes as long as one month, was noted. In 0.8% yeast extract medium the cells did not change to a brown colour which possibly indicated a capability to avoid oxidative darkening.

One of the most interesting observation made when using yeast extract as medium component to grow licorice cells was that the cells reached their maximum yield in only 10 days after inoculation. This incubation period was comparatively shorter than cells grown in PRL-4-CCM medium.

The PRL-4-YM medium, that is the PRL-4-C medium containing yeast extract, usually promoted a low pH at the time of cell harvest, in some cases, this pH was as low as

pH 2.

Generally speaking, the PRL-4-YM medium supported good cell growth and could replace the use of coconut milk. In addition the cells growing in this medium secreted considerable polysaccharide. The yield and the composition of the polysaccharide will be discussed in later section.

Table 6. Yield of licorice cells (gm/100 ml medium) in yeast extract and casein hydrolysate medium

PRL-4-C	1st transfer	2nd transfer	3rd transfer
0.8% YE	0.7890*	**	
0.5% YE	1.1922	1.1271	1.1356
0.3% YE	1.0733	0.9518	1.0127
0.2% YE	0.9182	0.7192	0.4223
0.5% CH	0.9120	**	
0.2% CH	0.9412	0.7907	0.5686
0.05% CH	0.8450	0.4339	
0.25% YE & 0.25% CH	0.9699	**	
0.10% YE & 0.10% CH	1.0219	0.7855	0.4160
0.05% YE & 0.05% CH	0.9052	0.7693	0.3007

* average of triplicate

** lost experiments due to the delaying of cell growth

V. Effect of ascorbic acid and l-gulono-lactone on the growth of licorice cells

An experiment was carried out to study the effects of ascorbic acid and l-gulono-lactone on the growth of licorice cells. PRL-4-CCM containing either 2 mg/l ascorbic acid (A) or 2 mg/l gulono-lactone (B) were used to grow the cells and a comparison was made with cells grown in the presence (C) and absence (D) of 2mg/l 2,4-D. Medium containing 2,4-dichlorophenoxyacetic acid supported the best growth, followed by ascorbic acid and gulono-lactone. The colour of the cells and the medium varied from light yellow through to deep brown, the more active 2,4-D stimulated culture was yellow whereas the slower growing cultures were a brown colour. The cell response to these treatments can be shown by the oxygen consumption as measured by the Gilson Differential respirometer. (Fig. 12)

In order to determine whether the concentration of the above compounds in the medium had an effect on cell growth, the compounds were studied at several different concentrations. A great variation within the treatment was apparent in the growth rate and yield of the cells, especially in ascorbic acid medium. The large growth variation which exists between flasks within the same treatment is rather interesting and cannot be explained.

In the paper of Naguib on Cunninghamella Sp.⁸¹ the function of ascorbic acid is probably related to the respira-

Figure 12. Oxygen uptake by 3.0 ml of 7 days cell suspension (approx. 50 mg dry weight)

symbols: circles, cells grown in PRL-4-CCM with 2 mg/l 2,4-D; squares, cells grown in PRL-4-CCM with 2 mg/l ascorbic acid; triangles, cells grown in PRL-4-CCM with 2 mg/l gulono-lactone; cross, control.

tion and carbohydrate metabolism. Ascorbic acid induces rapid sucrose consumption accompanied by increased keto acid content and respiration rate. Ascorbic acid also effect the glucosan and galactosan accumulation and nucleoprotein content.

The metabolic pathway of ascorbic acid and the function of 2,4-D in plant tissue is unknown. From these experiments, it is evident that ascorbic acid and gulono-lactone stimulated cell growth although at the concentration tested the stimulation is not as marked as 2,4-D.

Table 7. Yield of licorice cells (dry wt., gm/100 ml medium) in PRL-4-CCM with 2,4-D, l-gulono-lactone, ascorbic acid or absence of any growth regulator

PRL-4-CCM	Yield (dry wt., gm/100 ml medium)		
	Exp. No 1	Exp. No 2	Exp. No 3
2,4-D 2mg/l	1.3744	1.2988	1.3957
G. L. 2mg/l	0.6670	0.6835	0.5964
G. L. 5mg/l	0.5280	0.5939	0.5142
G. L. 10mg/l	0.6953	0.7032	0.5838
A. A. 2mg/l	0.1219	0.2132	0.9019
A. A. 5mg/l	0.1703	0.3015	1.4107
A. A. 10mg/l	0.2079	0.1890	0.8693
control	0.0239	0.1253	0.6096

PART II. Products from licorice single cell suspension
culture

I. Fruit smell

Licorice cell cultures removed from the shaker and permitted to stand without shaking developed a pleasant apple odour. The aromatic odour was present in cultures of cells grown in the media PRL-4-CCM, PRL-4-1%CM-GM and the medium with gulono-lactone, but did not occur in PRL-4-YM and PRL-4-CHM. The components of the apple aroma present in the head space above the culture were compared with the compounds found in a sample of natural apple essence by gas chromatography. Several of the constituents had the same retention time on the column as some of the components found on the licorice cell aroma (Fig. 13). One of the major peaks was ethanol, which was confirmed by enzymatic assay with alcohol dehydrogenase (table 8).

