GROWTH OF TISSUES OF HIGHER PLANTS IN CONTINUOUS LIQUID CULTURE AND THEIR USE IN A NUTRITIONAL EXPERIMENT

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FOREWORD

This research was carried out at Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York 10701, under Contract No. AF 33(616)-1355, administered by the Biomedical Laboratory, Aerospace Medical Research Laboratories, Aerospace Medical Division, Wright-Patterson Air Force Base, Ohio. Walter Tulecke, PhD, Associate Plant Physiologist, was the principal investigator for Boyce Thompson Institute. Alton E. Prince, PhD, Chief, Biospecialties Branch, Physiology Division, Biomedical Laboratory, was contract monitor. This work was begun on 1 January 1964 and completed on 30 April 1965.

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This technical report has been reviewed and is approved.

WAYNE H. McCANDLESS
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ABSTRACT

The purpose of this work was twofold: 1) to devise a continuous culture system for higher plant cells and 2) to produce a sufficient amount of plant tissue culture for a nutritional experiment with weanling mice. The overall purpose of this work is to provide information for the evaluation of plants as food sources in long term space missions and as possible future sources of supplementary protein. Rose tissue was the most suitable for growth in liquid culture and approximately 40 pounds (20 kg) fresh weight of sterile tissue was produced from seven cultures which were harvested periodically over a period of 222 days. The cultures were of 8 liters volume and this amount was maintained by replacing the medium which was harvested. The average yield was 112 g/l fresh weight (4.6 g/l dry weight)/day. Approximately 10 pounds (5.0 kg) of contaminated rose tissue was also produced. When incorporated into a test diet for weanling mice, the sterile rose tissue was a better food supplement than contaminated rose. A bacterial contaminant which grew well with the rose tissue and did not appreciably alter the growth rate was isolated and identified as Achromobacter liquefaciens. Since this was a reisolated type species for the genus, it was deposited in the American Type Culture Collection (ATCC No. 15716). Protein determinations on the rose tissue showed a high protein level for tissue grown in continuous culture as compared to tissue grown in flasks.

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Section I

INTRODUCTION

This work was undertaken for two primary reasons. First, it was desirable to devise and construct a system for the continuous culture of plant cells which would permit the repeated harvest of tissue over extended periods of time. This system, here named the phytostat, incorporated principles of the chemostat (ref 37, 38) and was used for plant cells. The second reason was to determine whether the plant tissue could serve as a food supplement for weanling mice. The harvested tissue was to be added to a standard protein test diet as part of a nutritional experiment. The selection of tissues for this work was based on the criteria of growth rate, protein content, edibility, friability of the tissue, and ability to grow in liquid media. Tissue cultures of rose, corn, tomato, and Ginkgo were tested for possible use because each filled two or more of these criteria. The requirement for sufficient quantities of tissue to feed 20 mice for three weeks precluded extensive work with more than one tissue. Difficulties with contamination and in devising a suitable culture apparatus dictated that the work be limited to the best growing tissue. The rose tissue satisfied all the above criteria, was the fastest growing tissue, and was therefore selected as the main subject for this work. It was grown in both sterile and contaminated culture; harvests of tissue were dried, incorporated into food pellets, and then used for the nutritional experiment.

Some determinations were made on the protein content of tissues as a function of age. The purpose of these studies was to determine the best conditions for the highest yield of protein. Other experiments were conducted with a bacterial contaminant which was found to grow in association with the rose cells. The organism was subsequently isolated and identified as Achromobacter liquefaciens. Its compatible growth with the rose cells was confirmed by a second phytostat culture.

Section II

THE SELECTION OF TISSUES

The best tissue for these studies would be one with a rapid growth rate, a loose friable texture, a high protein content of good nutritional quality derived from an edible plant part and preferably photosynthetic. Needless to say, this ideal has not been achieved. However, the above characteristics were kept in mind in attempting to select the best tissue for these studies.

At various stages in this work, the tissues of tomato stem (Lycopersicon esculentum Mill.), rhubarb (Rheum rhaponticum L.), potato tuber (Solanum tuberosum L.), carrot root (Daucus carota L.), Ginkgo pollen (Ginkgo biloba L.), corn endosperm (Zea mays L.), and rose stem (Rosa sp.) were tested for their suitability for production in large amounts. Corn

and tomato tissues were favored for reasons of edibility, but they proved to be difficult to grow in liquid media. Carrot and rhubarb grew in large aggregates but would not remain friable enough for continuous culture; potato tissue did not grow well in liquid. The Ginkgo tissue grew well but not as well as the rose tissue.

The rose tissue was selected for the phytostat culture system because it possessed several advantages: it grew well on a defined medium, was easily suspended in liquid, was homogeneous and undifferentiated, was not known to be toxic and could be considered a somewhat unusual edible plant material. Moreover, its protein content was adequate for the proposed feeding experiments, although it was not as high as some green tissue cultures such as tomato. In addition, rose tissues have been useful in other investigations on growth rate (ref 27), chlorophyll (ref 22), terpenoid (ref 63), ubiquinone and plastoquinone (ref 49), and organic acid, amino acid, and sugar content (ref 61).

The rose tissue was composed of parenchyma cells with prominent nuclei and few inclusions. The cells showed very active cytoplasmic streaming, contained many protoplasmic strands, and were highly vacuolated. The tissue was white to yellow in appearance and readily fragmented into small clusters of cells. No differentiation was observed in this tissue which was derived from stem callus. The process of nuclear and cell division in the rose tissue appears to be normal for plant cells. The cells are uninucleate and form clusters of a few to several hundred cells. During growth in the continuous cultures, some cells separate from the clusters and form new cell masses. Cultures from tomato stem (ref 45) were light green in color, loose and friable in texture and rapidly growing on agar media. Microscopic examination showed conspicuous chloroplasts. No differentiation was observed. The corn endosperm tissue (ref 46) and Ginkgo (ref 50) tissues were previously described.

Section III

MEDIA

Chemically defined media were used for all experiments. The rose tissue was grown on the medium given in Table I; this is the same medium used for a tissue derived from the female gametophyte of Ginkgo (ref 53). The male Ginkgo (ref 50, 57), corn (ref 46), and tomato (ref 51) tissues were grown on their respective media (see Tables II and III). The media for corn and Ginkgo were very similar and differed primarily in the single amino acid required as an organic nitrogen source.

The large quantity of medium required for rose cultures was prepared by passage through a sterilizing millipore filter. This method greatly facilitated the setting up of phytostat cultures by allowing the sterilization of 20 liters of medium at one time. This medium was kept in reservoirs and was used as needed.

TABLE I

COMPOSITION OF NUTRIENT MEDIUM FOR ROSE TISSUE

	Amount
Inorganic Salts	
Potassium chloride	900.0 mg/l
Potassium nitrate	80.0 "
Magnesium sulfate·$7H_2O$	760.0 "
Sodium nitrate	1800.0 "
Calcium nitrate·$4H_2O$	280.0 "
Sodium dihydrogen phosphate·H_2O	300.0 "
Sodium sulfate	200.0 "
Trace Elements	
Potassium iodide	0.5 "
Boric acid	0.2 "
Manganese sulfate·H_2O	0.8 "
Zinc sulfate·$7H_2O$	0.5 "
Copper sulfate·H_2O	0.02 "
Molybdic acid	0.01 "
Cobalt chloride·$6H_2O$	0.01 "
Vitamins	
Calcium pantothenate	1.0 mg
Pyridoxine·HCl	0.5 "
Thiamin·HCl	0.5 "
Nicotinic acid	1.0 "
Other Components	
Iron citrate	5.0 "
Sorbitol	100.0 "
Kinetin	0.5 "
Adenine	10.0 "
Naphthaleneacetic acid	1.0 "
Glutamine *	200.0 "
m-Inositol*	100.0 "
Cytidylic acid*	100.0 "
Guanylic acid*	100.0 "
Sucrose	20.0 g
Agar	8.0 "
pH	5.5

* Sterilized through a Seitz filter (ref 53).

TABLE II

COMPOSITION OF NUTRIENT MEDIUM FOR TOMATO TISSUE *

	mg/l
Ammonium nitrate	1,650
Potassium nitrate	1,900
Magnesium sulfate·$7H_2O$	370
Calcium chloride·$2H_2O$	440
Potassium dihydrogen phosphate·H_2O	170
Sodium EDTA	37.3
Ferrous sulfate·$7H_2O$	27.8
Boric acid	6.2
Manganese sulfate·$4H_2O$	22.3
Zinc sulfate·$4H_2O$	8.6
Potassium iodide	0.83
Sodium molybdate·$2H_2O$	0.25
Copper sulfate·$5H_2O$	0.025
Cobalt chloride·$6H_2O$	0.025
Nicotinic acid	0.5
Pyridoxine·HCl	0.5
Thiamin·HCl	0.1
m-Inositol	100.0
Glycine	2.0
Sucrose	30,000.0
Kinetin	0.32
2,4-Dichlorophenoxyacetic acid	0.05
pH	5.7

* cf. Murashige and Skoog, 1962 (ref 35).

TABLE III

COMPOSITION OF NUTRIENT MEDIA FOR GINKGO * AND CORN † TISSUES

	mg/l	
	Ginkgo	Corn
Magnesium sulfate·$7H_2O$	730.0	530.0
Calcium nitrate·$4H_2O$	280.0	325.0
Sodium sulfate	200.0	200.0
Potassium nitrate	80.0	80.0
Potassium chloride	65.0	65.0
Sodium dihydrogen phosphate·H_2O	165.0	165.0
Sodium molybdate·$2H_2O$	0.025	0.025
Copper sulfate·$5H_2O$	0.025	0.025
Manganese sulfate·$4H_2O$	3.000	3.000
Zinc sulfate·$7H_2O$	0.500	0.500
Boric acid	0.100	0.500
Ferric citrate	2.000	10.000
Thiamin·HCl	0.25	0.25
Pyridoxine·HCl	0.25	0.25
Nicotinic acid	1.25	1.25
Calcium pantothenate	1.00	0.25
Glycine	7.50	7.50
Naphthaleneacetic acid	0.10	—
Sucrose	20,000.0	20,000.0
l-Arginine·HCl	210.0	—
l-Asparagine	—	2,000.0
pH	6.0	6.6

* cf. Tulecke, 1960 (ref 50).

† cf. Straus, 1960 (ref 46).

