CHAPTER SEVEN

Biochemical Differentiation: The Biosynthetic Potentialities
of Growing and Quiescent Tissue

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I. Introduction

Growth may be visualized in terms of the progressive formation of
chemical compounds. It is such a familiar fact that many organs and
tissues of higher plants may have uniquely different chemical compo-
sition that the significance of this often passes unnoticed. However,

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2 The work which furnished much of the background on which this Chapter is
based was done with the support of PHS research grants (currently GM 09609)
from the National Institutes of Health.
if divisions from the zygote are strictly equational, then all its living
derivative cells should have all the genetic information to achieve
at any time all or any part of the biochemistry of the whole plant. The
lesson to be drawn from modern work on cell and tissue culture is
that living cells of higher plants are in the morphogenetic sense
potentially totipotent (cf. Chapter 8, pp. 329–376). Since this is so, they
should also be biochemically totipotent. Therefore, one now envisions
the process of growth and development in terms of the restraints which
are imposed on potentially totipotent cells, so that in their position in
the plant body they express only part of their innate potentialities as
they are controlled by extrinsic factors. Moreover, the factors that
control the morphology are frequently, if not invariably, associated
with different expressions of their biochemistry.

It is a subtle and difficult problem to know whether the conditions
that exist during growth and development prescribe the metabolism and
the biochemistry, so that the morphogenetic development follows, or
vice versa. If modern interpretations of biochemical genetics and the
genetic regulation of metabolism are adequate, it would seem that
the conditions that obtain during growth, differentiation, and morpho­
genesis prescribe that given genes become activated by the specific
conditions which occur at any given point along the time course of
growth. Then via the familiar sequence of gene → messenger RNA →
protein (enzyme), the genes prescribe the biochemical reactions that
occur. In view of this, the following questions may be posed. Is it
possible to reproduce at will the particular biochemistry of any par­
ticular organ, tissue, or cell? Can one induce potentially totipotent
cells to express the biochemistry that they normally achieve in a given
morphological setting, without the necessity of reproducing that setting
intact by the growth of the whole organism? These questions have an
important bearing on our interpretation of growth and development
on the one hand, and of the control of metabolism on the other. For
this reason they are appropriately considered here.

One should, however, recognize at the outset that the problems are
difficult. The difficulties are those that beset biology as it moves from
the phase of analysis to that of synthesis. It may be easier to under­
stand how cells proliferate than how they grow in an organized way. As
organization becomes more complex, “division of labor” permits special­
ization on the one hand and provides circumstances under which the
“whole is greater than the sum of its parts.” This applies at the cellular
level. We now know the individual chemical reactions and proc­
esses of which the organelles like mitochondria, chloroplasts, nuclei,
ribosomes, etc., are capable. Nevertheless the growing cell as a whole
far outdoes the isolated particles in giving effect to these processes. This is conveniently seen in terms of photosynthesis or protein synthesis, for no one would yet claim that the isolated biochemical system can compete with the intact growing cell in these respects. It may, therefore, not be surprising in the present state of knowledge that the specialized biochemistry of tissues and organs may be so intimately associated with these structures that it is still easier to achieve the necessary conditions by allowing the cells and organs to grow than to create circumstances which elicit their biochemistry without the need for the entire process of growth and development.

In short, in the present state of knowledge, molecular biology and biochemical genetics tell us much about what is feasible, but it still requires a knowledge of growth and organization, of morphogenesis and differentiation, to understand the circumstances which dictate what will occur. The ultimate aim, though still a distant goal, is so to understand the circumstances that make their impact upon cells during growth, differentiation, and morphogenesis that one may successfully achieve the chemical differentiation without the necessity of recapitulating the entire morphogenesis. Success in this field would have both practical application and profound biological significance. In this chapter work toward this end by the methods of cell and tissue culture will be summarized.

II. The Unique Composition of Cells, Tissues, and Organs

Examples of these phenomena abound; only a few representative ones can be cited. Even in the early studies of reserve carbohydrates, as in the work of Parkin (267) on monocotyledons, the organ specificity of given storage products was noted. It is a familiar fact that starch forms in the leaves of such plants as the Jerusalem artichoke (Helianthus tuberosus), whereas in the tubers inulin occurs. The common annual sunflower (H. annuus), a closely related plant, forms starch in its leaves, but its roots form starch not inulin. Grafting experiments have shown that the characteristic storage product is determined by the organ site to which the products of photosynthesis are translocated; it is not in any way determined by the photosynthetic organ in question. It is axiomatic that storage organs like seeds may store fat, protein, or starch according to the plant in question, and these storage products may be very different if the products of metabolism are mobilized by other active organs of the plant body.

The study of nitrogen metabolism, greatly stimulated in the modern period by the methods of chromatography and ready means of separa-
tion and isolation, (cf. Chapter 4, Volume IVA), has shown the multiplicity of simple compounds that occur and the extent to which they are often localized in special tissues or organs (such as fruits, seeds, bulbs, and rhizomes). Storage organs may be characterized by local accumulations of compounds which may be comparatively rare elsewhere in that plant, or even elsewhere in the plant kingdom. For instance, γ-methyleneglutamine and γ-methyleneglutamic acid have been isolated from tulip bulbs by Zacharius, Pollard, and Steward (418). An extensive survey of amino acids occurring in other species has shown that these new compounds are not generally distributed in plants, as are asparagine and glutamine, and aspartic and glutamic acids (cf. 103, 104, 338). Similarly Pollard (273) found that crown gall tumor tissue of Kalanchoe caused by Agrobacterium tumefaciens accumulated γ-hydroxyvaline. The occurrence of γ-hydroxyvaline and its lactone seems to be limited to only one species (K. daigremontiana) (277). Azetidine-2-carboxylic acid is accumulated especially in the rhizomes of Convallaria majalis (the lily-of-the-valley) (100, 342).

But among the organ- and tissue-specific compounds that are laid down during growth, the proteins have always received attention. That the proteins of seeds and storage organs of perennation had distinctive characteristics motivated their early use in protein biochemistry (e.g., edestin). Even prior to modern ideas about gene regulation of metabolism via the determination of protein and enzyme synthesis, botanists had speculated upon the sequential changes in protein formation that might accompany development (cf. 268), and such ideas were expressed in the 1950's by the views of Brown et al. (291) on the progressive change of proteins and enzymes characteristic of different stages of development as seen along the axis of roots. Studies of this sort, whether applied to the fractionation of the total protein complement, the recognition of enzymes or their several isozymes have, in recent years, been greatly facilitated by the use of starch gel electrophoresis (224) and even later by acrylamide gel electrophoresis, as in the studies on roots (351) and on the organs to be found in tulip bulbs (18). So much so, that the prevalence of organ specificity in the occurrence of the proteins, enzymes, or isozymes (cf. Fig. 48, Chapter 4, Volume IVA) laid down during growth in plants (18) may now be acceptable as an axiom so that the attention is more to be focused upon the means by which their occurrence is regulated than upon the fact of their existence.