The licorice cells after standing in their growth medium were found to contain 0.24% ethanol.

Figure 13. Graph of gas chromatography for apple aroma from licorice growth medium

speed 4, temp. 100°C, attenu. 1X, detect temp. 140°C, inject temp. 120°C.

Table 8. Ethanol content in the licorice cell aroma as measured by alcohol dehydrogenase ($\text{RCH}_2\text{OH} + \text{NAD} \rightleftharpoons \text{RCHO} + \text{NADH}_2$), use ethanol solution as standard

Reaction medium	Ethanol content
PRL-4-1%CM-0.5mg/l IAA	0.24%
PRL-4-1%CM-0.5mg/l kinetin	0.15%
PRL-4-1%CM-2mg/l G. A.	0.12%
PRL-4-1%CM-2mg/l 2,4-D	0.20%

II. The pectin-like material

The yeast extract medium not only supplied a good medium for cell growth, but also stimulated the production of large amounts of polysaccharide from the cells. The polymer could pass through Whatman No.1 filter paper; was positive in the carbohydrate Molisch reaction; formed a gel which disappeared on heating and could be precipitated by an equal volume of ethanol.

A. Yield

The growth medium from the licorice cell culture was filtered through the No.1 Whatman paper, and then dialyzed against running cold tap water for 16 hours.

The yield of the pectin-like material was measured by the phenol-sulfuric method. The results of the analyses are shown in table 9 and table 10.

Following the removal of the growth medium, the

cells were extracted with 30 ml of boiling water to obtain the hot water soluble cell pectin fraction. This fraction was dialyzed as described above, the yield of the pectin in this fraction and in the medium are compared in table 9. The results indicated that the pectin-like material was present not only in the growth medium of the culture but also in the cell extract.

The total pectin, i.e. medium pectin plus cell pectin, found in cell cultures at various culture ages and at various yeast extract concentrations are listed in table 10.

Table 9. Pectin-like material contained in the cold growth medium filtrate and hot water cell extract of cell cultures after 20 days incubation

	Culture				
	PRL-4-C 0.8%YE	PRL-4-C 0.5%YE	PRL-4-C 0.3%YE	PRL-4-C 0.2%YE	PRL-4-C 10%CM
medium	500 mg/l	760 mg/l	600 mg/l	600 mg/l	680 mg/l
cell	275	280	280	170	190

The amount of pectin-like material extractable from the cultures would appear to depend on the length of the culture incubation. The maximum total hot water extractable pectin from the cultures occurred after approximately one

Table 10. Total pectin-like material from yeast extract media at different ages (days) after inoculation*
yield represent by mg/1000 ml medium

age (days)	PRL-4-C						10%CM
	8g/1YE	5g/1YE	3g/1YE	2g/1YE	5g/1CH	2g/1CH	
13		675	550	510			480
17		690	550				500
20		690	625				500
24	613	720	680				600
27		980	840	754	612	550	800
30	775	1050					780
32		1100	880	770			770
36		950		650			650

* total media pectin + hot water soluble cell pectin.

month of culture incubation, table 10. The culture of this stage was not in active growth, figure 2. The maximum pectin concentration was produced in the medium containing yeast extract in place of coconut milk. The yield represent 10% of the dry weight of the cells.

Growth media from other plant tissue cultures, such as bush bean and tobacco, also contained pectin-like material. Therefore the pectin-like material in plant suspension cultures could be a universal phenomena.

The middle lamella, the area located between two adjacent plant cells is composed of calcium and magnesium pectates. The formation of single cells in the liquid medium may be due to the solution of the material of the middle lamella and thus possibly account for the accumulation of the

polysaccharide in the growth medium. As the cells aged, that is following active growth in culture, many of the cells appeared to be in various stages of cell degradation as viewed under the phase contrast microscope. This release of cell material may account for the increase in soluble polysaccharide. On the other hand it is entirely possible that this material may be synthesized from simple sugars by enzymes active at a late stage in the cell growth cycle.