The stock cultures were maintained in test tubes on their respective media. The temperature was 23-25° C and approximately 250 f c of light was provided by cool white fluorescent lamps in a 12 hour photoperiod. The tissues in Erlenmeyer flasks and dispensing bottles were agitated on a gyro-rotatory shaker under essentially the same conditions. The phytostat cultures were maintained in a laboratory where they could be easily attended. Diffuse light and a normal photoperiod prevailed; room temperature was maintained at about 23° C.

An attempt was made to grow quantities of both corn and tomato tissues by using solid media in fernbach flasks. The tissues were suspended in 10 ml of liquid medium and broken up into small clusters of cells. This inoculum was poured into a fernbach flask and distributed over the surface of the appropriate medium for corn, rose, or tomato. The tissues grew very well but the yield per liter of medium per unit of time was below that obtained for rose tissue in the phytostat. For example, 1.0 gram fresh weight of corn tissue from a test tube produced 9 grams after 3 weeks growth in a fernbach flask; for tomato, 1.0 gram yielded 29 grams in 3 weeks; 1.0 gram of rose tissue gave 28 grams after three weeks. However, rose tissue in the phytostat produced an average of 100 grams per liter per day as a sustained yield. Moreover, the cells from the liquid cultures were younger. The overriding disadvantage of the fernbach cultures was that they yielded older cells at the time of harvest. In addition, the protein content of the older tissues was lower than that of younger tissue (see Section VI).

Section IV

CONTINUOUS CULTURE SYSTEM: THE PHYTOSTAT

Plant cells have been grown in liquid media in Erlenmeyer flasks (ref 36), special Florence flasks or culture tubes (ref 30), roller tubes (ref 44), rotating flasks (ref 27), or carboys (ref 54, 55). However, these systems have not been used for periodic harvests and additions of medium for continuous cultures over an extended period of time. The development of such a continuous culture system, here named the phytostat, is described. The system has been in operation successfully for periods of over 2 months. As much as 4.5 kg (10 pounds) fresh weight of tissue per culture per month have been harvested.

The criteria for a useful continuous culture system would include simple and effective operation, a good yield of cells, safeguards against contamination, some adaptability to varying needs, and control of physical and nutritional factors. Most of these requirements have been met in the system described here.

The phytostat is composed of relatively simple components including some elements of the more expensive and sophisticated systems which are available commercially. The latter are used for microbial cell culture and are variously described as microfermentors, biogens, and fermacells. Other special apparatus has been used for specific purposes, such as the chemostat (ref 37), continuous flow systems for animal cells (ref 25), or dialysis cultures (ref 18, 20), each of which have special merit for their particular use.

The phytostat culture apparatus is shown in operation in Figure 1 as it was used for the production of rose tissue. Figure 2 shows the details of the system from the test tube culture to the 8-liter culture bottle.

The plant tissue cultures were maintained in test tubes by transferring at 2-4 week intervals. The tissue from tubes (Fig. 2 A) was inoculated into 100 ml of liquid medium in a 250 ml Erlenmeyer flask (Fig. 2 B) which was placed on a rotatory shaker. Adequate inoculum was needed to condition the medium and to minimize the lag period of growth. After one to two weeks the tissue and medium from one flask was transferred (by pouring or by use of a large bore needle and syringe) into a four-liter dispensing bottle containing 900 ml of fresh medium. The dispensing bottle (Fig. 2 C) was plugged at the bottom with a cotton tampon so that tissue would not collect there or be damaged by the motions of shaking. At the end of two weeks on the shaker, the contents of the dispensing flask were transferred to the culture bottle (Fig. 2 D).

The culture bottle was prepared with tubes for medium addition from the reservoir (Fig. 2 N), a harvesting port (Fig. 2 O), exhaust tubes (Fig. 2 K) and an aeration tube of Teflon. The tube ends were fitted (when necessary) with glass ball and socket joints. Medium additions occurred only via the reservoir (Fig. 2 N), which was connected to the culture bottle by a ball and socket joint.

In transferring the tissue from the dispensing bottle to the culture bottle, the ball joint connections (previously autoclaved and wrapped with aluminum foil) were joined. This was done by making the connection in the flame of a Meeker burner. Two spring clamps made the joint secure. After allowing the connection to cool, the dispensing bottle was agitated to suspend the tissue, the tampon plug was pulled out by means of an attached thread leading outside the flask, and the tissue was transferred to the culture bottle by gravity feed. Hemostats and hose clamps were used to close the hosing (such as between C and D in Fig. 2). When the joint was disconnected, the portion attached to the culture bottle was rinsed with 80% ethanol, flamed, and then capped with aluminum foil.

The air supply for aeration and agitation of the cultures was sterilized by a series of filters (Fig. 1). Compressed air was passed through a pressure reduction gauge which was equipped with a water and oil trap and the gauge was regulated to give about 0.7 kg per sq cm (10 pounds) pressure; passed vertically through Perlite to rid it of any residual oil from the compressor; and passed through an Air Hyperfilter (Fermentation Design). Later, a second filter (not shown in Fig. 1) was incorporated into the system. This second filter consisted of a 5.1 X 91.4 cm (2 in X 36 in) air sterilizing filter (Biochemical Processes, Inc.) packed with discs of furfural-resin bonded glass fibers. This unit was superior to the first filter and greatly aided the maintenance of a sterile air supply. It was engineered to handle quantities of air in excess of the flow rate used for the phytostat cultures, i.e. 12 liters/minute/culture. The air filters were autoclaved periodically prior to use.

FIG. 1. PHYTOSTAT CULTURES OF ROSE TISSUE.

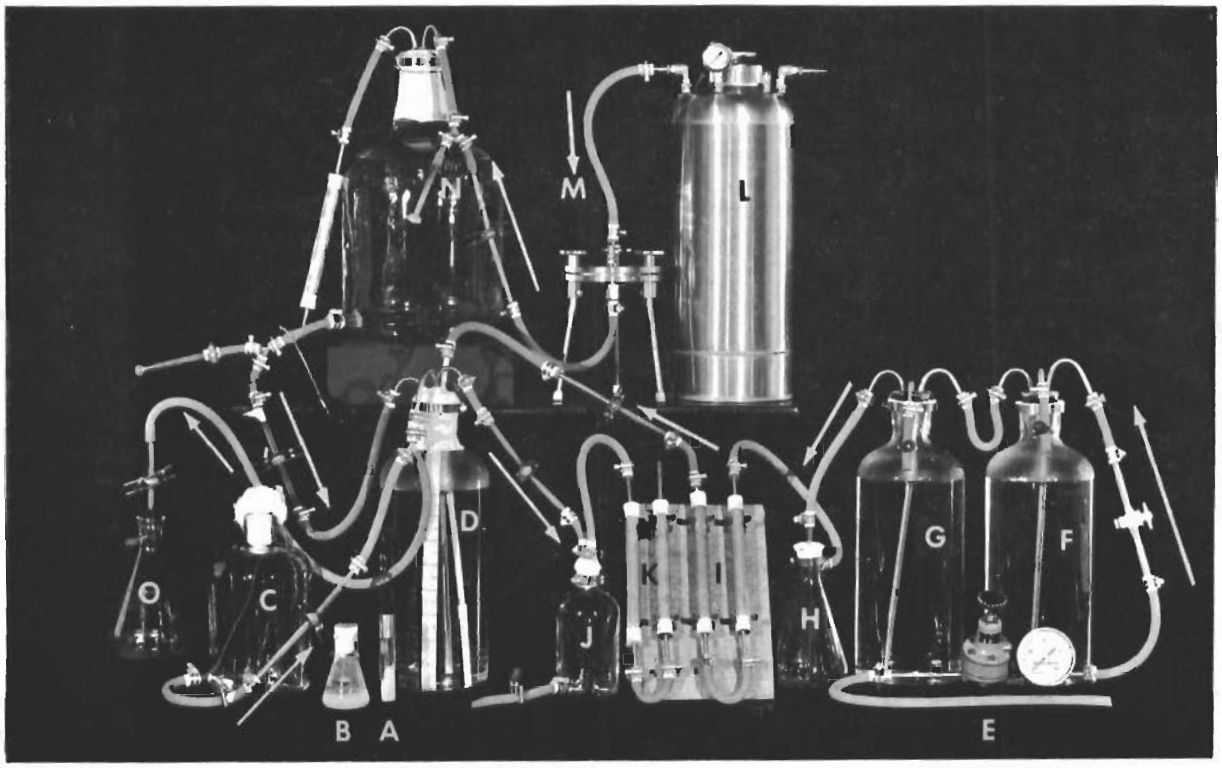

FIG. 2. DETAILS OF PHYTOSTAT CULTURE APPARATUS AND SCALE-UP. *

* A. Test tube culture of tissue on agar medium; B. Erlenmeyer flask (250 ml.) with 100 ml. of liquid medium and tissue; C. Dispensing bottle, used for inoculation of D; D. Phytostat culture bottle; E. Air inlet with reduction valve; F. and G. Air humidifiers; H. Safety trap; I. Air sterilization filters; J. Exhaust trap; K. Exhaust filter; L. Pressure tank for medium; M. Millipore filter; N. Reservoir of medium; O. Harvest flask.

The air stream from the filters was reduced by a valve (Fig. 2 E) and passed through two humidifying bottles (Fig. 2 F and G) which contain a one per cent copper sulfate solution. The latter solutions were replenished as evaporation occurred. A trap (Fig. 2 H) served to prevent accidental wetting of fiber glass filters (Fig. 2 I). Teflon was used for the aeration tube to prevent the adherence of cells; no sparger was used because large bubbles were desired to agitate the culture.

The reservoir (Fig. 2 N) and filter (Fig. 2 M) were sterilized separately and then connected by a ball joint and clamps. The prepared medium in the tank (Fig. 2 L) was pressure-filtered through a sterilizing millipore membrane (in the filter) and into the reservoir. The reservoir in turn was connected to the culture bottle (Fig. 2 D) by a ball joint. The latter was flamed before and after connecting, and then wrapped in aluminum foil and flamed again. The medium from the reservoir was fed to the culture by gravity. When this was done, the air supply was cut off with hemostats (between D and I, and G and H in Fig. 2). Additional joints (wrapped in aluminum foil until used) were connected as inlets at the top or as outlets from the dispensing end of the reservoir (see Fig. 2); these made it possible to replenish medium in the reservoir many times or use the reservoir for several culture bottles at the same time.