Such specialized tissue systems as laticifers (Figs. IE–G) are also associated with their own peculiar biochemistry, as the copious literature on opium, rubber (43, 250, 272, 331), chicle (cf. 408, 409), and papain testifies (161, 178). Secretory or glandular hairs, like those of Mentha (cf. Figs. 2A, B), are the seat of the production, or at least
the secretion, of extremely complex mixtures of essential oils and terpenes. The flavor and fragrance industries have exploited this ability of plants to make volatiles since essential oils are notoriously difficult mixtures to reproduce synthetically and at will (7).

Whole volumes have been written on the biochemistry of such organisms as fungi, and the fact that their storage products or their unique biochemistry is so often associated with spores, fruiting bodies, or host-specific parasitism, may here be merely mentioned (280, 384). But it is in the area of pharmaceutically important compounds and the ancient use of plants as sources of medicinal substances that the unique ability of plants to localize special features of their biochemistry in special organs is best seen. Some examples drawn from special categories of compounds follow.

A. Alkaloids

The elaboration of alkaloids is not generally considered to be localized in specific organs but appears to be a characteristic of all organs (including the seeds), although it must be emphasized that not all organs of any one species display such functions. Noteworthy among organs which are devoid of alkaloids are the seeds of *Nicotiana* sp. (tobacco) and of *Papaver somniferum* (the opium poppy) even though in other parts of the plant they may be produced abundantly. Although the seeds of these plants do not store detectable quantities of alkaloids, upon germination alkaloids are to be found even in the very young seedling. Although alkaloids of annuals may be localized in various organs, there may also be marked fluctuations of alkaloid content in any of the organs throughout the growing season; for example the period of maximum output of alkaloids in *Papaver* appears to coincide with the early flowering stage. With increased age, for example in biennials and perennials, alkaloids appear to be more localized in a few organs. The bark of trees is generally richer in alkaloids than are the leaves or shoots, and this may be attributed to their accumulation in the bark year after year.

Although it is difficult to generalize, there are certain evident tendencies in alkaloid production and distribution. Their concentration is normally high in (a) very active centers such as growing fruits, (b) epidermis, piliferous layer, and the layers just below them, (c) vascular sheaths and other parenchyma adjoining and penetrating the vascular tissue, (d) latex vessels when present. It is also noteworthy that the cells just behind the shoot and root meristems, and adjacent to the cambium and cork phellogen, which are so frequently reported as accumulating alkaloids, are in the stage of active vacuolization. Fre-
Fig. 1A–C. See legend on p. 234.
FIG. D–F. See legend on p. 234

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FIG. 1. Biochemistry and morphology: some specialized structures and their products. (A) Section of mustard (*Brassica nigra*) seed coat (testa) and embryo through radicle and cotyledons. (B) Section of seed coat to show cells which secrete mustard oil. Oil may be synthesized in the inner layer, secreted via the columnar palisade cells to accumulate in giant cells beneath the epidermis. (C) Section of linseed (*Linum usitatissimum*) showing cotyledons and testa. (D) Cross section of seed coat and outer region of endosperm. (E–G) The nonarticulated anastomosing laticiferous system of *Carica papaya* at various magnifications. The latex containing proteolytic enzymes constitutes a sap of the coenocytic multinucleate elements. (A–D, photos from slides supplied by Dr. A. Der Marderosian, Philadelphia College of Pharmacy and Science.)

sequently, epidermal hairs contain high concentrations of alkaloid. [For citations to, and confirmation of, the above observations, reference may be made to such major sources as James (164) and Mothes and Romeike (246).]

Grasses which form hordenine (\(N,N\)-dimethyltyramine) provide a clear illustration of the brief, but localized, appearance of inductive enzymes. No alkaloid is present in any part of the resting grain (e.g., as in barley, *Hordeum vulgare*). After a few days of germination, when the radicle emerges, determinable amounts equal to about 0.5\% of the dry weight are to found. The \(N,N\)-dimethyltyramine is, however, restricted to the meristematic cells at the root tip (cf. 221, 330).

**B. Volatile Oils**

The cells in which aromatic oils are deposited and the circumstances under which oil formation takes place have been extensively studied
The observation has been made that some of the cells or spaces in plants are filled with oil droplets, difficult to distinguish in situ from fats. The oil secretion often appears in localized areas and in very different cell types, and distinctions have been made between external and internal secretory cells. The external glands may be epidermal cells or modifications of these, such as the secretory hairs which occur in Mentha and other Labiatae. The secretion product is usually accumulated outside the cell between a cuticle and the rest of the cell wall. The cuticle is a thin layer which covers the secretions, and a slight touch suffices to break this (cf. Figs. 2A and B).

Internal glands may be located throughout the plant body; they are often formed by deposition of the oils between the walls of the cells, that is in intercellular cavities. This schism of cells has been called a schizogenous formation. If this is followed by dissolution of the surrounding cells, they are called schizolyssigenous glands. Often these intracellular glands grow to form long canals, lined on the inside with a layer of thin-walled cells. This layer is said to have a double function, namely the separation of other tissues from the oils and the formation of oils and resins. In such cases, the secretion forms in the epithelial cells or in the membranes and passes through the cell wall into the interior of the gland (Figs. 1A and B and 2C-G).

The literature on the exact site of formation of substances like terpenes, shows that secretion vacuoles may suddenly appear in the cell, then increase in number and size, whereupon the nucleus and cytoplasm are said to degenerate simultaneously (180). These oil globules seem often to be enveloped by a membrane. Some observers have seen small droplets of oil, formed in or near the chloroplast, which later coalesce to form larger drops. Other plants seem to accumulate the oil in the membranous layers adjoining the secretion pockets (2-4).

There are many data to emphasize that varying the environment greatly influences the quality and yield of volatile oils. It is less well known, however, that the different parts of the plant may simultaneously contain oils which are very different in chemical composition. As an extreme case, the composition of the oil of Ceylon cinnamon (Cinnamomum zeylanicum) may be cited, (cf. also Figs. 2C and E). Bark oils are high in cinnamic aldehyde, leaf oils are comprised primarily of eugenol, and the root oil contains a high percentage of camphor. Orange (Citrus sinensis) and lemon (Citrus limon) in flower and fruit contain oils of very different composition, and in numerous examples only certain of the plant parts contain the oil. Iris (Iris sp.), valerian (Valeriana sp.) and calamus (Acorus calamus) oils occur only in the roots; in sweet birch (Betula sp.) and cinnamon (Cinnamomum sp.)
Fig. 2A–C. See legend on p. 239.
Fig. 2D–E. See legend on p. 239.
Fig. 2F, G. See legend on facing page.
oils are found in the bark, whereas in the case of sandal (Santalum album) and cedar (Cedrus sp.) the heartwood contains the volatile oils.