B. The components of the pectin-like material

Purified pectin-like material was hydrolyzed with 3% (v/v) nitric acid containing 0.05% (w/v) urea for 3 hrs or 18 hrs at 100°C. The hydrolysate was neutralized by diluted NaOH solution.

The sugar components were identified by descending paper chromatography. The hydrosate was developed in solvent A, B, and C respectively, using glucose, galactose, fructose, arabinose, rhamnose, cellubiose, xylose, galacturonic acid, glucuronic acid as known standards. Hydrolysis for a period of 18 hours did not visibly increase the number or the amount of free sugars over that found after 3 hours hydrolysis as shown in figure 13.

Following development with the ethyl acetate-pyridine-water solvent, the silver nitrate reagent indicated four major and one minor zone with a positive reaction.

In order to examine the composition of these latter sugar zones, the pectin hydrolysate was applied as a streak

on one or more chromatogram sheets. The chromatograms were developed with solvent A for 16 hours. A reference strip removed from the side of each of the chromatogram sheets was sprayed with silver nitrate reagent to locate the zones. On the remaining unsprayed portion of the chromatogram, strips were removed corresponding to the four major and one minor zones (Fig. 14). The sugars were isolated from the strips by eluated with water.

Eluates of zones A-E were chromatographed in two or three different solvent systems, the results are shown from Fig. 15 to Fig. 17.

The following conclusion could be drawn for the possible components contained in the pectin-like material hydrolysate.

Zone A: galactose

Zone B: glucose, fructose and arabinose

Zone C: arabinose and fructose

Zone D: glucose, fructose and xylose

Zone E: galacturonic acid and glucuronic acid

Thus it would appear that the pectin-like material from licorice cells contained glucose, fructose, galactose, arabinose, xylose, galacturonic acid and glucuronic acid.

Fig. 14 Paper chromatogram run in solvent A (ethyl acetate-pyridine-water 8:2:1) for 16 hours.

S₁, S₂: Pectin-like material hydrolysates

Sugar standards: 1. glucose 2. galactose 3. fructose
 4. arabinose 5. rhamnose
 6. cellobiose 7. xylose
 8. galacturonic acid
 9. glucuronic acid

(The results shown that the hydrolysates do not contain rhamnose or cellobiose)

Fig. 15. Paper chromatograms for eluate zone A
 I. run in solvent C; II. run in solvent A;
 III. run in solvent D.

Standards: 1.glucose 2.galactose 3.fructose 4.arabinose
 5.xylose.

Fig. 16. Paper chromatograms for eluate zone E
 I. run in solvent A; II. run in solvent C;
 III. run in solvent D.

Standards same as in fig. 15

Fig. 17. Paper chromatograms for eluates B, C and D.

left, run in solvent C; middle left, run in solvent H;
right, run in solvent A; middle right, run in solvent A.

(The results shown: B contain arabinose, fructose, glucose.
C contain arabinose.
D contain glucose, fructose, xylose.)

III. Steroids, triterpenoids and glycyrrhizinic acid

A. Steroids

The cell steroids were extracted with chloroform and the extracts were examined by thin-layer chromatography on silica gel. The chloroform extracts of the cells grown in different media were applied to the silica gel and developed in the desired solvent systems. The plates were i. observed under ultra-violet light; ii. sprayed with antimony pentachloride or iii. sprayed with Liebermann-Burchard reagent. The results are shown in figure 18 to figure 21.

The extracts contained many components which could be located with UV light and with indicator reagents. In addition, the cell extracts obtained from cells grown on different medium indicated mainly the presence of same steroids although there were some minor differences. Five to six fluorescent spots and one or two absorbent spots were located under UV light of 258 m μ . Five to six colour spots were indicated with Liebermann-Burchard reagent, and about four colour spots with antimony pentachloride reagent.

As shown by TLC, at least tentatively, there were fewer components contained in the CHCl₃ extract of licorice stick, than in similar extracts of plant cell culture. In addition, the components contained in the plant cell extract reacted quite different from those found in licorice root extract.

Figure 18. TLC run in solvent iii (isopropanol-methanol 2:1) observation under UV, all the spots are fluorescent.

L: licorice stick chloroform extract

1-7: chloroform extract of cells grown in different media

Figure 19. TLC run in solvent iii, after sprayed with Liebermann-Burchard reagent and heated at 105°C for 5 min.

L: licorice stick chloroform extract

1-7: chloroform extract of cells grown in different media

A: pink purple

C: green

E: brown

B: purple

D: faint purple

F: deep brown

Figure 20. TLC run in solvent i (chloroform-methanol 2:1) observation under UV, all the spots are fluorescent.