Harvests of the culture were obtained by using a side-arm Erlenmeyer (Fig. 2 O) connected to stainless steel tubing which runs to the bottom of the culture vessel (Fig. 2 D). While air was agitating the culture, a vacuum was applied to the side-arm Erlenmeyer, the clamps closing this harvest tube were released, and a measured amount was removed. The harvest tube was clamped in two places, one near the ball joint and the other near the bottle, then the ball joint connection was broken. The end of the harvest tube was washed three times with 80 per cent ethanol and capped with aluminum foil. This tube was used for daily harvests and the flow was in an outward direction only. For some studies, it may be important to take into account the fact that some tissue and medium remain in the harvest tube between harvests. The harvested tissues were filtered, weighed, and then frozen. A VirTis Unitrap freeze-dry unit was used to dehydrate the tissues from the frozen state.

The trap on the exhaust line (Fig. 2 J) collected some moisture and this was removed as required by opening the dispensing tube. The trap served to keep the exhaust filters free of liquid. The exhaust filter on the reservoir (Fig. 2 N) admitted sterile air as medium was withdrawn.

The general procedure adopted for daily use with the phytostat cultures was as follows: 1) The culture was harvested by appropriate methods; 2) the harvest was checked for color, odor, pH change, and then observed microscopically for condition of cells and possible contamination; 3) medium was added to replenish the harvested volumes; 4) the harvested volume was placed in a graduated cylinder and a settled cell volume was taken after five minutes; 5) the tissue was harvested by using Vaculine (Johnson & Johnson) milk filter discs in a Büchner funnel, and a fresh weight of tissue was obtained; 6) the tissue was freeze-dried, a dry weight obtained, and the tissue stored at -30° C.

As a precaution, the culture bottle was aerated for 24 to 48 hours before adding the inoculum; this assured that the system was clean. For the same reason, the reservoirs of media were allowed to stand for two days prior to use and were inspected daily for sterility before medium was added to the culture.

Preliminary experiments on the growth of tissues in an aerated liquid culture were run by using the techniques of the larger bottles, but adapting them to 1-liter graduated cylinders. The latter permitted several replicates to be run from the same aeration system and it also facilitated comparisons between media or between tissues. Settled cell volumes could be taken as daily readings on the cylinders, providing that the tissues settled out within a reasonable length of time. These graduated cylinder cultures were useful for evaluating tissues as to their suitability for growth in aerated liquid cultures; this system could also be adapted for agitation with various gas mixtures, such as increased CO_2.

Antifoam Q (Dow Corning) was used to reduce foaming without affecting growth. However, some difficulty was experienced in sterilizing this viscous substance and it was used only when absolutely necessary. In the case of the rose tissue, extensive foaming occurred only when the culture was contaminated.

The size of the inoculum was somewhat variable from one phytostat culture to another. The average inoculum was 25-60 g/l fresh weight, 1-2 g/l dry weight. In addition, the inoculation was sometimes done by starting with a 1:1 dilution, i.e., one liter from the dispensing flask and one liter of fresh medium and then additional medium was added in liter amounts over 4-10 days to bring the culture to 8 liters volume. In a few instances, the dilutions were as much as 1:7.

Approximately one week was needed from the time of inoculation to the first harvest. Thereafter, depending on the growth rate, the tissue was harvested usually every day or every second day. It was found that the growth rate could be controlled by the frequency and amount of the harvests.

The results from nine phytostat cultures of rose tissue, two contaminated and seven sterile, are shown in Table IV. The contaminated cultures ran for a total of 62 days; 29 liters of medium containing 4,245 grams fresh weight (132 g dry weight) of tissue were harvested. The average yield per liter was 149 g fresh weight and 4.5 g dry weight. These yields were as good as those obtained from the sterile cultures. In the latter, the total days of culture were 222; 163 liters of medium containing 19,481 g fresh weight (734 g dry weight) of tissue were harvested. The average yield per liter was 112 g fresh weight and 4.6 g dry weight. The sterile rose tissue was the equivalent of approximately 43 pounds wet weight (20 kg) and one and one-half pounds dry weight (0.7 kg); the contaminated tissue represented about 10 pounds fresh weight (4.5 kg) and one-third pound dry weight (0.15 kg).

The contamination rate was rather high in the early phase of this work. Nine phytostat cultures of rose tissue (No. 1, 2, 4, 6, 8, 9, 10, 11, and 18) were contaminated within the first week and they yielded only small amounts of tissue. Phytostat cultures No. 18 and 20 were experimental runs

TABLE IV

PHYTOSTAT CULTURES OF ROSE TISSUE

Culture No.	Total days	Liters harvested	Total tissue		Average harvest	
			Fresh weight, g	Dry weight, g	Fresh weight, g/l	Dry weight, g/l
3 *	25	13	2,271	60	175	4.6
5 *	37	16	1,974	72	123	4.5
Total	62	29	4,245	132	298	9.1
Average	31	15	2,122	66	147	4.5
7 †	52	26	3,987	137	153	5.3
12	55	44	5,803	200	133	4.6
13	43	33	4,835	169	146	6.0
14	8	3	246	12	82	4.0
15	46	40	2,984	140	75	3.5
16	12	8	1,121	44	140	5.5
17	6	9	505	32	56	3.5
Total	222	163	19,481	734	785	32.4
Average	31	23	2,783	105	112	4.6

* Contaminated with Achromobacter liquefaciens.
† Sterile rose tissue.

and were not included in the tabulation (Table IV). The contaminated tissue used in the feeding experiments was derived from cultures No. 3 and 5 (contaminated with Achromobacter) plus the terminal harvests of other cultures which became contaminated.

The yield of rose tissue from a representative culture is shown in Table V. A total of 27 harvests was made over a 55 day culture period with an average yield of 133 g/l fresh weight (4.6 g/l dry weight). The original inoculum of one liter of suspended rose tissue was added to 7 liters of fresh medium to give an 8 liter system. At the 31st day the volume was reduced to 7 liters because of foaming and was maintained at this level until the 55th day when the entire culture was harvested. The pH remained stable at about 5.3. Some of the variability in fresh weight was due to the filtering system which consisted of a Büchner funnel, filter pad and applied vacuum. It was difficult to standardize this procedure.

The close correlation between the total fresh and dry weight of tissue harvested is shown in Fig. 3. Also noticeable are the changes in the slope of the lines at day 9, 16, 23, 30, 37, 42, and 51. Each change in the slope

TABLE V

YIELD OF ROSE TISSUE FROM A REPRESENTATIVE CULTURE (NO. 12)

Days	Time of harvest	Culture harvest, l	pH	Medium added, l	Yield, g/l	
					Fresh wt	Dry wt
0	0900	--	5.5	8	60 *	2.0 *
7	0900	1	5.6	1	139	3.4
8	0900	1	5.5	1	152	4.4
9	0830	2	5.5	1	334	7.8
12	1600	1	5.5	2	239	7.0
14	0900	1	5.5	1	175	6.5
15	0900	1	5.5	1	172	5.8
16	0830	1	5.3	1	173	6.5
20	0830	1	5.4	1	220	6.0
21	0830	1	5.3	1	207	5.5
22	0930	1	5.5	1	107	3.5
23	0900	2	5.3	2	333	12.0
26	0830	1	5.5	1	155	4.9
27	1000	1	5.3	1	105	4.0
29	0900	1	5.4	1	105	3.5
30	1130	2	5.3	1	176	6.7
33	0930	1	5.3	1	115	4.0
34	0900	1	5.4	1	113	4.3
35	0900	1	5.3	1	123	4.1
36	1330	1	5.3	1	111	5.5
37	1600	2	5.2	2	233	9.0
40	0830	1	5.3	1	116	4.9
41	0900	1	5.2	1	109	5.0
42	0930	5	5.3	5	589	22.5
49	0830	1	5.3	1	151	5.3
50	0900	1	5.6	1	135	5.4
51	0900	2	—	2	296	10.7
54	0930	1	5.0	1	98	3.8
55	0900	8	4.5	—	762	28.5
Total	—	44	—	43 **	5,803	200.5
Average	—	--	5.3	—	138	4.6

* These figures represent the estimated fresh and dry weight of the inoculum; they were calculated from settled cell volumes and are not included in the totals.

** This total is lower than the harvest total of 44 liter because at the 30th day 2,000 ml was harvested and only 1,000 ml of medium added; thereafter, the culture was run as a 7-liter system.

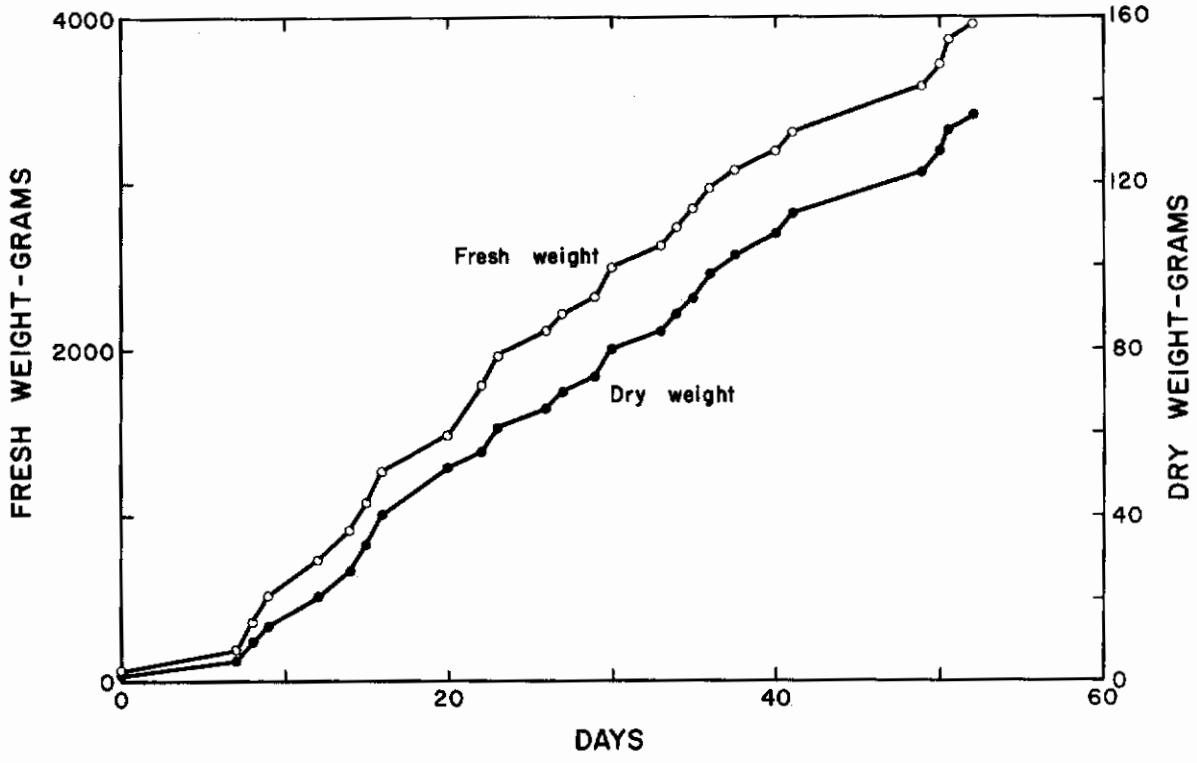

FIG. 3. TOTAL ROSE TISSUE HARVESTED FROM PHYTOSTAT NO. 12.

reflects a change in the conditions of the phytostat system. For example, the changes at day 9, 23, 30, 37, and 42 are the result of larger than normal harvests and medium additions (see Table V). The changes in slope at day 16 and 51 are the result of extended periods of growth without harvest or additions of medium. Essentially, these results demonstrate the sensitivity of the system to harvests and the replenishment of nutrients.