C. GLYCOSIDES

Since the discovery in 1830 of salicin in the bark of willow (Salix), many glycosides from angiosperms have been isolated and characterized [cf. (5, 158, 228) for a discussion of these compounds]. The glycosides are usually present in the vacuole and are sometimes localized in particular cells (cf. 151), but more usually, they are accumulated in specific organs. Although many examples could be cited (cf. 266), the following case of the mustard oil glycosides will serve to make the point.

The mustard oil glycosides are a class of natural products which occur in a large variety of higher plants belonging to a relatively small number of plant families. They are characterized by the ability to undergo enzymatic hydrolysis to isothiocyanates ("mustard oils"), (cf. Figs. 1A and B) sulfuric acid, and \( \alpha \)-glucose. Invariably, the latter has been found to be the sugar moiety of the more than 50 individual compounds so far recorded, justifying their designation as glucosides (cf. 181–183).

Seeds of Iberis amara contain only glucoiberin, furnishing, on hydrolysis of the glucoside, the mustard oil \( \text{CH}_3\text{SO(CH}_2\text{)}_3\text{NCS} \); whereas seeds of I. sempervirens have a mixture of three glucosides, viz., glucoibervirin, glucoiberin, and glaucouerin giving rise to \( \text{CH}_3\text{S(}\text{CH}_2\text{)}_3\text{NCS} \), \( \text{CH}_3\text{SO(CH}_2\text{)}_3\text{NCS} \), and \( \text{CH}_3\text{S(}\text{CH}_2\text{)}_4\text{NCS} \), respectively. It has been noted however that root material of I. amara contains glucoibervirin rather than glucoiberin. Mention will be made later concerning the examination of cultures of Iberis for its mustard oil glycosides.

In all these diverse systems, whether the resultant product occurs extra- or intracellularly, or even whether it remains after the cells have succumbed, the central problem remains: namely, what factors predispose a given set of cells to do what they do?

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Fig. 2. Biochemistry and morphology: some specialized structures and their products. (A) Glandular hairs of peppermint (Mentha piperita) which secrete essential oils shown in section and surface view. (B) Early and late stages in the development of glands of Mentha. (C) Cross section through the bark of Ceylon cinnamon (Cinnamomum zeylanicum) which contains the oil. (D) Section through the unopened flower bud of clove (Eugenia caryophyllata), which contains oil in the glands shown. Oil is synthesized in the peripheral cells and secreted into the cavity of the gland. (E) The oil glands of cinnamon bark (cf. C). (F) Cross section of leaf of Pinus strobus showing a ring of resin-containing ducts in the parenchyma. (G) A resin duct showing central cavity surrounded by secretory cells. [A and B after (555); C and D photos from slides supplied by Dr. A. Der Marderosian, Philadelphia College of Pharmacy and Science.]
It should be recognized, however that the characteristic or unique biochemistry elicited in organs and tissues during growth and development may be a function of nutrition as well as environment. Again this has been shown by references to a variety of nitorgenous compounds that are produced by plants. One can change the soluble nitrogen compounds of mint plants very greatly by controlling such environmental factors as their mineral nutrition and the factors of length of day and night temperature, which interact with nutrition to determine how the genetically determined biochemistry shall be expressed. This has been documented (332, 355). Other examples of this phenomenon could also be quoted (cf. 338).

Nevertheless, although nutrition and environment may greatly modify biochemical expression, there are recognizable sequences which are dictated by the growth and differentiation of the organ in question. The technique of gel electrophoresis, which more sensitively reveals the composition of the soluble protein fraction in cells than earlier procedures, has permitted this fact to be brought out in certain systems (351). Passing from the apex of the root of pea (Pisum sativum) along the axis, changes in the protein composition occur concomitantly with differentiation. In the tulip (Tulipa) bulb such morphogenetic modifications of leaves as bulb scales, petals, sepals, even anthers and ovules have their distinct protein complements, though presumptively these are all determined in cells and by nuclei which are genetically similar (18).

Proteins with characteristic properties as enzymes are well known to accumulate in specific situations, even though their enzymatic qualities may not there be utilized. Urease in the cotyledons of the jack bean (Canavalia ensiformis) is an outstanding example (cf. 310), and papain in the laticifers of Carica papaya (cf. Figs. 1E–G), ficin in figs (Ficus carica), and bromelin in pineapple (Ananas comosus) are equally striking (cf. 307).

The fact that unique composition is the normal outcome of the growth and development of cells, organs, and tissues is therefore obvious. The question is now as follows. Can the separate culture of the tissues or cells that are associated, in the intact plant body, with a characteristic biochemistry successfully recapitulate the conditions that obtain in the intact plant?

III. Cultured versus Quiescent Tissues: The Events of Growth Induction

When previously inactive or senescent cells spring into active growth, as in the phenomenon of wound healing, many events occur. Characteristically, respiration increases, metabolic activity passes from the low level
that obtained in the mature storage organ, water and salt uptake ensue, cells divide, and characteristically storage products (in which the mature cells were rich) tend to disappear and to be drawn upon for the new growth that occurs. A typical example of this sort of situation is to be found in the cells of the tissue of the potato (Solanum tuberosum) tuber; information on this system has previously been referred to in this treatise (Vol. II, Chapter 4, p. 335 et seq.). Even more striking are the changes which ensue when hitherto resting or quiescent cells are made to grow and to divide actively in the freely suspended state. The cells as they exist and grow free, frequently bear but little resemblance to the comparable cells as they existed in the intact system. This has been shown for cells of carrot (Daucus carota var. sativus), potato tuber, and the storage parenchyma in the banana (Musa) fruit (240, 335).