L: licorice stick chloroform extract
 1-7: chloroform extract of cells grown in different media.

Figure 21. TLC run in solvent i, observation after sprayed with antimony pentachloride chloroform solution and heated under 105°C for 5 min.

L: licorice stick chloroform extract

1-7: chloroform extracts of cells grown in different media.

A: brown

C: deep brown

B: pale brown

D: yellowish brown

B. Glycyrrhizinic acid

Glycyrrhizinic acid is the most important commercial component contained in the licorice plant. The assay methods developed within the literature for glycyrrhizinic acid can be divided into three fundamental groups.

i. A measure of the weight of the precipitates formed by adding acid or certain salts into the crude licorice extracts.

ii. A measure of the sugar moiety.

iii. A determination of the quantity of the aglycon, glycyrrhetic acid, by colorimetric or polarographic method.

The first two methods are very unspecific, as other carbohydrate constituents of the extract may be simultaneously determined or may interfere with the assay. The third method, is much more specific than the other two, although it does not differentiate between structurally similar compounds which might be present in the cell extract.

The following procedure for the analysis of glycyrrhizinic acid was developed to avoid as many analytical ambiguities as possible.

i. The taste of the purified material; true glycyrrhizinic acid gives a very pronounced sweet taste, reported to be 50 times as sweet as sucrose. In this study, purified glycyrrhizinic acid was prepared from licorice root stick of commerce and was used as standard.

ii. The hydrolysis of glycyrrhizinic acid to

glycyrrhetic acid with 6 N. H_2SO_4 for six hours, followed by extraction the mixture with CHCl_3 . The free glycyrrhetic acid can be identified by

- (a) the R_f on TLC
- (b) the colour reaction with antimony pentachloride
- (c) the colour reaction with Liebermann-Burchard reagent

Glycyrrhetic acid gives positive colour reaction (brown) with antimony pentachloride but no reaction with Liebermann Burchard reagent.¹⁷

- (d) the reduction of the glycyrrhetic acid with LiAlH_4 in ether solution. The reduced glycyrrhetic acid gives a pink colour to Liebermann-Burchard reagent¹⁷.

The results of the analyses for licorice in the cell plant cultures are listed in table 11.

From these results it can be seen that glycyrrhizinic acid did not occur in the licorice plant cell culture. Either there was no glycyrrhizinic acid produced by the cells or the assay method was not sensitive enough to indicate the possible presence of small amount of glycyrrhizinic acid.

C. Triterpenoids

When the freeze-dried licorice plant cells were treated according to the procedure for purifying glycyrrhizinic acid, a product (CGZ) was obtained which appeared visibly to be quite similar to the purified glycyrrhizinic acid

Table 11. The results of glycyrrhizinic acid assay

	glycyrrhethinic acid	licorice root stick	licorice cells from suspension
a.colour of fraction A, fig. 1		brown	yellow
b.colour of fraction B, fig. 1		deep brown	deep brown
c.colour and viscosity of fraction C, fig. 1		deep brown high viscosity	deep brown high viscosity
d.colour of fraction D, fig. 1		reddish brown	reddish brown
e.colour of fraction E, fig. 1		yellow powdery	yellow powdery
f.taste of the final product in fig. 1	not sweet	strong licorice sweet	salty
g.Rf of the abs. spots of the CHCl ₃ extract from hydrolysa- te on TLC, $\star$ under UV	0.65, 0.78, 0.92 (n-hexane- acetone)	0.92 (n-hexane- acetone)	—
h.colour reaction with SbCl ₅ in CHCl ₃	positive brown	positive brown, yellow	positive brown
i.colour reaction with Ac ₂ O/H ₂ SO ₄	negative	negative	5 positive spots
j.colour reaction of the reduced material with Ac ₂ O/H ₂ SO ₄	positive	positive	—

from licorice sticks (LGZ). However glycyrrhizinic acid could not be found in the preparation when tested by taste and by TLC. After CGZ was hydrolyzed (CGZH) and fractionated on TLC, two brown coloured and one yellow coloured compounds were produced when sprayed with SbCl_5 chloroform solution, and five colour compounds (brown and purple) were produced when sprayed with Liebermann-Burchard reagent. None of the compounds corresponded either to glycyrrhizinic acid or to glycyrrhetinic acid and therefore were considered to be some other triterpenoids.