Several counts of cell populations were made using various techniques. Hemacytometric counts were not reproducible, nor were dilution series and aliquot counts of samples taken directly from the harvests. The best method was to sample the harvested tissue and medium with a large bore pipette (10 ml from 1000 ml). These aliquots were placed in centrifuge tubes, fixed in Randolph's chrom—acetic-formalin fixative, processed for staining in Heidenhain's hematoxylin, and dehydrated to alcohol. Aliquots were taken for a dilution series in alcohol and finally transferred to xylol and diluted in Permount mounting medium. One-tenth ml portions were placed on slides and total cell counts were made of all cell clusters. Some estimates of cell numbers were made because of the clumping of cells. The average yield of tissue from the phytostat culture appears to be greater than that reported for other tissue cultures grown by batch methods. Yields of tobacco cells were reported (ref 17)

as 60 mg/ml fresh weight, 3 mg/ml dry weight, 2 X 10^5 cells/ml, with a generation time of 1.5 days. The rose tissue gave average yields of 112 mg/ml fresh weight, 4.6 mg/ml dry weight, approximately 4 X 10^5 cells/ml and a generation time of about one day.

In general, the contaminants were bacterial and they were usually detrimental to the growth of the rose tissue. One exception to this was the organism which infected phytostat culture No. 3. Both the rose and the bacterium grew well together for more than two weeks. The bacterium was isolated and inoculated into culture No. 5. Again the tissue and the bacterium grew well together for more than 5 weeks. The organism was identified as Achromobacter liquefaciens and was shown to have the characteristics given in Table VI and Fig. 4 (which confirms the organism as peritrichous flagellate). Furthermore, the bacterium was found to be the type species of the genus and it had not, apparently, been reisolated and authenticated for many years since its isolation by Eisenberg (ref 14), although it was described in Bergey's Manual (ref 5). The culture was deposited with the American Type Culture Collection (#15716) (ref 56).

The growth of rose tissue in the presence of Achromobacter liquefaciens in phytostat No. 5 is shown in Fig. 5. This culture was purposefully contaminated with the organism isolated from culture No. 3. The dry weight at 12-21 days is higher than the dry weight from later harvests and this result is similar to that obtained from sterile rose culture No. 13 (see Fig. 6). The tissue fresh weight from early harvests is higher in both cultures (5 and 13). This could represent an actual increase in cell hydration in the early harvests, but data to substantiate this are not available. Also shown in Fig. 5 are the bacterial counts for the first 17 days. The figure demonstrates that for the period from the 10th to the 17th day the bacterial count, pH and dry weight of tissue were relatively stable. These results indicate that, at least for periods of up to one week, the rose tissue and Achromobacter liquefaciens grow in a symbiotic relationship in the phytostat culture.

Four large volume cultures of corn endosperm tissue were set up. One was successful for 68 days; one was maintained for 48 days without appreciable growth and two were lost to contamination. In the successful culture the tissue did not grow for the first five weeks. This was determined by taking settled cell volumes at frequent intervals. Over this period of time an apparently new form of tissue evolved and began to grow. In the succeeding weeks approximately 186 grams fresh weight (9 g dry weight) of tissue was harvested from 13 liters. The average yield was 14 g/l fresh weight (0.690 g dry weight).

Three large volume cultures of Ginkgo pollen tissue of a total of 76.1 volume yielded 3,208 g fresh weight (234 g dry weight) of tissue for an average of 42 g/l (3.0 g/l dry weight). The best culture ran for 76 days, yielded 2,029 g fresh weight (148 g dry weight) from 45 liters. The average yield was 45 g fresh weight (3.3 g dry) per liter.

One interesting characteristic of these tissues is the odor which they emit at different stages of their growth. The rose tissues in phytostat culture, for example, would fill the room with a slight but noticeably

TABLE VI

CHARACTERISTICS OF THE BACTERIUM, ACHROMOBACTER LIQUEFACIENS (EISENBERG) BERGEY

* Oxygen requirements	Aerobic
* Gelatin	Slow liquefaction, putrid odor
* Motility	Motile
* Temperature	No growth at 37° C
* Potato slant	Light yellow
† Gram stain	Negative
† Flagellation	Peritrichous (as shown by electron micrograph)
† Broth	Some sediment; slight pellicle and turbidity
† Indole	Negative
† Nitrate	No reduction to nitrite
† Litmus milk	Unchanged to slightly alkaline, no coagulation
Shape	Rod
Width	0.5 - 0.8μ
Length	2.0 - 3.0μ
Spores	Negative spore stain (also killed in 1/2 hr. boiling water)
Colonies	Circular, smooth, white to grey
Medium	Nutrient agar plus 0.5% yeast extract
Growth	Spreading, somewhat viscid
pH	Neutral optimum; will grow at 5.5
Citrate	Utilized
Acetomethylcarbinol	Positive
Sucrose	Slightly acid, no gas
Fructose	" " " "
Glucose	" " " "
Lactose	No acid or gas
Raffinose	" " " "
Mannitol	" " " "
Starch	No hydrolysis
Catalase	Positive
Blood agar	β-hemolysis
Trimethylene oxide reduction	Negative
Hydrogen sulfide (Kliger's)	Negative
Urea	Negative

* These tests are diagnostic for Achromobacter liquefaciens according to Eisenberg (ref 14).

† These tests are added characters of Bergey (ref 5).

Other tests indicate additional characters as determined in this laboratory.

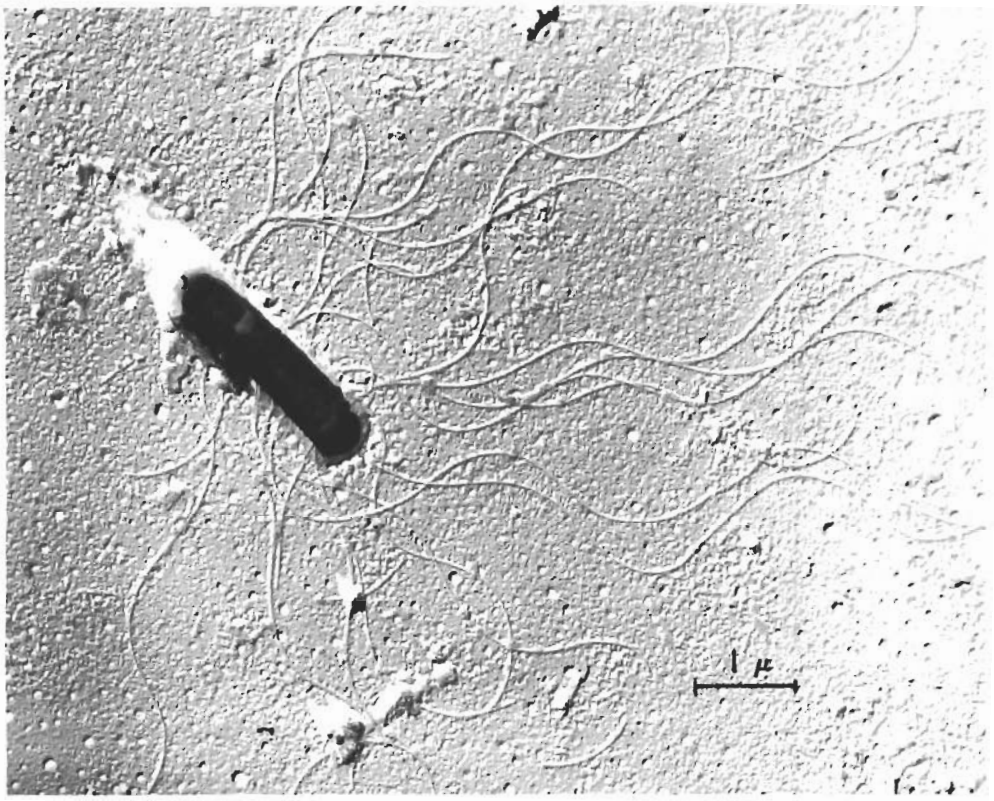

FIG. 4. ELECTRONMICROGRAPH OF ACHROMOBACTER LIQUEFACIENS (BY DR. STEFAN ORENSKI AND DR. EISHIRO SHIKATA)