Many other examples of this contrast between resting, storage, normally developed tissue and freely suspended, actively growing cells could be cited, but the interesting point emerges that freely suspended rapidly growing cells in liquid media, caused to grow under the influence of such growth factors as those to be found in coconut milk, all tend to resemble each other rather strikingly, even though they differ in appearance from the cells in their tissue of origin (cf. Fig. 3). But again the point of resemblance concerns chiefly the absence of storage materials in the growing cells, and this applies not only to such visible structures as starch grains, but also to the characteristic soluble nitrogen compounds which, commonly rich in the storage tissue, are maintained at very much lower levels in the actively growing cells (353). Moreover, the relative composition of the soluble nitrogen as it exists in the growing cells is usually very different from that of the resting counterparts. Such nitrogen-rich substances as asparagine or arginine frequently tend to disappear, and the only such nitrogen-rich substance that tends to persist in the growing tissue, but at a much lower level than in the nongrowing, is often glutamine (cf. Table I). Thus the ratio of protein nitrogen to alcohol soluble (non-protein) nitrogen is very much greater in the growing than in the nongrowing tissue (Table II), and, were we able fully to fractionate the protein into its individual constituents, it is certain that the protein of the rapidly growing cells might be very different from the complement of protein to be found in the resting cells. Indeed, the information already available indicates the the bulk protein of the growing tissue is at least richer in the basic amino acids, so-called histone bases, as indeed it is also richer in a protein moiety that contains far more hydroxyproline than is common in the protein of resting cells (275, 341, 348).

Braun and co-workers (52–54) have also called attention to the
Fig. 3. Contrasts between quiescent cells and their active counterparts growing free. (A) Resting cell of the secondary phloem of carrot root, showing carotene in chromatophores. (B) Resting cell of potato tuber showing abundant starch grains. (C) Resting cell of banana fruit pulp showing abundant starch grains. (D) Free cell of carrot grown in liquid medium containing coconut milk (10%) (Compare
A). (E) Free cells of potato tuber that have grown in a liquid medium containing coconut milk (10%) and 2,4-dichlorophenoxyacetic acid (2,4-D) at 6 ppm (Compare B). (F) Free cells of banana fruit activated to grow by coconut milk and naphthaleneacetic acid (NAA) (Compare C). From Steward, Canad. J. Botany 39, 441-460 (1961). Reproduced by permission of the National Research Council of Canada. See also Steward et al. (335).
metabolic differences in rapidly growing and resting cells with reference to tumor and gall development (10, 309). Attention may also be drawn to differences in susceptibility to gamma irradiation. Resting, quiescent carrot tissues prior to the induction of growth are very vulnerable to the indirect effects of radiation. By contrast, if growth by cell division has occurred, the cells become surprisingly resistant to much higher dosages of radiation (cf. 159). At the same time, resting quiescent cells of carrot tissue are more resistant to cyanide and carbon monoxide than are the postinduction cells; the converse is true of their susceptibility to such uncoupling agents as the nitrocresols.

Other examples could also be cited: for example actively growing cells maintain a lower internal concentration of Cs137 than do non-

\[ \text{Aspartic acid} \]  
\[ \text{Glutamic acid} \]  
\[ \text{Serine} \]  
\[ \text{Glycine} \]  
\[ \text{Asparagine} \]  
\[ \text{Threonine} \]  
\[ \text{Alanine} \]  
\[ \text{Glutamine} \]  
\[ \text{Lysine} \]  
\[ \text{Arginine} \]  
\[ \text{Methionine} \]  
\[ \text{Proline} \]  
\[ \text{Valine} \]  
\[ \text{Leucines} \]  
\[ \text{Phenylalanine} \]  
\[ \text{Tyrosine} \]  
\[ \text{γ-Aminobutyric acid} \]

**TABLE I**

**COMPARISON OF THE NITROGENOUS COMPOSITION OF THE ALCOHOL-SOLUBLE FRACTION OF MATURE, NONGROWING, AND TISSUE-CULTURED PLANT TISSUES**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Potato tissue</th>
<th>Carrot tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-growing</td>
<td>Cultured</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>11.22</td>
<td>1.44</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>16.95</td>
<td>12.73</td>
</tr>
<tr>
<td>Serine</td>
<td>8.81</td>
<td>7.28</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.15</td>
<td>8.00</td>
</tr>
<tr>
<td>Asparagine</td>
<td>291.5</td>
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</tr>
<tr>
<td>Threonine</td>
<td>11.83</td>
<td>4.50</td>
</tr>
<tr>
<td>Alanine</td>
<td>20.75</td>
<td>21.50</td>
</tr>
<tr>
<td>Glutamine</td>
<td>579.0</td>
<td>15.72</td>
</tr>
<tr>
<td>Lysine</td>
<td>12.1</td>
<td>2.62</td>
</tr>
<tr>
<td>Arginine</td>
<td>114.5</td>
<td>15.46</td>
</tr>
<tr>
<td>Methionine</td>
<td>7.74</td>
<td>0.0</td>
</tr>
<tr>
<td>Proline</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Valine</td>
<td>29.1</td>
<td>6.81</td>
</tr>
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<td>Leucines</td>
<td>11.15</td>
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<tr>
<td>Phenylalanine</td>
<td>11.71</td>
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</tr>
<tr>
<td>Tyrosine</td>
<td>9.38</td>
<td>3.66</td>
</tr>
<tr>
<td>γ-Aminobutyric acid</td>
<td>40.75</td>
<td>88.3</td>
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</table>

* From Steward et al. (353).

* Values are expressed as micrograms of nitrogen per amino acid per gram fresh weight.

\[ ^1 \text{Wood et al. (415) now believe they have recognized the substance in cells which causes autonomous, tumorous growth. The substance in question is not a substituted aminopurine though it is now said to be produced by many cells and tissues in response to treatment with kinetin.} \]
proliferating tissues which absorb and store Cs\textsuperscript{137} in vacuoles, although the absorption of Cs\textsuperscript{137} by actively growing cultures occurs progressively throughout their growth period (235).

All this amounts to the recognition that, despite the constancy of the genetic information in potentially totipotent living cells, its expression in terms of the biochemistry may be very different, according as the cells are actively growing and dividing, or as they exist in the

TABLE II

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Carrot</th>
<th>Potato</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Initial non-</td>
<td>Final non-</td>
</tr>
<tr>
<td></td>
<td>growing</td>
<td>growing</td>
</tr>
<tr>
<td>Fresh weight (mg/explant)</td>
<td>3.0</td>
<td>107.8</td>
</tr>
<tr>
<td>Nonprotein nitrogen ((\mu g/g) fresh weight)</td>
<td>801</td>
<td>124</td>
</tr>
<tr>
<td>Protein nitrogen ((\mu g/g) fresh weight)</td>
<td>741</td>
<td>898</td>
</tr>
<tr>
<td>Nonprotein nitrogen ((\mu g/g) explant)</td>
<td>2.4</td>
<td>13.4</td>
</tr>
<tr>
<td>Protein nitrogen ((\mu g/g) explant)</td>
<td>2.2</td>
<td>96.6</td>
</tr>
<tr>
<td>Ratio of nonprotein to protein nitrogen</td>
<td>1.07</td>
<td>0.138</td>
</tr>
<tr>
<td>Protein nitrogen synthesized ((\mu g/explant))</td>
<td>94.7</td>
<td>97.2</td>
</tr>
<tr>
<td>Ratio of final to initial protein nitrogen</td>
<td>44</td>
<td>31</td>
</tr>
</tbody>
</table>

*a From Steward et al. (353).*

mature, quiescent state in the intact plant or organ in which they normally occur. Thus it is not surprising that it has proved a difficult, but still challenging, task to induce cells in culture to form the characteristic metabolites and storage products which develop normally in the intact organ. This difficult problem requires that the cells in culture be brought, by external means, under the same kind of restraints that apply to them in their normal situation. While this has not yet been possible in many instances, it is worthwhile to document the kinds of attempts that have been made to achieve these ends.

