(i) that they are actually involved in electron transport; (ii) that they are facilitating an optimal rearrangement of the reacting components; (iii) that they are removing a surface-active agent which has become attached to the enzyme and has inhibited it. The last possibility is rendered unlikely by the result that removal of deoxycholate with resin from an S3 supernatant does not lead to reactivation.

These results explain a discrepancy observed in our laboratory in 1950. When studying the copper content of cytochrome oxidase (*7*), we noted that there was poor correlation in many instances between the oxygen uptake catalyzed by a fraction and the height of the *alpha*-absorption peak in the reduced state. The ratio, O_2 uptake : 601-mμ absorption, decreased as succeeding fractions were made by extracting heart muscle particles with 1 percent deoxycholate. It may now be suggested that the third and fourth fractions were probably deficient in the lipide or in the lipide-soluble substance being discussed here (*8*).

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Production of Gibberellin-like Substances by Plant Tissue Cultures

One of the most discussed as well as one of the most investigated problems today in the fields of plant physiology and plant agriculture generally is that of the gibberellins and their action on plant growth (*1, 2*). That this group of fungal metabolic products has many very dramatic effects on plants has been amply demonstrated (*2, 3*). How these chemicals act and exactly how valuable they will be to agriculture are two questions which still remain unanswered.

Recently, investigators have reported gibberellin-like activity in extracts from several plants (*4–6*). Most of this type of investigation has been carried out by Phinney and his colleagues. In surveying numerous plants and plant parts, a surprising number of active extracts was found. This led Phinney to suggest that gibberellin-like compounds are widespread and might be universal in their occurrence among flowering plants (*6*). The greatest activity was found in the green seeds, young fruit, and endosperm of several plant species.

The loss of activity as seeds reach maturity, the location of active extracts in stem tips (*7*), and the general activity of immature tissues indicate that immature tissues—rapidly dividing meristematic areas—are the sites of synthesis of these gibberellin-like materials. If this is the case, plant-tissue cultures should be an excellent place to look for such activity. If it is found, this should lend much weight to the hypothesis of universal occurrence in the plant kingdom. Moreover, tissue-culture techniques should lend themselves to investigations of such problems as rate and site of synthesis, nutritional factors affecting synthesis, and control of synthesis.

This is a preliminary report of the results of our survey of plant tissue cultures as sources of gibberellin-like factors.

The tissues used in this study are well-established cultures which have been maintained in our laboratory for several years. The conditions under which they have been grown have been discussed in previous publications (*8*). Extractions were made, in each case, of 20 g (wet weight) of tissue about 4 weeks after its latest subculture. The method of extraction was patterned after that used by Phinney and his co-workers (*5*) and consisted of (i) extraction of diced tissue with acetone-water (1:1) on a shaker for 24 hours; (ii) filtration of the solvent, and (iii) reduction in volume over a steam bath to 5 ml. This 5-ml sample was sprayed with a deVilbiss atomizer on five dwarf pea plants (*9*) (1 ml per plant) which had been planted in sand 1 week previously. Growth of the plants was measured 3, 5, and 7 days after application, and the results were compared with measurements of control plants and of plants sprayed with standard gibberellin solutions.

Table 1. Plant tissue-culture extracts which have gibberellin-like effects on dwarf pea seedling growth. (LP) Nickell low-phosphate medium (*11*); (24) Burkholder and Nickell high-phosphate medium (*12*); (CM) coconut milk (18 percent by volume); (YE) Mead Johnson yeast extract (5 g/l); (2,4-D) 2,4-dichlorophenoxyacetic acid (0.6 part per million); (*p*Cl) *p*-chlorophenoxyacetic acid (0.6 part per million). Effects listed below are related to untreated plants (O) and gibberellin-treated (0.5 part per million) plants (++++).

Plant source	Common name	Plant part	Type tissue	Medium used	Relative effect
Vinca rosea	Periwinkle	Stem	Crown gall	LP	++
Helianthus annuus	Sunflower	Petiole	Crown gall	LP	+++
Melilotus officinalis	Sweet clover	Stem	Crown gall	LP-YE-*p*Cl	+
Melilotus officinalis	Sweet clover	Root	Virus tumor	24-YE-*p*Cl	+
Melilotus officinalis	Sweet clover	Stem	Virus tumor	24-YE-*p*Cl	+
Melilotus officinalis	Sweet clover	Root	Callus	LP	+
Agave toumeyana	Century plant	Leaf	Callus	White-2,4-D-CM	++
Ilex aquifolium	Holly	Stem	Callus	White-2,4-D-CM	+
Phaseolus vulgaris	Pinto bean	Cotyledon	Callus	White-2,4-D-CM	+

It should be noted that positive growth effects were obtained with extracts of both monocots and dicots, as well as with both leguminous and nonleguminous dicots. Activity is not limited by the type of tissue or by the plant part from which it originated, for stems, roots, leaves, and cotyledons are all represented, as well as nonpathological callus tissues and tissues of virus tumor and crown gall origin. This represents a wide distribution of plant material from many points of view: taxonomical, physiological, morphological, and pathological.

Responses of the magnitude obtained with the standard gibberellin solutions and with the plant extracts were not obtained with any other solutions of specific chemicals tested. These included several common auxins, purines, numerous antibiotics, organic acids, amino acids, and various types of chelating agents. Nor did the media on which the tissues were grown cause this response.