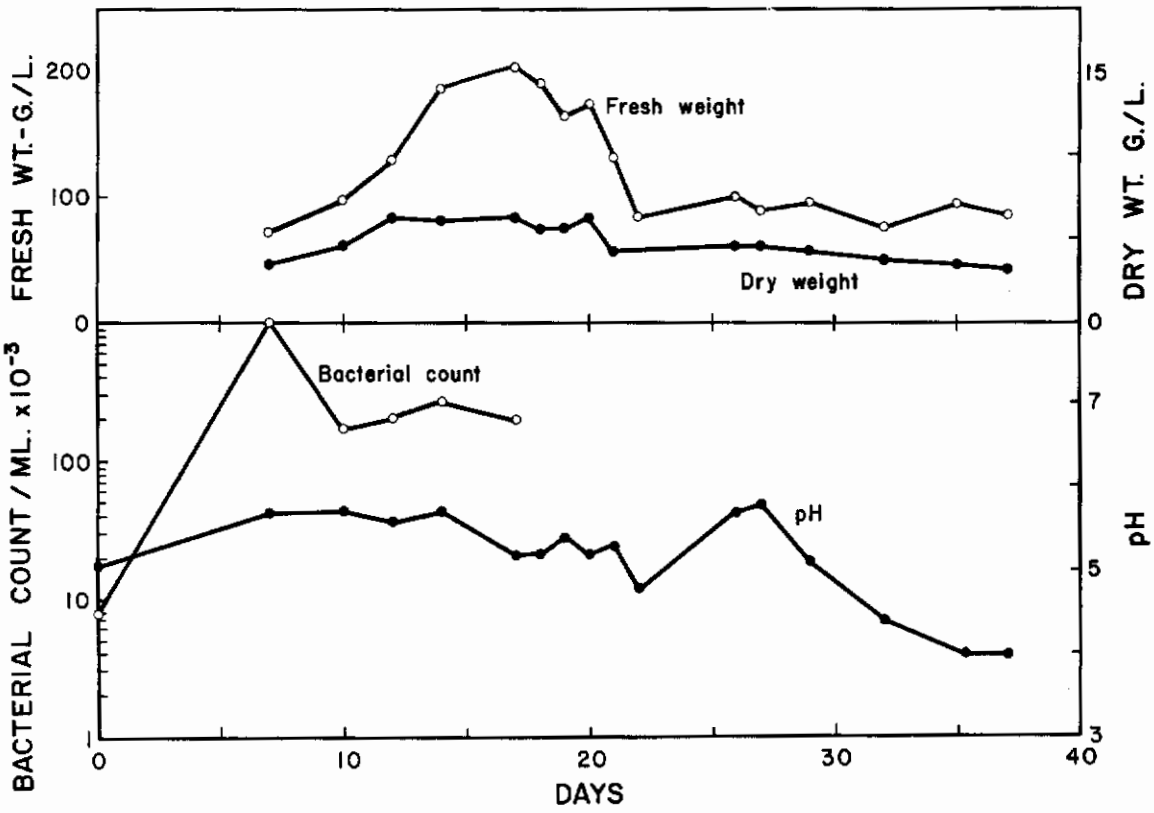

FIG. 5. GROWTH OF ROSE TISSUE IN PRESENCE OF ACHROMOBACTER LIQUEFACIENS IN CULTURE NO. 5.

sweet aroma. This was especially true if the room was closed up for a day with no ventilation. The Ginkgo tissue also had a faintly sweet odor. In fernbach cultures, the tomato tissue gave off a somewhat putrid odor as it aged, even though the tissue appeared very healthy. The corn tissue had the odor of boiled corn which was very obvious when the cotton plug was removed during the harvesting procedure. These observations suggest that there are some interesting volatile compounds produced by these tissues; some of these may be similar to naturally occurring components of the same plants.

Section V

STRAINS

The results from two phytostat cultures suggest that there is a selection of cell types occurring during growth. Culture No. 13 of the rose tissue was grown for one month. The tissue grew in rather large granular nodules, some of which were hollow and others doughnut-shaped. This characteristic appeared early in the growth; this strain was isolated and placed on agar media in test tubes. The culture was sterile and was later used as inoculum for another 8-liter culture (No. 15) which ran for about six weeks; the growth of tissue was also granular and contrasted to the normal strain which was of loose texture and easily friable. The fresh and dry weight yields of culture No. 15 did not equal those obtained from the original 13. Apparently the transfer to agar medium caused the loss of some capacity for growth in liquid culture.

The daily harvests of fresh and dry weight rose tissue obtained from phytostat No. 13 are shown in Fig. 6. As with most of the cultures, there is a higher yield from early harvests (12-21 days) than from later harvests (25-45 days). This is shown quite well by the curve for dry weights of harvested tissue. The consistent higher yields in early harvests suggest that the medium is depleted of one or more essential components and that the rate of medium addition is insufficient to sustain the higher initial growth rate. In effect, the growth of the tissue in phytostat culture is limited by components of the medium, a situation analogous in principle to the chemostat of Novick and Szilard (ref 38) and Monod (ref 31).

The second instance of selection occurred in a phytostat culture of corn tissue. In this case, the corn tissue was inoculated into the culture vessel and grew very poorly. Inoculation took place on 21 October but no harvest could be made until 25 November when only 6 g/l were obtained. Growth improved noticeably after about six weeks when harvests of 15-20 g/l were obtained. This culture was terminated after 68 days. The strain of corn tissue which evolved over this long period of time scarcely grew when isolated and placed on agar media in test tubes. The culture was sterile, but apparently was adapted to the aerated liquid conditions of growth. Growth was so poor that it appeared to be dead. After several transfers, however, it recovered and resumed growth. Normally, a tissue reisolated from liquid medium grows as well as the original tissue.

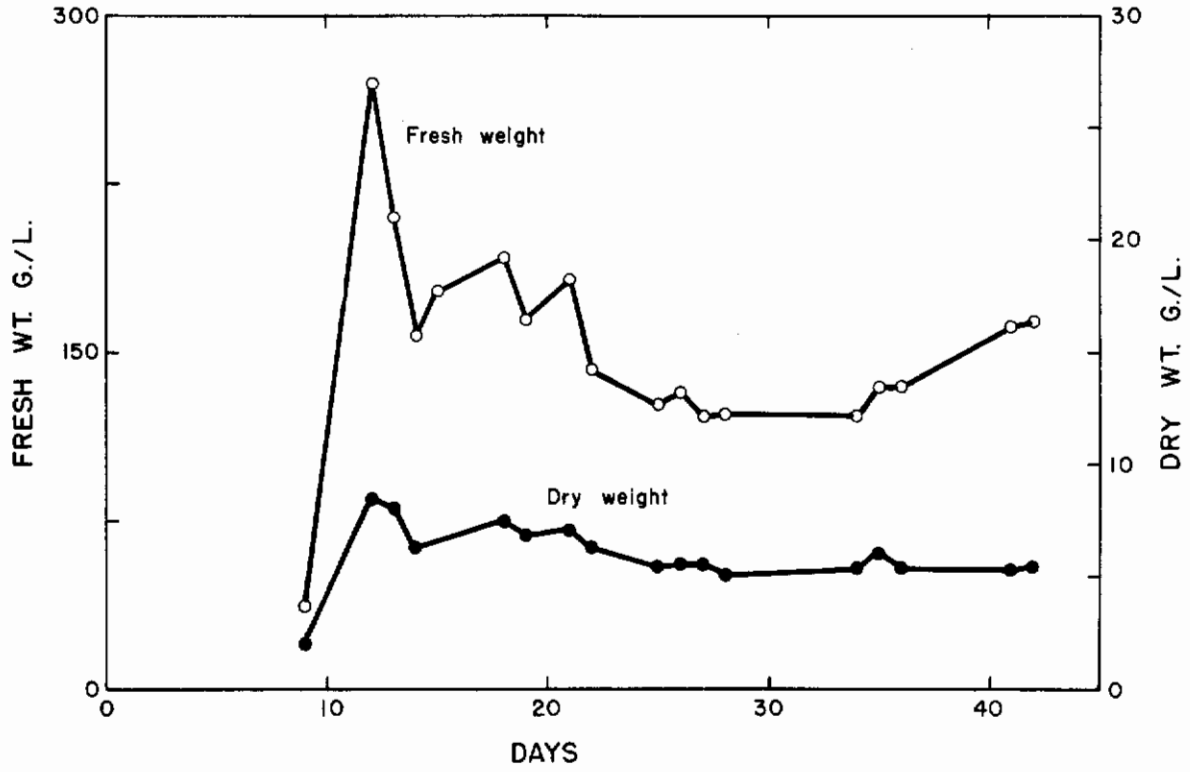

FIG. 6. GROWTH CURVE FOR ROSE TISSUE IN PHYTOSTAT CULTURE NO. 13.

These two strains (or mutants) furnish additional evidence from plant cells for the occurrence of evolution in mass cultures, a phenomenon which was described for microbial systems by Novick and Szilard (ref 38). These changes represent selective adaptations to a relatively constant environment and they signify that the medium and conditions are not optimal for the original inoculated cells. More than that, they emphasize that one of the dangers of continuous culture is the development of new strains. On the other hand, these changes also show that some controls are needed to restrict the occurrence of variants and strains. Conversely, the changes themselves may be useful in obtaining a speeded up evolution in vitro, perhaps in directions which are desirable and partly predictable.

Section VI

PROTEIN CONTENT

One of the main arguments for using a continuous culture system (such as the phytostat) is that the cell population is composed of a high proportion of active young cells. Since young cells synthesize more protein than older cells, it follows that the continuous culture systems should produce cells with a relatively high protein content. This depends on the rate of harvest, the dilution rate, the temperature, and many other factors. However, if conditions are regulated correctly, a high proportion of young actively metabolizing cells should yield more protein than an older population (ref 45). To test this assumption, an experiment was set up in the following manner. Tissues of rose, corn, and tomato were inoculated on agar media in fernbach flasks. At the end of each week, a harvest was made and the protein of the alcohol insoluble residue was determined (ref 39).

Fig. 7 gives the results of the experiment with data expressed as percentages of protein of the dry weight. It is evident that the protein content of rose and corn tissue is highest after one week while tomato reaches a maximum protein content in the second week. In interpreting these figures it is obvious that the protein could remain constant, and the nonprotein matter could increase, to give an apparent decrease in protein. The alcohol insoluble residues do show some variation in percentage of the dry weight of tissue, but this is negligible. The cell wall could increase to cause the apparent decrease in protein; this is one possibility, especially if growth is by cell enlargement. In the absence of cell counts for the tissues, however, it is not possible to plot the protein content on a cellular basis.

The protein content of tissue grown in the phytostat system was 16 per cent. This included harvests from cultures which were maintained at high cell densities. Protein levels of 19 per cent were obtained from less dense cultures and it is possible that slightly higher values could be obtained by controlling harvests and the medium. The fact that sustained yields of tissue were obtained over extended periods of time with a high level of protein is good evidence that the cells are in an active state of proliferation and that the population is high in percentage of young dividing cells.