The means that have been employed to convert the metabolism of
the actively growing cultured system into that of the more mature organ can be summarized in principle as follows:

1. Consideration has been given to the natural maturation of cells, etc. (cf. 25a), due to the removal or exhaustion of essential nutrients, organic or inorganic, or due to the accumulated "staling products" which the metabolism of the tissue itself produces.

2. One can imagine that to produce a given metabolite, a given complement of enzymes might need to be formed in the growing cell; the techniques of enzyme induction have been employed by adding what seemed to be an appropriate substrate to the medium.

3. There is a difficulty in comparing nutritional requirements \textit{in vivo} with the components of a culture medium, for the synthetic ability of the intact plant is significantly more complete that is that of tissue explants. Thus, it is probable that growing cells and tissues in culture require growth factors over and above those essential to the intact plant from which they were derived. One can assume that the cultured free cells might lack some essential metabolite in a given biosynthetic sequence which in fact might be supplied from other cells or organs through translocation. Thus one could furnish to potentially starch-forming cells glucose-1-phosphate or uridinediphosphate glucose in the hope that this might foster starch formation. However, such experimentation cannot usefully be carried very far in some instances, for the permeability of plant cell membranes under the conditions of tissue culture has not been specifically tested, and a substance may seem of no avail in a medium when, in fact, the reason for its apparent inactivity is that it cannot reach the active sites.

4. Such devices as those made familiar under the principle of metabolic feedback offer possibilities of inducing drastic change in the metabolism of cells as they grow and develop. For example, feedback inhibition or activation of enzyme activity by intermediates and end products of the pathway involve rapid interactions between small molecules and macromolecules. The time required for such a system to return to the equilibrium state once it has been disturbed is very short; of the order of a few milliseconds or, for complex systems, a few minutes at most. The binding of an "effector" molecule to a site other than the active site of the enzyme can greatly change the relationship between the speed of the reaction and the substrate concentration. The response of the enzyme to such binding has been termed an \textit{allosteric effect} (242). The effector molecule may be an end product or an intermediate in the pathway or be some other small molecule; it may activate rather than inhibit, and there may be more than one effector for a given allosteric protein.
5. But it is also possible that cultures may be called upon to perform fewer synthetic functions than the intact plant and may be able to dispense with certain substances in the medium. To this end, media which would encourage a more autotrophic metabolism have been used, e.g., the lowering or elimination of a reduced carbon source. Some tissues (e.g., tobacco) will grow in the complete absence of sucrose from the medium, since green strains are capable of photosynthesis (32).

IV. The Use of Tissue and Cell Cultures to Study Metabolism and to Produce Specific Compounds

Many papers have emphasized the potential of plant tissue cultures, but few have critically evaluated the pertinent work with the specific intention of stating precisely the problem and the difficulties to be overcome to achieve full control of their growth and metabolism (cf. 64, 185, 257, 325, 328, 363, 364, 379).

With the mounting evidence of "totipotency" [cf. (334, 349, 352) and Chapter 8 of this volume] there is every reason to believe that use of the synthetic abilities of cultured cells and tissues could be exploited in the same way as those of microorganisms (20, 280, 384). Although the organization of the plant body itself places certain restrictions upon the continued growth of many of its cells and tissues, these restrictions may be circumvented if the cells are grown free and in isolation. The problem is to evoke the synthetic potentialities. An attempt will now be made to consolidate work done in this area.

A. Nonprotein Nitrogen Compounds

That cultured plant tissues synthesize the usual (e.g., protein) amino acids should occasion no surprise; in fact, it would be surprising if this were not so. Despite this, many casual references have been made to the synthesis of such compounds by cultured tissue (cf. 64, 257, 394, 406).

Since the use of paper chromatography on an alcoholic extract of the potato tuber, many new nitrogen compounds have been disclosed (cf. 338, 340, 342, 343). The significance of all these compounds is not yet clear, but the idea that the soluble nitrogen pool merely comprises the prefabricated compounds needed for protein synthesis must now be discarded (103, 104, 336, 342). Many nonprotein nitrogenous compounds exist, and often occur in large amount. Their presence suggests that many normal metabolic routes have passed unsuspected. One obvious
A. D. KRIKORIAN AND F. C. STEWARD

approach to the problems posed by the presence of these nitrogen compounds is that of tissue culture. The synthesis and behavior of these special compounds can be studied under controlled conditions with the idea of relating the appearance or disappearance of them to some overall metabolic scheme (cf. 22, 405, 406).

1. **γ-Glutamyl Compounds of Peanut** (*Arachis hypogaea*) **Cultures** (199)

In 1952, a new amino acid amide and its corresponding dicarboxylic amino acid [γ-methyleneglutamine (γ-MG) and γ-methyleneglutamic acid (γ-MGA)] were isolated from the vegetative parts of peanut plants (85). The same substances were also isolated from tulip bulbs (418). The parent substance from which the methylene compounds might be derived is γ-hydroxy-γ-methylglutamic acid (339); this has been recognized in several sources (101, 138, 354).

Fowden (99) found that the compounds exist in homogenates of stems, leaves, hypocotyls, and roots of peanut; but, the new compounds could not be detected in seedlings less than 2 days after germination. However, they always occurred after the differentiation of the first leaf. The stem, root, and hypocotyl of seedlings germinated and maintained in the dark for 12 days all contained γ-MG. It could not be detected in immature or ripe seeds, or in the hydrolyzates of a commercial sample of the protein arachin. Thus, the compound which appeared after germination, occurred in all the vegetative tissues examined except the cotyledons.

The existence of these gamma-substituted compounds in a few distantly related plants suggests that they form a closely related metabolic group. However, the enzymes that catalyze the interconversion of γ-MGA and γ-MG (35), have not been demonstrated although experiments in which labeled pyruvic acid or alanine was supplied to peanuts (105) indicated a common metabolic precursor.