IV. The reducing substances of the licorice plant cell growth medium

Ascorbic acid is found universally in the differentiated tissues of all plants. It is also found in some quantity in certain plant cultures⁶. The high reducing ability of the medium of licorice cells as indicated by the reduction of Fehling solution suggested the presence of reducing substances. The reducing substance did not appear to be ascorbic acid as this acid could not be found by paper chromatographic analysis. In an attempt to determine whether ascorbic acid can accumulate in the growth medium, ascorbic acid, l-gulonolactone, were added to the medium. Both ascorbic acid and l-gulonolactone remain stable in the medium for at least several days.

l-gulonolactone was not converted to ascorbic acid

Figure 22. TLC run in isopropanol-95% ethanol 2:1
observation under UV

Gly.t.: glycyrrhitinic acid

LGZ: isolated glycyrrhizinic acid from licorice
stick

CGZ1,2: isolated "glycyrrhizinic acid" from cells

LGZH: stick licorice hydrolysate

CGZH: cell licorice hydrolysate

Figure 23. TLC run in isopropanol-95% ethanol 2:1 observation after sprayed with antimony pentachloride chloroform solution and heated under 105 C for min.

Gly.t.: glycyrrhetic acid

LGZ: isolated glycyrrhizinic acid (licorice) from licorice stick

CGZ1,2: isolated "licorice" from cells

LGZH: stick licorice hydrolysate

CGZH: cell licorice hydrolysate

Figure 24. Two dimension TLC for cell licorice hydrolysate
 1st dimension was run in n-hexane-acetone 2:1
 and then observed under UV
 2nd dimension was run in isopropanol-95% ethanol
 2:1, and then sprayed with antimony pentochloride
 chloroform solution and heated under 105 C for
 5 min.

(a)

(b)

Figure 25. TLC run in solvent v. (n-hexane-acetone 2:1)
 (a) observation under UV
 (b) observation after sprayed with Liebermann-Burchard reagent

LGZH: stick licorice hydrolysate
 CGZH: cell licorice hydrolysate

as indicated by paper chromatographic analyses.

As forementioned, the growth medium of licorice cells gave two major and one or two minor silver nitrate reducing spots when it was analyzed by the paper chromatographic method. It was found the two major silver reducing spots showed only one spot when the chromatogram was developed with aniline phosphate (Fig. 26).

The spot that could not be shown with aniline phosphate was tentatively identified as sorbitol. As shown in fig. 27 and fig. 28, it did not appear to be mannose, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, nor was it originally contained in PRL-4-CCM medium. It was not dulcitol, inositol, glucosamine, gluconolactone, but appeared to be sorbitol or mannitol. A paper chromatogram (Fig. 29) which was run in solvent G for 50 hours and then was developed with silver nitrate reagent resulted in good resolution of the compound. By comparing the three spots shown in M+GM (mannitol+growth medium) and the two spots shown in S+GM (sorbitol+growth medium) column, it would

appear that the unknown sugar was sorbitol.

The other major spot on the paper chromatogram which was developed with aniline phosphate reagent was identified as fructose as shown in fig. 30.

The sucrose in the medium disappeared long before the cell maximum yield was reached (Fig. 27I, 28II). on further incubation, fructose was found to be completely absent leaving relatively small quantities of sorbitol (Fig. 31). Finally on continued incubation, sorbitol also disappeared.

Figure 26. Paper chromatograms run in solvent A (ethyl acetate-pyridine-water 8:2:1)

M: licorice growth medium

Standards: glucose, galactose, fructose, arabinose, xylose.

left: developed in silver nitrate reagent

right: developed in aniline phosphate reagent

(I)

(II)

Figure 27. Paper chromatograms run in solvent A (ethyl acetate-pyridine-water 8:2:1)

CM: PRL-4-CCM medium before inoculation

GM: licorice growth medium

Standards: galactose, glucose, fructose, arabinose, xylose.

left sheet of I and II: developed in silver nitrate reagent

right sheet of I and II: developed in aniline phosphate reagent

(I)

(II)

Figure 28. Paper chromatograms run in solvent A (ethyl acetate-pyridine-water 8:2:1)

In fig.(I), material applied: dulcitol, sorbitol, PRL-4-CCM medium, licorice growth medium, mannitol, inositol, glucose.
developed in silver nitrate reagent

In fig.(II), materials applied: sorbitol, mannitol, PRL-4-CCM, medium, licorice growth medium, glucose, galactose, fructose.
left sheet, developed in silver nitrate reagent
right sheet, developed in aniline phosphate reagent.