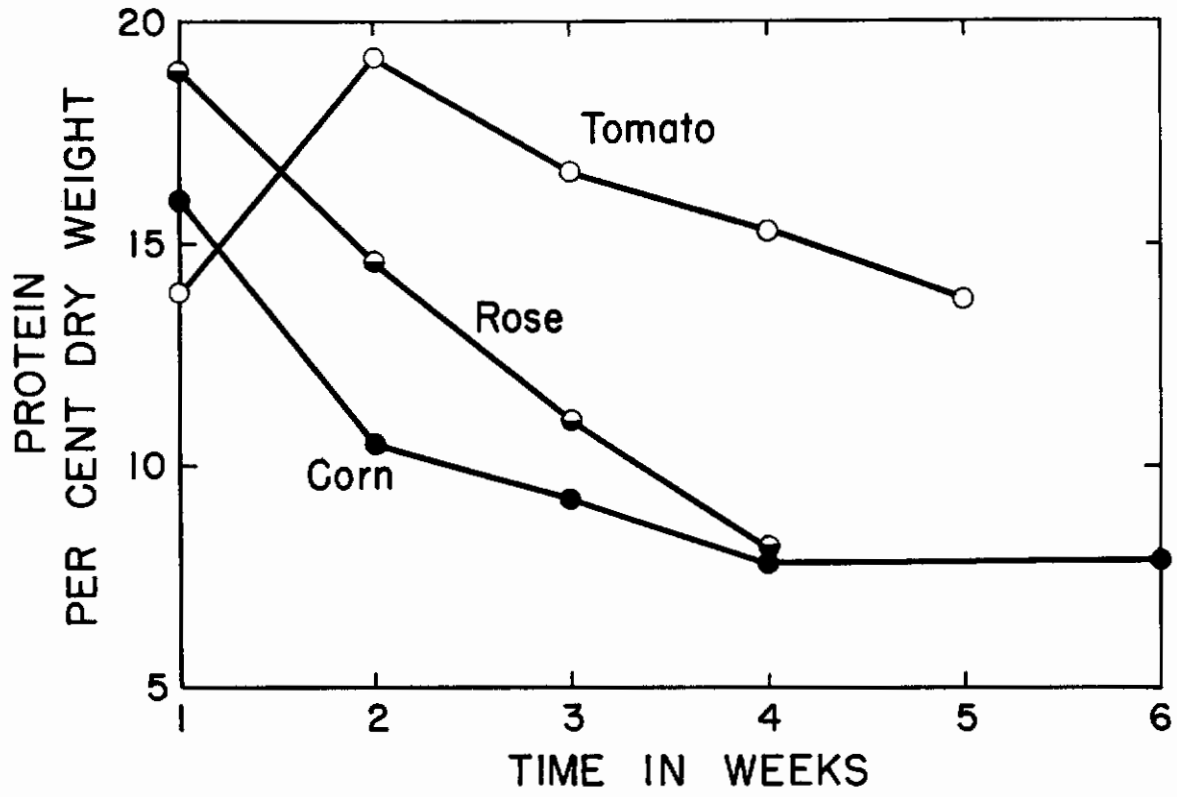

FIG. 7. PROTEIN CONTENT OF ROSE, CORN, AND TOMATO TISSUES AS A FUNCTION OF AGE.

The amino acid content of the rose tissue was reported previously (ref 61). Both the free and combined amino acids were determined for the tissue harvested at different periods in its growth on a solid medium containing coconut water. The rose tissue was harvested from test tubes at 8, 15, 22, 29, and 43 days. The percentage protein was calculated to be 11.3, 7.9, 5.0, 3.6, and 2.4 respectively. This suggests that there was an early synthesis of protein by the rose tissue and that this fell off as the culture aged. However, this result might also be explained by an increased content of cellulose in the older tissues, which would increase the weight of the sample of residue without a comparable increase in protein. That the latter is not an adequate explanation is evident from the per cent dry weights and the alcohol insoluble residues. The dry weight at 8 days is 6.7 per cent and it decreases to 4.4 per cent at 43 days. The alcohol insoluble residue as per cent of the dry weight was 24.4 at 8 days and 44.1 at 43 days. This means that the alcohol insoluble residue at 8 days probably consists of less wall material (by 50 per cent) than at 43 days; hence, a sample at 8 days would be higher in protein. The converse would be true at 43 days when more cell wall and less protein would be present. Thus, the difference of 11.3 per cent protein at 8 days and 2.4 per cent at 43 days was partly due to the low cellulose content of the residue at 8 days. However, there is a fourfold difference in protein between these two samples and only a twofold difference in alcohol insoluble residue, hence, there is a higher protein content in 8-day as against 43-day-old tissue. It should also be mentioned that these levels of protein were calculated from the amounts of protein amino acid present in hydrolyzed samples. Because of the nature of the determinations, the protein levels are probably somewhat higher than indicated above. The difference of 11.3 per cent protein from 8-day-old rose tissue (ref 61) and 18.9 per cent protein from rose tissue grown in fernbach flasks (Fig. 7) may be attributed to the difference in media used, or possibly also to the selection of a different more rapidly growing strain of tissue.

Another possible factor is that the rate of maturation of the cells is important, since it has been suggested by Blakely and Steward (ref 2) and Boll (ref 4) that the mature nondividing cells nourish the younger dividing ones. This is a reasonable explanation in the sense that dividing cells need specific metabolites which may not be furnished by the medium; these metabolites could be produced by cells in an active metabolic state and be made available to cells undergoing rapid division. Under these circumstances, the higher protein could come from the metabolically active cells and/or the dividing cells. However, there is no evidence from the phytostat cultures to indicate which of these assumptions (or other hypotheses) is correct.

The fact that most of the phytostat cultures of rose cells show a rapid growth to a peak of fresh and dry weight and then level off indicates that growth is probably limited by one or several components of the synthetic medium. This condition was pointed out by Novick (ref 37, 38) and Monod (ref 31) for chemostat cultures of bacteria. One constituent of the medium can be limiting to growth and thus can serve as a control lever to regulate growth. A similar regulation of growth of the rose tissue appears to occur in the phytostat cultures, but the nature of the limiting component is not known.

Section VII

NUTRITIONAL EXPERIMENT

The adequacy of rose tissue protein as a supplement to the diet of weanling mice was tested in a nutritional experiment.

Preliminary tests with the tissues of rose, corn, tomato, and Ginkgo had already shown that the tissues were acceptable to mature mice when incorporated into food pellets. These acceptability tests were run for 2-3 days and did not involve measurements of change in body weight (gain or loss). Contaminated rose tissue was also fed and no untoward effects were noted.

The pellets for the nutritional experiment were formulated by using the standard U.S.P. diet (ref 40) shown in Table VII. This diet was modified for pelleting as shown in the Test Diet. The formulation was made to these specifications by General Biochemical Co., Chagrin Falls, Ohio. The Test Diet was then supplemented with casein to give two control diets of 17.6 per cent and 8.8 per cent protein. The experimental diets consisted of Test Diet with 8.8 per cent protein, plus a supplement of sterile or contaminated rose tissue to bring the protein to 11 to 14 per cent. The composition of these diets is given below:

Diet A	Test diet with 20 per cent casein (18 per cent protein).
Diet B	Test diet with 10 per cent casein (9 per cent protein).
Diet C	Test diet with 9 per cent casein and 41 per cent sterile rose tissue (14 per cent protein).
Diet D	Test diet with 9 per cent casein and 41 per cent contaminated rose tissue (11 per cent protein).
Diet E	Wayne Lab-blox feed for mice with 24 per cent protein.

The standard laboratory control diet of Wayne Lab-blox pellets (Diet E) contained 24 per cent protein, 4 per cent fat, and 4.5 per cent crude fiber. It contained all the salts and vitamins known to be required and also unknown factors from liver meal, fish meal, corn meal, ground oats, soybeans, wheat germ, hay molasses, alfalfa meal, soybean oil, and brewers yeast.

The pellets containing rose tissue (formulations C and D) were prepared in this laboratory by thoroughly blending the ingredients in a mortar, moistening, casting into molds and freeze drying. The pellets containing casein (formulations A and B) were prepared by General Biochemical Co. All of these pellets with the Test Diet had a somewhat disagreeable odor, probably from the cod liver oil. The pellets were fed to mature mice in a preliminary feeding test and were found to be acceptable.

The feeding experiments with the five diets were conducted by Carworth Laboratories, Inc., New City, N. Y. Ten female mice, each approximately 13-14 grams in weight and four weeks old were used for each diet. Pellets and water were provided ad libitum; each mouse was in an individual cage and the temperature was 22-23° C. The weights of the mice and the food intake were recorded daily for three weeks. About 8-10 additional mice were placed on the casein and rose supplemented diets (A through D) in a second experiment. The results were similar to those reported below.

TABLE VII

DEPLETION DIET FOR PROTEIN BIOLOGICAL ADEQUACY TEST (REF 40)

Ingredients	U.S.P. XV diet (ref 39) (powder), g/100 g	Test diet (for pellets), g/100 g
Dextrin	83.900	2.000
Corn oil	9.000	9.000
Sucrose	—	66.900
Corn starch	—	15.000
Salt mix	4.000	4.000
Agar	2.000	2.000
Cod liver oil	1.000	1.000
Vitamin supplement		
Choline chloride	0.1500	0.1500
i-Inositol	0.1000	0.1000
Calcium pantothenate	0.0020	0.0020
Niacinamide	0.0015	0.0015
Riboflavin	0.0018	0.0018
Pyridoxine	0.00025	0.00025
Thiamin	0.0002	0.0002
Para-aminobenzoic acid	0.0002	0.0002
Folic acid	0.0002	0.0002
Menadione	0.0002	0.0002
Biotin	0.00002	0.00002
Salt mixture		
Sodium chloride	139.3	139.3
Potassium biphosphate	389.0	389.0
Magnesium sulfate, anhydrous	57.3	57.3
Potassium iodide	0.79	0.79
Calcium carbonate	381.4	381.4
Ferrous sulfate	27.0	27.0
Manganese sulfate	4.01	4.01
Zinc sulfate	0.548	0.548
Cupric sulfate	0.477	0.477
Cobaltous chloride	0.023	0.023

Microkjeldahl determinations (ref 39) of the nitrogen content of the alcohol insoluble residue of dry rose tissue were made; the protein content of the tissue was calculated as 16 per cent. This value represented an average from pooled samples of tissue from several phytostat cultures. This per cent was used in formulating the pellets for the nitritional experiment.

There were problems with pellet formulation that were concerned with dilution of the test diet and a lowering of the protein level by the supplements of rose tissue. These can best be described by giving the actual formulations of the diets. The A diet consisted of 80 g of Test Diet plus 20 g of casein; the B diet was 90 g of test diet and 10 g of casein. Since the casein consisted of 88 per cent protein, the A diet contained 17.6 per cent protein and the B diet of 8.8 per cent proteins. The C and D diets were formulated with 90 g of B diet (containing 7.9 g of protein) and 62 g of rose tissue (containing approximately 10 g of protein). The total protein content was approximately 17.9 g/152 g of formulation, or 11.8 per cent protein. Pellets were made of this composition.