All the evidence from this laboratory is that γ-MG and γ-MGA are sluggish metabolites, very difficult to label with C\textsuperscript{14} and appear to have very low rates of turnover (102, 279). Although γ-MG and γ-MGA are not present in the seed proteins, they do rapidly appear after the protein breakdown that accompanies seed germination.

The cultures established in this laboratory for the purpose of studying the metabolism of these compounds, were derived either from peanut cotyledons or stem. These cells were originally recalcitrant, but they now grow vigorously on a basal medium supplemented with coconut milk and casein hydrolyzate. Examination of peanut cell cultures has not yet disclosed γ-MG and γ-MGA. Additional growth supplements to the media did bring about some changes in the soluble (cf. Table III)
and alcohol-insoluble content of the cultures. The cells grown in media supplemented with coconut milk, i.e., without other synergists, contained more soluble nitrogen than the cells grown in other media. The other media, in decreasing order for the amount of soluble nitrogen they induced contained benzthiazoloyloxyacetic acid (BTOA), indoleacetic acid (IAA), naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,3,6-trichlorophenylacetic acid (2,3,6-TPAA).

In none of the cultured peanut cells was there any chromatographically detectable γ-MG or γ-MGA. There were, however, a number of familiar amino acids (Table III), the amides, asparagine, and glutamine were notably inconspicuous, and there were other unidentified compounds, but these were in very minute quantities.

### TABLE III
**Alcohol-Soluble Nitrogen of Peanut Cells Cultured in Various Media over a Period of 30 Days**

<table>
<thead>
<tr>
<th>Compound</th>
<th>CM</th>
<th>CM + BTOA</th>
<th>CM + IAA</th>
<th>CM + NAA</th>
<th>CM + 2,4-D</th>
<th>CM + 2,3,6-TPAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>3.0</td>
<td>5.5</td>
<td>2.4</td>
<td>9.5</td>
<td>1.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.0</td>
<td>9.4</td>
<td>4.7</td>
<td>4.6</td>
<td>5.1</td>
<td>5.6</td>
</tr>
<tr>
<td>Serine</td>
<td>5.0</td>
<td>2.2</td>
<td>5.3</td>
<td>3.6</td>
<td>1.9</td>
<td>2.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.7</td>
<td>3.3</td>
<td>7.2</td>
<td>2.3</td>
<td>4.0</td>
<td>4.2</td>
</tr>
<tr>
<td>Asparagine</td>
<td>8.2</td>
<td>5.4</td>
<td>6.6</td>
<td>5.3</td>
<td>21.7</td>
<td>22.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.4</td>
<td>9.5</td>
<td>4.7</td>
<td>11.5</td>
<td>7.6</td>
<td>5.4</td>
</tr>
<tr>
<td>Alanine</td>
<td>21.2</td>
<td>6.9</td>
<td>11.7</td>
<td>6.6</td>
<td>12.6</td>
<td>11.7</td>
</tr>
<tr>
<td>Glutamine</td>
<td>3.9</td>
<td>10.7</td>
<td>6.7</td>
<td>10.2</td>
<td>5.6</td>
<td>6.3</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.4</td>
<td>4.2</td>
<td>2.1</td>
<td>2.6</td>
<td>3.33</td>
<td>2.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.9</td>
<td>4.9</td>
<td>6.6</td>
<td>5.7</td>
<td>5.5</td>
<td>5.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>8.3</td>
<td>8.1</td>
<td>9.3</td>
<td>7.7</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Proline</td>
<td>4.9</td>
<td>5.0</td>
<td>6.6</td>
<td>4.7</td>
<td>6.7</td>
<td>10.6</td>
</tr>
<tr>
<td>Valine</td>
<td>5.3</td>
<td>6.2</td>
<td>4.0</td>
<td>7.3</td>
<td>3.3</td>
<td>4.0</td>
</tr>
<tr>
<td>Leucine(s)</td>
<td>8.4</td>
<td>9.2</td>
<td>11.6</td>
<td>9.4</td>
<td>3.3</td>
<td>3.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.8</td>
<td>2.2</td>
<td>4.0</td>
<td>2.6</td>
<td>4.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.9</td>
<td>4.3</td>
<td>3.4</td>
<td>4.2</td>
<td>6.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Pipecolic acid</td>
<td>1.0</td>
<td>2.2</td>
<td>2.0</td>
<td>1.1</td>
<td>2.6</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Total μg amino acid nitrogen (μg/g fresh weight of tissue)  
94.6 78.3 78.1 71.0 56.2 44.2

---

*a Nitrogen of each compound as percentage of total soluble nitrogen in compounds determined by ninhydrin. The basal medium consisted of White's basal medium, coconut milk (CM) 10%, and casein hydrolyzate 200 mg/liter plus one of the following: BTOA 10 mg/liter, IAA 1 mg/liter, NAA 5 mg/liter, 2,4-D and 2,3,6-TPAA, 5 mg/liter.*
Although no treatment tried induced γ-MG or γ-MGA to form, it was, nevertheless, possible to modify the composition of the free nitrogen compounds in the peanut cells by altering the nutrient medium. The changes so induced however, were more quantitative than qualitative.

### TABLE IV

**Alcohol Soluble and Insoluble Nitrogen of Peanut Cells Cultured in Light and Dark over a Period of 16 Days**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Light grown</th>
<th>Dark grown</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble[^b]</td>
<td>Insoluble[^c]</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>7.3</td>
<td>6.4</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>6.0</td>
<td>17.2</td>
</tr>
<tr>
<td>Serine</td>
<td>4.4</td>
<td>4.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.6</td>
<td>7.0</td>
</tr>
<tr>
<td>Asparagine</td>
<td>12.5</td>
<td>—</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.3</td>
<td>5.8</td>
</tr>
<tr>
<td>Glutamine</td>
<td>9.1</td>
<td>—</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.8</td>
<td>5.9</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.5</td>
<td>7.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.4</td>
<td>15.9</td>
</tr>
<tr>
<td>Proline</td>
<td>7.3</td>
<td>9.4</td>
</tr>
<tr>
<td>Valine</td>
<td>8.2</td>
<td>4.7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>—</td>
<td>3.1</td>
</tr>
<tr>
<td>Leucine(s)</td>
<td>4.1</td>
<td>5.6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>6.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Pipelic acid</td>
<td>1.3</td>
<td>—</td>
</tr>
</tbody>
</table>

| Total amino acid nitrogen (μg/g fresh weight of tissue) | 69.1 | 1115.0 | 42.7 | 1124.0 |

[^a]: Nitrogen of each compound as percentage of the total soluble or insoluble nitrogen in compounds determined by ninhydrin. The medium consists of White’s basal medium plus coconut milk 10% and casein hydrolyzate 200 mg/liter.