Figure 29. Paper chromatograms I. run in solvent F (80% phenol)
 II. run in solvent G (isopropanol-ethyl acetate-
 water 7:1:2) for 50 hours.

materials applied: mannitol sorbitol, mannitol
 licorice growth medium, mannitol, growth medium,
 sorbitol, sorbitol growth medium, fructose.

developed with silver nitrate reagent

Figure 30. Paper chromatograms I. run in solvent G II. run in solvent A III. run in solvent F. materials applied: sucrose, sorbose, CM, GM, glucose, fructose, galactose.

Figure 31. Paper chromatograms run in solvent A materials applied: No. 1-19 licorice growth medium, (PRL-4-CCM) 1. medium with 2,4-D, 13 days old; 13,17,18,19, medium with 2,4-D, older cells; 2,3,4, medium with gulonic lactone; 5-10, medium with ascorbic acid, 11,12, medium without growth factors; 5-7,11, cells and medium changed to brown. developed with silver nitrate reagent.

DISCUSSION

I. Licorice cell culture

Licorice cells were cultured successfully in PRL-4-CCM and PRL-4-YM media. In both cases, a high level of a complex nutrient, coconut milk or yeast extract, was needed.

The liquid endosperm, coconut milk, has been found to be required by many normal callus and suspension tissues¹⁴³. Considerable interest has been shown in the identification of the active growth constituents present in coconut milk. Pollard, Shantz and Steward (1961)⁹³ have divided the active components of coconut milk into three fractions, namely, a nitrogenous component (consisting of reduced nitrogen compounds particularly amino acids and their amides), a neutral component (in which the most prominent constituents are m-inositol, scyllo-inositol and sorbitol) and an active component which has resisted analysis but is regarded as including auxin or auxin precursors. In later evidence it was found that the effects of the active components of coconut milk cannot be replaced by kinetin or its analogues¹⁴³. There is also evidence that the active component contains biologically active substances possibly leucoanthyanin-like compounds, chemically distinct from the known kinins.

The culture medium, successfully used by White¹³⁵ (1934) for the continuous culture of excised tomato roots,

contained a balanced solution of inorganic salts, sucrose and 0.01% of yeast extract. The extract was essential for continued root growth. Robbins and Bartley (1937)⁹⁷ and White¹³⁷ (1937) showed that the yeast extract could be partially replaced by crystalline thiamine hydrochloride. Robbins and Schmidt (1939)^{98,99,100} then reported that a mixture of thiamine and pyridoxine would permit the indefinite culture of excised tomato roots and therefore concluded that the growth promoting activity of yeast extract was due to its content of these vitamins. Further addition of other B vitamins failed to enhance the growth of tomato roots of the strain used by Robbins and Schmidt. This was confirmed by Bonner (1943). However, Bonner and Devirian (1939)¹⁶ and Robbins (1941)¹⁰¹ showed that the growth of other tomato root clones was further enhanced by addition of niacin (nicotinic acid). White (1937) found that, with a clone of tomato roots, the yeast extract could be fully replaced by thiamine plus a mixture of nine amino acids and later reported that the nine amino acids could be fully replaced by an appropriate concentration of glycine^{136,137,139}.

Yeast extract was reported to be an extremely good source of the B-vitamins (B₁₂ excepted). These vitamins are nicotinic acid, pantothenic acid, thiamine, p-amino-benzoic acid, riboflavin, folic acid, pyridoxine and biotin.

In the experiments reported in this investigation, it was found that the 10% coconut milk in the PRL-4-CCM medium could be replaced by 0.5% yeast extract. It would seem

possible that the high level of B-vitamins may play an important role in licorice cell growth and division. The amount of B-vitamin added from yeast extract to the medium which supported growth was calculated to be about 100-1500 times that contained in PRL-4-C medium and about 50 times the concentration contained in White's medium. Due to the complexity of the yeast extract, one has to be very careful in drawing any conclusion about the effective factors present, but it is clear that the high level of B-vitamin as contained in 0.5% yeast extract did not show any inhibition to the cell growth. Because the PRL-4-YM supported satisfactory cell growth, the possibility of formulating a new defined medium for licorice cells is suggested.

Licorice cells vary in growth rate and colour. The cells required a considerable time to adopt to the culture medium; once the cells have adopted to the new medium, the cell growth rate and yield increases with the number of culture transfers.

During the experiments with cells grown in the media with the added factors, ascorbic acid and l-gulonolactone, a number of flasks of cells and growth medium changed to dark brown colour. This colour response appear to be indication of culture failure. On the other hand, in other light coloured cultures, both the ascorbic acid and the gulonolactone media supported an much better growth than the cells grown in the medium without added growth factors.