The protein content of the formulated pellets was checked by analyzing for alcohol insoluble nitrogen (ref 39) and then calculating the per cent protein (X 6.25). The results from analyses gave the following per cent protein: A-18.1, B-8.5, C-13.9, D-10.9, and E-25.0. These figures compare reasonably well with the calculated formulation of the pellets as per cent protein: A-17.6, B-8.8, C-11.8, D-11.8, and E-24. The major difference is in diet C, which gave analyses which were higher than expected. A partial explanation for this result lies in the fact that the sterile rose had a slightly higher level of protein than the contaminated rose.

From the above data, it can be seen that the composition of the pellets was altered by the addition of either casein at 20 and 10 per cent or the addition of rose tissue. These additions also diluted the Test Diet. Furthermore, the bulk furnished by the rose tissue was not compensated for by using inert roughage in the casein supplemented diets. As a result, the addition of rose tissue caused a dilution of the protein from casein as well as a dilution of rose protein in the Test Diet. This accounts for the low amount of protein in the C and D diets. It was not feasible (with the amounts of rose tissue available) to increase the amount of protein to the level of the A diet. The dilution factors mentioned above precluded this being done. Thus, the prepared diets represent an arbitrary composition to test the effect of rose tissue as a dietary supplement. As a consequence, the results of the nutritional experiment must be evaluated with all these factors in mind, in addition to the fact that the protein content of the diets was constant and well-defined.

The results of the nutritional experiment are summarized in Fig. 8. The Wayne Lab-blox control diet (E) was the best; the diet with 10 per cent casein plus sterile rose tissue protein (C) was the best experimental diet. The latter was superior to either contaminated rose tissue (D) or the diets supplemented with casein alone (A and B). The best response of the C group is shown as the average of three mice ([C]). These mice showed no toxicity symptoms and, when placed on the E diet (after termination of the three week experiment), they soon gained weight and were as healthy and vigorous as the mice on the E diet from the beginning of the experiment. At the end of eight weeks these mice appeared normal in all respects.

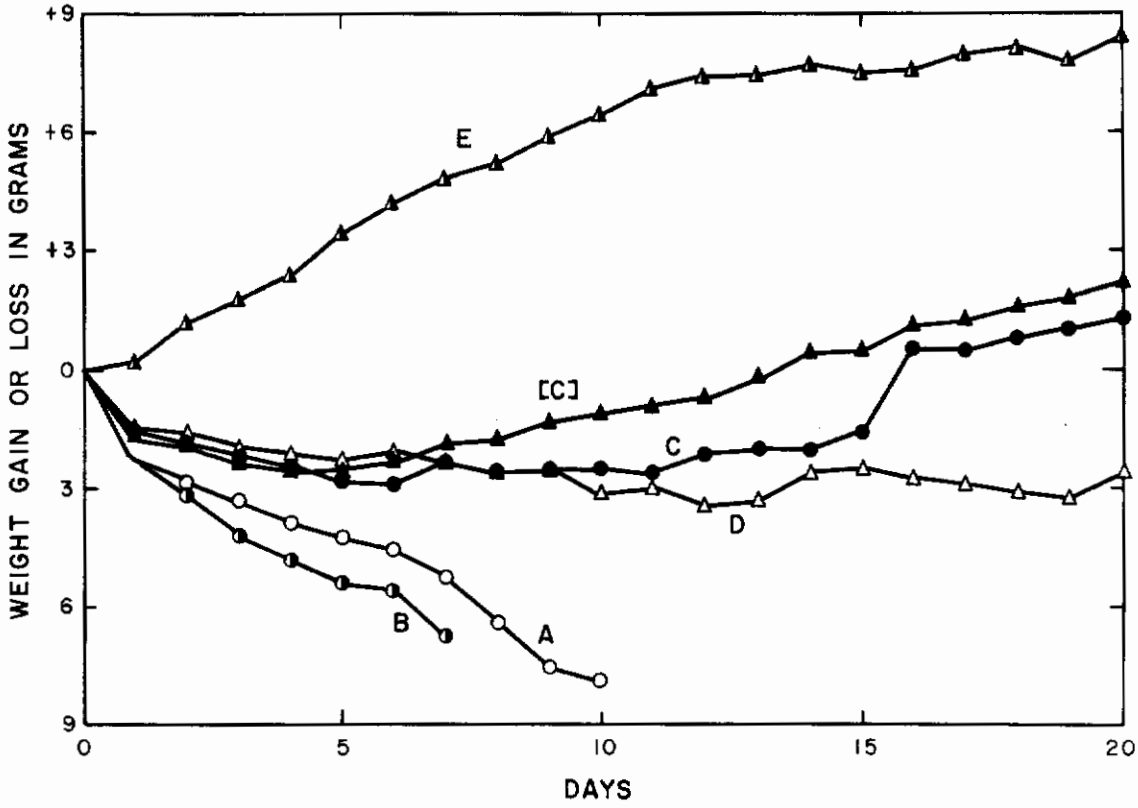

FIG. 8. GROWTH OF WEANLING MICE ON DIETS SUPPLEMENTED WITH ROSE TISSUE. *

* A. 20 Per cent casein (18 per cent protein); B. 10 per cent casein (9 per cent protein); C. 6 per cent casein plus 41 per cent rose tissue (14 per cent protein); D. 6 per cent casein plus 41 per cent contaminated rose tissue (11 per cent protein); [C] the average growth of three mice which gained weight; E. Wayne Lab-blox control diet (24 per cent protein). All averages (other than [C]) are from the number of mice indicated in Table X.

The average gain or loss of weight for 10 mice fed the five diets is shown in Table VIII.

TABLE VIII

AVERAGE WEIGHT GAIN OR LOSS OF WEANLING MICE FED A DIET SUPPLEMENTED WITH ROSE TISSUE CULTURE

Day	Weight gain or loss (grams) *				
	A	B	C	D	E
1	-2.2	-2.3	-1.5	-1.5	+0.2
2	-2.8	-3.2	-1.8	-1.7	+1.2
3	-3.3	-4.2	-2.2	-1.9	+1.8
4	-3.9	-4.8	-2.5	-2.1	+2.4
5	-4.3	-5.4 (3)	-2.8	-2.3	+3.4
6	-4.6 (7)	-5.6 (2)	-2.9	-2.1	+4.2
7	-5.3 (7)	-6.7 (2)	-2.4 (9)	-2.3	+4.9
8	-6.4 (5)	Dead	-2.6 (9)	-2.6	+5.3
9	-7.5 (3)		-2.6 (9)	-2.5 (9)	+5.9
10	-7.9 (1)		-2.5 (8)	-3.1 (9)	+6.5
11	Dead		-2.6 (8)	-3.0 (8)	+7.1
12			-2.1 (7)	-3.4 (8)	+7.4
13			-2.0 (7)	-3.3 (7)	+7.4
14			-2.0 (7)	-2.6 (5)	+7.7
15			-1.6 (7)	-2.5 (4)	+7.6
16			+0.5 (5)	-2.7 (4)	+7.6
17			+0.5 (5)	-2.9 (4)	+8.0
18			+0.8 (5)	-3.1 (4)	+8.1
19			+1.0 (5)	-3.2 (4)	+7.8
20			+1.3 (5)	-2.6 (3)	+8.4

* Average of 10 mice unless otherwise indicated in parentheses.

A - Test diet plus 20 per cent casein (18 per cent protein).
B - Test diet plus 10 per cent casein (9 per cent protein).
C - Test diet plus 6 per cent casein plus 41 per cent sterile rose tissue (14 per cent protein).
D - Test diet plus 6 per cent casein plus 41 per cent contaminated rose tissue (11 per cent protein).
E - Wayne Lab-blox feed for mice (24 per cent protein).

The casein supplements alone were very poor and by the eighth day all of the mice on the B diet and half of those on A diet were dead. At the same time, only one mouse from the sterile and contaminated rose tissue supplemented diets had died, although they had lost weight. By the end of the experiment, 5 mice on the C diet (sterile rose tissue) and 3 on the D diet (contaminated rose tissue) had survived, but only those on the C diet showed any gain in weight. When compared to highly supplemented E diet, the results appear rather poor. However, it is necessary to take into account the fact that the C diet contained about half of the protein of the E diet and, because of the greater bulk of the C diet, the mice on the C diet ate a little more than

half as much food as those on the E diet. This is shown quite well in Table IX which gives the average consumption of food and protein for mice on the five diets.

TABLE IX

FOOD AND PROTEIN CONSUMPTION COMPARED TO BODY WEIGHT (GAIN OR LOSS) OF MICE FED A TEST DIET SUPPLEMENTED WITH ROSE TISSUE

Test diets *	Average food consumption, g/day/mouse	Average protein consumption, g/day/mouse	Average gain or loss in body weight, g
A	1.0	0.18	-4.8
B	0.7	0.06	-4.6
C	2.1	0.29	-1.6
D	1.4	0.15	-2.4
E	3.7	0.89	+5.8

* A - Test diet plus 20 per cent casein (18 per cent protein).
B - Test diet plus 10 per cent casein (9 per cent protein).
C - Test diet plus 6 per cent casein plus 41 per cent sterile rose tissue (14 per cent protein).
D - Test diet plus 6 per cent casein plus 41 per cent contaminated rose tissue (11 per cent protein).
E - Wayne Lab-blox feed for mice (24 per cent protein).

The mice on the E diet ate the most food, gained weight, and had the highest protein intake. The next best group was the mice on the C diet which was supplemented with sterile rose tissue. In contrast, the mice on the B diet had the lowest food consumption and protein intake and did not eat the food, even though it was provided ad libitum. Autopsies of this group showed empty stomachs and an intestinal tract which contained mucous. The mice were anemic and their tails became white and later black and atrophied. These animals also showed a mucoid diarrhea with traces of blood. The animals on the A diet ate slightly more food and consumed three times as much protein, but their average loss in body weight was about the same as for the mice on the B diet.

These results suggest that the edibility of the A and B diets was low and that it was markedly improved by the addition of rose tissue. The gain in body weight of mice fed the C diet also indicates that rose protein was being utilized. The poorer performance of mice on the D diet further indicates that the contaminated rose is inferior to the sterile rose as a supplement. Thus, it can be concluded that the sterile rose tissue has some value as a food supplement for weanling mice.