[^b]: Quantitative paper chromatographic data.

[^c]: Beckman Spinco amino acid analyzer data.

Since none of the standard growth promoters caused the formation of γ-MG and γ-MGA in the cultured peanut cells, the effects of light and dark were tested (cf. Table IV).

The cells grown in light had slightly more free soluble amino acids than those grown in the dark, but there was no difference in the
amount of alcohol-insoluble nitrogen. Again, however, none of the cells so grown contained any detectable \( \gamma \)-MG or \( \gamma \)-MGA. Hence the cultured peanut cells do not readily respond to changes in the light and dark regime during growth by the synthesis of these compounds.

The outstanding effect on the soluble fraction related to asparagine and glutamine. The glutamine N content was greater in the light (9.1% of soluble N) than in the dark (3.9% of soluble N) whereas the asparagine N content was greater in the dark (35.5% of soluble N) than in the light (12.5% of soluble N). These differences are in a now familiar direction, for light often favors glutamine-N content of leaves and darkness often favors asparagine [cf. Steward (332) for a discussion of the effects of light and darkness on nitrogen metabolism]. To this extent the cells cultured from cotyledons (and stems) behaved like leaves. Some other free amino acids seemed to be relatively favored by light (aspartic, serine, threonine, valine) whereas others (proline, glycine) seemed to be favored by darkness. In spite of this degree of modification to the free nitrogen compounds the \( \gamma \)-methylene compounds did not appear as a result of these treatments, nor were any significant effects observed on the composition of the bulk protein.

2. \( \gamma \)-Hydroxyvaline in Kalanchoe daigremontiana (199)

Pollard (273) observed certain unidentified compounds on chromatograms of the alcohol soluble fraction of *Kalanchoe daigremontiana*. It subsequently appeared that these substances were related as a free hydroxyamino acid (hydroxyvaline) and its lactone (277, 342). This amino acid does not enter into proteins as such, and is present only in the soluble amino acid pools.

Table V lists the analysis of the alcohol-soluble nitrogen of young plants of *Kalanchoe daigremontiana* and compares the amino acid composition of the leaves, stems, and roots with cultured cells derived originally from stem pith. Table VI compares the protein amino acid composition for the same materials.

Although there was relatively little soluble nitrogen in the intact plant organs, there was present a relatively large amount of \( \gamma \)-hydroxyvaline and \( \gamma \)-aminobutyric acid. By contrast, the cultured tissue contained no \( \gamma \)-hydroxyvaline and only a trace of \( \gamma \)-aminobutyric acid. No great difference between the bulk protein composition of the cultured and noncultured tissue was found.

Although Pollard (273) found that crown gall tumor tissue of *Kalanchoe* contained more hydroxyvaline and hydroxyproline than nontumorous tissue, it appears that this difference does not apply generally to cultured tissues (cf. Table VI).
3. The Metabolism of Cultured Haplopappus gracilis (199)

The main difference between the relative composition of the soluble-N compounds in the intact plant and in cultured tissue of *Haplopappus* lies in the relative emphasis upon amides (and γ-aminobutyric acid) in the whole plant and upon alanine in the cultured tissue. Alanine often tends to be a more conspicuous feature of the free nitrogen compounds of the cultured cells than of their resting counterparts; this is also true of cultured carrot and potato tissue (34).

The composition of the alcohol insoluble fraction of cultured and normal *Haplopappus* tissue also appears to be quite similar. Whereas hydroxyproline was missing from the protein of the intact plant, there was a detectable amount present in the protein of the cultured cells; this feature has been noted in other cultures (cf. carrot and potato).

**TABLE V**

Comparison of the Alcohol-Soluble Nitrogen of Leaves, Stems, and Roots of *Kalanchoe daigremontiana* with Cells Cultured in a Basal Medium, Coconut Milk 10%, and NAA 5 mg/liter for 40 Days in Continuous Light*$^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cultured tissue</th>
<th>Leaves</th>
<th>Stems</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>5.6</td>
<td>3.3</td>
<td>1.9</td>
<td>7.6</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>8.9</td>
<td>5.3</td>
<td>10.7</td>
<td>15.1</td>
</tr>
<tr>
<td>Serine</td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>1.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>Present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>18.1</td>
<td>32.5</td>
<td>42.2</td>
<td>31.7</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amides</td>
<td>27.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>3.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>10.0</td>
<td>3.2</td>
<td>4.7</td>
<td>5.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>Trace</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>4.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>4.7</td>
<td>Trace</td>
<td>Trace</td>
<td>2.3</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-Aminobutyric acid</td>
<td>Trace</td>
<td>39.1</td>
<td>29.0</td>
<td>25.9</td>
</tr>
<tr>
<td>β-Alanine</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-Hydroxyvaline</td>
<td>None</td>
<td>16.3</td>
<td>11.1</td>
<td>11.5</td>
</tr>
</tbody>
</table>

Total amino acid nitrogen (µg/g fresh weight) | 109.0 | 53.4 | 67.5 | 54.8

*$^a$ Nitrogen of each compound as percentage of total soluble nitrogen in compounds determined by ninhydrin.
7. Biosynthetic Potentialities of Tissue

There were no conspicuous differences among cultured strains except with reference to the leucines (Table VII) which also tended to be richer in the cultured cells than in the protein of the intact plant.

4. Nitrogenous Compounds of Jack Bean (Canavalia ensiformis)

Krikorian (199) examined cultured tissue of jack bean cotyledons. Table VIII presents the amino acid analysis of cultured cotyledonal tissues of jack bean for comparison with that of the normal tissue. The cultures were grown for a period of 40 days on a basal medium supplemented with coconut milk 10% and 2,4-D 5 mg/liter. The following features may be noted. There was much less total nitrogen in the cultured tissues; the intact cotyledonal tissue contained large amounts of both free and combined amino acids. In addition to the difference in the overall quantity of nitrogen present, the relative composition of the soluble amino acids also varied. Foremost in this respect was the absence of canavanine in the cultures. This was so apparent that there could be no doubt that the canavanine present in the original tissue had somehow been utilized during the culture period and it was not replaced by the growing tissue from the exogenous sources of nitro-

![Table VI](image)

Comparison of the Alcohol-Insoluble Nitrogen of Leaves, Stems, and Roots of Kalanchoe daigremontiana with Cells Cultured in a Basal Medium, Coconut Milk 10%, and NAA 5 mg/liter for 40 Days in Continuous Light