II. The products from licorice single cell suspension culture

The products readily detected in licorice single cell suspension culture included a volatile apple aroma, a polysaccharide pectin-like material, steroids and triterpenoids and reducing substances all of which are secreted by the cells into the medium during growth.

The volatile apple aroma was composed of in part by ethanol and some esters which were formed by cells under apparent anaerobic condition. Most of the components of this volatile apple aroma have not been identified. The mechanism of ethanol formation in these cells is unknown.

The monosaccharides found in the pectin-like material hydrolysate include glucose, fructose, galactose, arabinose, xylose, galacturonic acid and glucuronic acid. The yield of the pectin-like material reached a maximum of 1.1 mg/ml one month after inoculation. These results are similar to the results reported in a very recent paper of Olson et al. 88. The latter investigators found the components of the polysaccharide in tobacco suspension culture medium to be arabinose, xylose, glucose, galactose, mannose and galacturonic acid. The tobacco cell cultures have a maximum yield of the polysaccharide of 1.2 mg/ml. The time needed to reach the maximum polysaccharide yield varied with the incubation temperature.

Large amounts of pectic substances are known to be involved in holding plant cells together. Thus, the pectic

substances present in the media from cell suspensions suggested that these are likely derived from the intercellular cements or a precursor thereof.

In 1966, Stoddart, Barrett and Northcote reported the analyses of the pectins of sycamore callus tissue,¹¹⁵ they found that the pectic substances were more heterogeneous and complex than those of apple fruit. The polysaccharide composition of the cell walls of sycamore cambium and sycamore callus tissues were found to be directly comparable; both contained galactose, glucose, mannose, arabinose, xylose, rhamnose and galacturonic acid. They supposed that the differences observed in the pectins from callus, cambium and fruit appeared not to be that of species difference but a characteristic of the nature of the growth and growth conditions of the cells and therefore related to the problems of the control and the mechanism of plant cell growth and differentiation.

A considerable number of the components of the licorice root have been isolated and identified^{34,35,103,105}. Among these components steroids and triterpenoids occur in considerable amount. It is not surprising therefore that steroids and triterpenoids should occur in licorice cell culture suspension. However, the assay method applied in this investigation did not indicate the presence of glycyrrhizinic acid. The identification of the steroids or triterpenoids present in the cell extract is important not only because of the commercial interest but also because of the interest in the genetic relationship between the intact plant tissue and

single cell suspension culture.

Sorbitol and fructose were found to be the two major sugars present in the medium of licorice cells after 12 days incubation. Burstrom demonstrated that excised wheat roots hydrolyzed sucrose in liquid media,¹⁸ Tulecke, et al.¹²¹ and Ball⁷ gave similar illustrations in their papers. Wang and Staba analyzed that in carboy I of the dual-carboy system of spearmint, sucrose was completely hydrolyzed to dextrose and fructose by the third day of culture, both dextrose and fructose were absent in the culture medium after 8 days¹³⁰.

This investigation is the first record of the occurrence of sorbitol in plant cell suspension cultures. The role that sorbitol plays in carbohydrate metabolism and the synthesis of and metabolic pathways of sorbitol present a very interesting research subject.

Acyclic polyhydric alcohols such as sorbitol and mannitol occur in many species of plant, although in the majority of cases neither quantitative data on the amounts present nor information on their metabolic role are available.

Donen (1939)²⁷ suggested that sorbitol is formed in plum fruit when the concentration of hexoses reaches a critical level, whereas Kid, West, Griffith and Potter (1938)⁶⁷ found that much of the sorbitol in pears was replaced by hexulose (probably fructose) as the fruit ripens. Sorbitol could also be utilized for polysaccharide synthesis in leaves of Rosaceae and Gramineae (Barker 1955⁸, Altermatt and Neish 1956²) but nothing was known of the extent of this conversion

under natural conditions. Andrew and Hough (1958)⁵ in the experiments with $^{14}\text{CO}_2$, originally designed to yield information on the biosynthesis of leaf polysaccharides, indicated that the sorbitol in plum leaves has an important role in the metabolism of these leaves. The incorporation of ^{14}C into sorbitol was considerably greater than into starch. Further investigation of sorbitol metabolism in plum leaves by Anderson, Andrew and Hough (1961, 1962)^{3,4} indicated that despite the large amounts of sorbitol present in the leaves, sorbitol was rapidly equilibrated with the primary products of photosynthesis. When D-(1 ^{14}C) and D-(6 ^{14}C)-glucose were metabolized by plum leaves, about 40% of the ^{14}C detected in the leaves after 5 hours was in sorbitol. Labelling patterns showed that the conversion of D-glucose into sorbitol probably occurs without rupture of the carbon chain. To a small extent, D-glucuronic acid appeared to be converted into sorbitol when fed to plum leaves, only about 20% of the activity of the acid was detected in the leaves after 5 hours and 80% of this was present in sorbitol.