The production of substances which elicit a response in test plants similar to that elicited by the fungal metabolic products, the gibberellins, has been demonstrated for tissue cultures of higher plants—in many cases tissues which have been maintained for years on a synthetic medium. The wide variety of plants represented by these cultures lends strong support to the hypothesis that the

gibberellins, or at least gibberellin-like substances, are universal in the plant kingdom and might play a vital role in the control of plant growth (*10*).

Further work with those tissue cultures which produce the greatest amount of activity is being completed (*10*).

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New Maintenance Medium for Cell Culture

Although animal serum has been a necessary constituent of most media for continuous cell cultures (*1*), many sera contain "inhibitors" to a wide range of viruses (*2*). It would be desirable to replace serum with another substance which does not contain nonspecific inhibitors or antibody and which would sustain cells in a condition sensitive to viral effects during the necessary observation periods (*1*).

Having observed that boiled skim milk contributed to the maintenance of fully grown LAC cells (*3*), we undertook a study to determine the nutritive value of skim milk in cell culture maintenance media.

Skim milk was prepared by dissolving Pet instant nonfat dry milk in distilled water, according to instructions on the container, in order to reconstitute original volume. The milk was either boiled for 5 minutes or autoclaved at pressure of 7 lb. in.^{2} for 15 minutes. The *p*H was adjusted to 7.2 with sterile 5-percent sodium bicarbonate, and 150-ml aliquots were frozen at – 20°C. This material was designated as 100-percent skim milk.

Basal medium [medium 199 (*4*), or Eagle's basal medium (*5*), at *p*H 7.3 to 7.6] was mixed with varying amounts (0 to 100 percent final concentration) of skim milk and fed to LAC cells and HeLa cells (Gey). Maintenance was very satisfactory in the presence of skim milk (10 to 40 percent) in either basal medium. Final concentration of 20 percent of skim milk in medium 199 was selected for further experimental use. Cell strains monkey heart (Salk) and KB (Eagle) and rhesus monkey kidney cells were maintained in this medium for periods of up to 4 weeks when fed at 7-day intervals. Rhesus monkey kidney may also be maintained in Earle's balanced salt solution containing 20 percent skim milk and either 3 percent Travamin (*6*) or 0.5 percent lactalbumin hydrolyzate (*7*). A twofold concentrate of any one of these media may be mixed with agar for plaque overlay of rhesus monkey kidney cells. As with serum-containing maintenance medium, the degree of cell support by basal medium containing skim milk varies with different cell culture lines and different cell culture strains. Rounded cells and debris may accumulate, especially over the densest areas of cell cultures. Exceptionally dense areas of tube cultures may actually degenerate, leaving a wide margin of clear viable cells, which are suitable for observing viral effects.

The activity of skim milk appears to be associated with one or more of the milk proteins; the activity is not dialyzable and not sedimentable at 78,000g for 1 hour. A material of this type which retains its cell nutrient capacity after exposure to high temperature is rather unusual. Thus, skim milk is the first material which, at least partly, replaces the serum protein necessary for culture of HeLa cells, KB cells, and other continuous cell lines (*5*).

Skim milk medium is turbid, due to suspended casein (*8*), and it is rather striking to observe clearing of the milk medium under certain conditions. Trypsin, stool suspension, and some bacterial contaminations will clear the medium. Monkey kidney cell cultures also clear the medium after 3 to 5 days' incubation at 36°C, while HeLa, LAC, and KB cells do not, in the absence of serum. Proteolysis of the suspended casein is thought to be the mechanism of clearing.

The viral sensitivity of a cell culture in a maintenance medium is relative to its sensitivity in the presence of an established medium on the same cell culture line. In comparative titrations, 4- to 100-fold serum-inhibition of poliovirus was observed when calf-serum-containing maintenance medium was compared with serum-free medium on monkey kidney cells. A number of lots of heated skim milk were tested in this manner, at 20- and 30-percent concentration, without detection of decreased sensitivity. Similar determinations were made with monkey-kidney adapted Coxsackie B1, B2, B3, B4, B5, A7, A9 and vaccinia virus (*9*). There was no evidence that skim milk medium decreased cell culture sensitivity to these viruses. Preliminary studies indicate that HeLa cells in the presence of Ginsberg's medium are tenfold more sensitive to adenovirus 3 and 4 than HeLa cells in the presence of skim milk medium.

To determine whether the heating of skim milk would completely destroy any "antibodies" in milk (*10*), 1 ml of human serum containing antibodies to all three types of poliovirus was added to 9 ml of skim milk, and a 5-ml aliquot was autoclaved in the manner used to sterilize skim milk. The autoclaved aliquot was free of poliovirus inhibitors, demonstrating that the heating process completely destroyed the activity of the added poliovirus antibodies in skim milk. These results indicate that skim milk medium provides an environment which allows relatively full sensitivity to most of the viruses tested. Additional advantages are simplicity of preparation, low cost, and reduction in requisite frequency of feeding the cells.

Skim milk maintenance medium appears applicable as a standard medium for (i) comparative assay of a variety of viruses on a number of different cell cultures, (ii) safety testing of virus vaccines, (iii) isolation of viral agents which were not previously cultured, due to neutralization by serum-containing maintenance media, and (iv) detection of proteolytic activity of cell cultures.

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