Section VIII

DISCUSSION

These experiments are probably the first attempt to produce sufficient amounts of a plant tissue culture for nutritional use. Other plants such as duckweed (Lemna minor) (ref 62) or Chlorella (ref 58) have been produced in culture systems and used as food supplements for experimental animals (ref 28), including man (ref 10). All of these experiments are largely exploratory at the present time, but they are beginning to provide information for the evaluation of plants as food sources in long term space missions (ref 10) and as possible future sources of supplementary protein. Such uses for tissue cultures of higher plants are hypothetical at the present time and highly speculative. However, as techniques and sophistication in the use of plant tissue cultures improve, it is quite likely that plant cells themselves (ref 48) or their products, enzymes, or capabilities (ref 24) will be used for specific purposes.

The most significant finding in the nutritional experiments on weanling mice was that the sterile rose tissue was a much better dietary supplement than bacterial contaminated tissue. More mice survived, gained weight, and surpassed their original weight when fed the diet supplemented with sterile rose tissue than when fed the diet with contaminated rose. This is in agreement with nutritional studies reported for weanling rats (ref 58), where it was found that the nutritional value and digestibility of sterile algal supplements were better than contaminated algal supplements. Although neither of the latter diets equaled the standard Wayne Lab-blox control diet, both were better than the two diets supplemented with casein alone, i.e., the 20 per cent casein and the 10 per cent casein. Both of the latter diets should have been adequate to sustain the mice, since similar diets have been recommended (ref 34) or used in other tests (ref 28, 58). Thus far, no adequate explanation for this result has been obtained. One possibility is that the roughage content of the casein control diets was low and therefore unacceptable. The fact that the mice on the 10% casein diet died of starvation and internal hemorrhage suggests an acute deficiency in this diet, possibly in the amount of cystine (ref 34). As a very low base line, the 10 per cent casein diet emphasized the usefulness of the sterile rose tissue as a supplement. In terms of survival and weight gain the Wayne Lab-blox diet was best, followed by the diet with sterile rose tissue, contaminated rose tissue, 18 per cent protein and finally the diet with 9 per cent protein. If viewed in this way, the experiment was successful in demonstrating that rose tissue can be utilized as a food supplement.

The protein amino acids of the rose tissue are compared to the Food and Agriculture Organization (FAO) human diet standard, the growth needs of albino rats, and to casein and soybean protein in Table X. From these data it can be seen that the rose protein is high in arginine, lysine, leucine, threonine, valine, glycine, and alanine as compared to casein or soybean meal; it is about equivalent in amounts of serine and aspartic acid and is low in histidine, methionine, cystine, phenylalanine, tyrosine, tryptophan (not determined for rose), glutamic acid, and proline. Compared to the growth needs

TABLE X

PROTEIN AMINO ACIDS OF ROSE TISSUE COMPARED TO THE FAO PATTERN, GROWTH NEEDS OF ALBINO RATS, CASEIN, AND SOYBEAN MEAL

Amino acid	g Amino acid/16 g N				
	Rose tissue (ref 61) *	FAO pattern (ref 15)	Growth needs of albino rats (ref 15)	Casein (ref 3)	Soybean meal (ref 3)
Arginine	7.4	—	3.8	4.2	7.0
Histidine	—	—	2.3	3.0	2.5
Lysine	16.6	4.2	7.2	8.2	6.6
Leucine }	22.2	4.8	8.7	10.1	7.6
Isoleucine }		4.2	6.3	6.6	5.8
Methionine	—	2.2	—	3.3	1.1
Cystine	—	—	—	0.4	1.2
Total sulfur amino acids	—	4.2	6.2	3.7	3.3
Phenylalanine	0.4	2.8	5.9	5.8	4.8
Tyrosine	1.9	2.8	3.3	6.3	3.2
Threonine	5.3	2.8	4.5	4.5	3.9
Tryptophan	—	1.4	1.4	1.5	1.2
Valine	9.0	4.2	7.5	7.4	5.2
Hydroxyproline	0.05	—	—	—	—
Serine	5.9	—	—	6.3	5.6
Glutamic acid	12.7	—	—	23.6	18.5
Aspartic	7.2	—	—	6.5	8.3
Glycine	7.0	—	—	2.1	3.8
Alanine	8.0	—	—	3.1	4.5
Proline	3.6	—	—	12.3	5.4

* Data calculated from analyses of 8-day-old rose tissue.

of albino rats and the FAO pattern, the rose tissue is low in sulfur and aromatic amino acids, but adequate in the other amino acids.

In making these comparisons, it is important to note that the content of lysine, proline, and hydroxyproline in the rose tissue protein varies with age (ref 61). The lysine content decreases as the tissue gets older and the proline and hydroxyproline increase. Tyrosine also decreases over the 43 day growth period; phenylalanine rises and then decreases to trace amounts. The eight day level was chosen for comparison because it is believed to be most comparable to the young actively growing cells from the phytostat cultures. Amino acid analyses on the tissue from the phytostat have not been done.

The simultaneous growth of rose tissue and Achromobacter liquefaciens in phytostat cultures for periods of more than a month is considered a significant result. In general, the other bacterial contaminants of the cultures were destructive to the tissue and soon became the dominant organism; Achromobacter liquefaciens was an exception.

Fallot (ref 16) has cited the stimulatory effect of Bacillus megatherium on the induction of growth in grape stem segments; Burkholder (ref 7) has also called attention to the role of bacterial products in regulating the growth of higher plants; and effects of Rhizobium species and Agrobacterium are well known. Other bacteria (ref 60) or bacterial products (ref 21) are inhibitory or destructive to tissues in culture. In relation to these studies the symbiotic growth of rose tissue and Achromobacter in phytostat culture is of interest and deserves further study.

Previous work with the growth of plant (ref 8, 13, 17, 30, 32, 36, 41, 42, 43, 47, 52, 59), animal (ref 9, 12, 20, 25), and microbial (ref 11, 19, 23, 29) cells in liquid media has made it possible to devise a continuous culture system of simple design for plant cells: the phytostat. This system has served for the production of more than 40 pounds (20 kg) of sterile rose tissue (fresh weight). The apparatus is considered a prototype, because the experience gained in its use suggests further improvements. For example, a separate air supply instead of the compressed air from the general laboratory lines would help to reduce contamination. A prefilter on the air intake of the compressor, air incineration, new filters made of polyvinyl alcohol (ref 1), or Snyder filters of the type used for gnotobiotic experiments might be used to increase the certainty of a sterile air supply. Automatic harvests and additions of medium undoubtedly could be made, but they would have to be programmed by some measurement of cell density. In turn, this depends upon having a tissue which grows as single cells or as clumps of easily suspended cells; such a tissue would permit the use of some density monitoring system. Eventually, a synchronous type of culture such as has been worked out for Chlorella (ref 32) may be obtained for plant cells; this would be an ideal system for certain purposes, such as studying the events of the cell division cycle.

Even with an improved culture apparatus, however, there would remain certain difficulties which are inherent in the biological aspects of the cell generating system. Foremost among these are the variations which have been observed in microbial systems (ref 6); the mutations which may eclipse the original inoculated organism (ref 26, 37); and the modulations of cell function which may be brought about by the nutrient medium (ref 33). These are the hazards of continuous cultures; they make it difficult to control cell populations or their activities. Nevertheless, the continuous culture of mass populations of cells offers other advantages in analyzing cell growth, composition, biochemistry, and physiology (ref 29).

At the present time, there is insufficient information from the continuous cultures of plant cells to analyze the dynamics of the system on a cellular basis. Greater appreciation of events and their control will be forthcoming when there is information on cell generation time, the cell cycle, and similar phenomena. Analysis is further complicated by a number of variables. For example, the cells of plant tissues grown in vitro are generally larger, more vacuolate, and hence more hydrated (ref 43) than cells from the original explant. In addition, the intercellular spaces are much greater because the cells grow in the absence of an outer limiting tissue such as an epidermis or bark. Superimposed on these factors are such things

as the change in osmotic value of the medium as the cells grow, the rate of cell expansion during the cell cycle and the rate of cell division. Much more study is required before these factors can be evaluated and better understood.

The culture of plant cells in phytostats should prove useful for many future studies. Investigations on cell metabolism, cell products, or the special functions of cells may use continuous culture systems of this type. Other work might involve the induction or transformation of cells in such a system, perhaps with the intent of obtaining evolution in vitro and selecting out desired cell strains. Chemically induced differentiation (cellular or metabolic) may be studied by manipulating the environment, including the medium. Additional novel uses might involve virus propagation, mixed cell cultures from unrelated plants, and bacterial, fungal or nematode cultures with selected plant cells.

Section IX

CONCLUSIONS

The following conclusions can be drawn from this work:

1. Plant cells can be grown continuously in a liquid culture system called a phytostat.

2. A tissue culture derived from rose stems can be used as a food supplement by weanling mice.

3. The sterile tissue of rose is a better food supplement than the bacterial contaminated tissue.

4. The rose tissue culture will grow well in the presence of a bacterium, Achromobacter liquefaciens.

5. The protein content of plant tissue cultures is higher in young tissues than in old tissues, and higher in phytostat cultures than in test tube cultures.

Section X

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13 ABSTRACT

The purpose of this work was twofold: 1) to devise a continuous culture system for higher plant cells and 2) to produce a sufficient amount of plant tissue culture for a nutritional experiment with weanling mice. The overall purpose of this work is to provide information for the evaluation of plants as food sources in long term space missions and as possible future sources of supplementary protein. Rose tissue was the most suitable for growth in liquid culture and approximately 40 pounds (20 kg) fresh weight of sterile tissue was produced from seven cultures which were harvested periodically over a period of 222 days. The cultures were of 8 liters volume and this amount was maintained by replacing the medium which was harvested. The average yield was 112 g/l fresh weight (4.6 g/l dry weight)/day. Approximately 10 pounds (5.0 kg) of contaminated rose tissue was also produced. When incorporated into a test diet for weanling mice, the sterile rose tissue was a better food supplement than contaminated rose. A bacterial contaminant which grew well with the rose tissue and did not appreciably alter the growth rate was isolated and identified as Achromobacter liquefaciens. Since this was a reisolated type species for the genus, it was deposited in the American Type Culture Collection (ATCC No. 15716). Protein determinations on the rose tissue showed a high protein level for tissue grown in continuous culture as compared to tissue grown in flasks.

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