Whetter and Tapper (1966)¹³² reported sorbitol in germinating apple seed, and in all organs of the developing seedling including the root. Photosynthesizing cotyledons appeared to be a site of synthesis of sorbitol. Sorbitol was considered to be a reserve form of carbohydrates in seeds and cotyledons of apple. When spur shoots of the apple tree with or without fruit were enclosed in plastic bags and exposed to $^{14}\text{CO}_2$, Hansen (1967)⁵⁰ found approximately 90% of the ^{14}C

taken up by the leaves was transferred to nearby fruit within 5 days. Immediately following application, the leaves contained 58-80% of the added ^{14}C as sorbitol, 7-9% as sucrose and 1-4% in the form of glucose. Within five days, the amount of sorbitol ^{14}C was greatly reduced while the glucose ^{14}C increased. The sorbitol ^{14}C and total sorbitol and glucose were highest in leaves from shoots without fruit. Williams, Martin and Stahly (1967)¹⁴² applied uniformly ^{14}C labelled sorbitol or sucrose to the underside of the leaves on young vigorously growing Delicious apple trees in the green house and studied the rates of absorption and translocation. Sorbitol ^{14}C moved readily in the tree and ^{14}C could be found in sugars, amino acids, and other organic compounds in plant. Phlorizin became radioactive soon after the application of the labelled sorbitol. Sorbitol was absorbed and translocated at a faster rate than sucrose and continued to move into apple fruits after watercore began to develop. Wieneke (1969)¹⁴⁰ also reported that after assimilation of $^{14}\text{CO}_2$ or absorption of sorbitol ^{14}C from the leaves, sorbitol and also sucrose were transported in the bark to the apple fruit. After one minute, sorbitol was the most labelled sugar.

The exact role of sorbitol in carbohydrate metabolism is still in confusion, but it is clear that sorbitol plays an important role since it is easily transported and incorporated into glucose, fructose, polysaccharides and cell material.

The enzymes, aldose reductase $(\text{R-CHO} + \text{TPNH} + \text{H}^+ \rightleftharpoons$

$\text{RCH}_2\text{OH} + \text{TPN}$) and sorbitol dehydrogenase ($\text{sorbitol} + \text{DPN} \rightleftharpoons \text{fructose} + \text{DPNH} + \text{H}^+$) have been demonstrated in animals and bacteria, but not in plant tissues. However, it is believed the two enzymes are also present in plant tissue due to the incorporation of sorbitol into glucose, fructose and other carbohydrates.

SUMMARY

The cells of the Licorice plant, Glycyrrhiza glabra, were cultured as a "single cell" suspension. Their growth behaviour, yield and metabolic products were studied.

The suspension cultures of the licorice plant were established from the friable calluses obtained from the radicle, cotyledon and hypotyl of the germinated seeds. The single cells, regardless of their origin showed little difference in cell size and morphology. After an apparent adjustment to the medium, the cells required 11-13 days of incubation to reach the maximum cell yield of 1.2 gm/100 ml medium, dry weight.

During the growth period, the pH of the growth medium decreased from pH 5.6 to pH 4.7 in first few days and then increased to about pH 6.0. A level of 10% coconut milk in PRL-4-CM medium was found to support the best cell growth; the lower the coconut milk level, the longer the growth period required to reach the maximum cell yield. It was also found that 0.5% yeast extract could be used to replace the coconut milk in the PRL-4-CCM medium.

The metabolites detected and examined in the licorice single cell suspension culture included a volatile apple aroma, a polysaccharide pectin-like material, and some steroids and triterpenoids.

The analyses of the licorice cell volatile apple aroma found under anaerobic conditions indicated the presence

of ethanol and some related esters.

The monosaccharides found in the pectin-like polysaccharide hydrolysate were glucose, fructose, galactose, arabinose, xylose, galacturonic acid and glucuronic acid. The pectin-like material in the cell preparations reached a maximum yield of 1.1 mg/ml after one month of growth.

Glycyrrhizinic acid, the common licorice constituent found in the root, could not be detected in the suspension cultures. However, several other related compounds which gave typical steroid and triterpenoid reactions were found.

Sorbitol and fructose were found to be the two major sugars which accumulated in free form in the licorice cell medium.

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