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cultured tissue</th>
<th>Leaves</th>
<th>Stems</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>7.3</td>
<td>9.4</td>
<td>8.4</td>
<td>12.7</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.2</td>
<td>6.9</td>
<td>8.3</td>
<td>9.5</td>
</tr>
<tr>
<td>Serine</td>
<td>7.0</td>
<td>5.9</td>
<td>4.6</td>
<td>4.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.8</td>
<td>8.4</td>
<td>7.9</td>
<td>8.4</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.5</td>
<td>6.0</td>
<td>5.9</td>
<td>6.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.7</td>
<td>9.5</td>
<td>8.0</td>
<td>7.4</td>
</tr>
<tr>
<td>Lysine</td>
<td>8.6</td>
<td>11.3</td>
<td>8.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>12.4</td>
<td>21.7</td>
<td>15.9</td>
<td>13.8</td>
</tr>
<tr>
<td>Proline</td>
<td>10.8</td>
<td>3.7</td>
<td>11.7</td>
<td>11.2</td>
</tr>
<tr>
<td>Valine</td>
<td>5.8</td>
<td>7.3</td>
<td>5.9</td>
<td>7.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.2</td>
<td>10.9</td>
<td>13.1</td>
<td>9.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>Trace</td>
<td>Trace</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Total amino acid nitrogen (µg/g fresh weight)</td>
<td>874.8</td>
<td>794.4</td>
<td>686.3</td>
<td>631.4</td>
</tr>
</tbody>
</table>

* Nitrogen of each compound as percentage of total insoluble nitrogen in compounds determined by ninhydrin.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Alcohol-soluble nitrogen</th>
<th>Alcohol-insoluble nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact plant</td>
<td>DS</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5.2</td>
<td>2.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.4</td>
<td>6.7</td>
</tr>
<tr>
<td>Serine</td>
<td>3.5</td>
<td>8.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.3</td>
<td>4.0</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.2</td>
<td>2.7</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.4</td>
<td>26.7</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Amides</td>
<td>33.8</td>
<td>13.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Lysine</td>
<td>3.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Arginine</td>
<td>11.9</td>
<td>10.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Proline</td>
<td>2.3</td>
<td>8.8</td>
</tr>
<tr>
<td>Valine</td>
<td>6.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>5.4</td>
<td>2.3</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.7</td>
<td>1.7</td>
</tr>
<tr>
<td>β-Alanine</td>
<td>0.5</td>
<td>—</td>
</tr>
<tr>
<td>7-Aminobutyric acid</td>
<td>6.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Pipolic acid</td>
<td>3.2</td>
<td>Trace</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Total nitrogen (μg/g fresh weight of tissue) 468.8 107.2 122.6 115.1 285.8 107.2 113.8 116.1

* The data presented show the analyses of the intact plant extract and the ninhydrin reactive compounds of strains DS, G, and F as grown in cell culture where DS = dark, red pigmented strain; F = friable strain; G = green strain (see page 282 and Fig. 11.) Data presented as percentage of nitrogen per amino acid per gram fresh weight of tissue. A peak which corresponded to ethanolamine was present in all cultured samples but was absent in the intact plant.

b The entire plant was extracted.
TABLE VIII

COMPARISONS BETWEEN THE ALCOHOL-SOLUBLE NITROGEN AND ALCOHOL-INSOLUBLE NITROGEN OF NORMAL AND CULTURED JACK BEAN COTYLEDONS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Normal nitrogen</th>
<th>Insoluble nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble</td>
<td>Insoluble</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Cultured</td>
</tr>
<tr>
<td></td>
<td>Total nitrogen</td>
<td>(mg/g fresh weight)</td>
</tr>
<tr>
<td></td>
<td>22.0</td>
<td>41.2</td>
</tr>
<tr>
<td></td>
<td>Total amino acid (mg/g fresh weight)</td>
<td>80.4</td>
</tr>
</tbody>
</table>

* Nitrogen of each compound as percent of total soluble or insoluble nitrogen in compounds determined by ninhydrin.

b Both amides were present although not individually determined on the amino acid analyzer.

gen. Williams and Hunt (410) have suggested that canavanine synthesis actually does not occur in developing seeds, but rather the canavanine is accumulated there after transport from the fruit which is the site of synthesis [cf. also Johnstone (168)].

5. Nitrogenous Compounds of Cultured Papaya (Carica papaya)

Fruit Tissue (199)

Cultures developed from explants of papaya fruit grown on a high salt basal medium (252) supplemented with coconut milk 15% and
2,4-D 5 mg/liter for a period of 90 days were analyzed for both alcohol-soluble and insoluble nitrogen compounds (see Table IX).

The total soluble nitrogen (milligrams of nitrogen per gram fresh weight) was slightly less than that of the younger fruit wall (976 μg of nitrogen in a young—one-eighth of mature size—in contrast to 765 μg of nitrogen in the cultured tissue) but was more than that found in the older (one-fourth to one-half the mature size) fruit wall.

There were several other conspicuous differences between the non-cultured and cultured fruit wall.

The normal fruit contained a substance, encountered elsewhere (e.g., in Phlox and Hemerocallis) (cf. 342), which is known to be a sub-

<table>
<thead>
<tr>
<th>TABLE IX</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alcohol-Soluble Nitrogen of Cultured and Noncultured Fruit Wall</strong></td>
</tr>
<tr>
<td>of <em>Carica papaya</em> &quot;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cultured</th>
<th>Noncultured</th>
<th>Noncultured</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1/2 mature</td>
<td>3/4 to 1/2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fruit</td>
<td>mature fruit</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>8.62</td>
<td>8.05</td>
<td>9.85</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.96</td>
<td>3.00</td>
<td>3.13</td>
</tr>
<tr>
<td>Serine</td>
<td>5.62</td>
<td>1.97</td>
<td>3.52</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.31</td>
<td>1.85</td>
<td>2.31</td>
</tr>
<tr>
<td>Asparagine</td>
<td>9.01</td>
<td>6.05</td>
<td>5.56</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.13</td>
<td>3.81</td>
<td>3.71</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.84</td>
<td>17.1</td>
<td>14.5</td>
</tr>
<tr>
<td>Glutamine</td>
<td>10.1</td>
<td>10.4</td>
<td>8.24</td>
</tr>
<tr>
<td>Histidine</td>
<td>7.05</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.22</td>
<td>6.42</td>
<td>6.20</td>
</tr>
<tr>
<td>Arginine</td>
<td>12.8</td>
<td>11.4</td>
<td>16.1</td>
</tr>
<tr>
<td>Proline</td>
<td>4.05</td>
<td>3.08</td>
<td>3.54</td>
</tr>
<tr>
<td>Valine</td>
<td>4.83</td>
<td>4.85</td>
<td>3.73</td>
</tr>
<tr>
<td>Leucines</td>
<td>5.49</td>
<td>6.17</td>
<td>4.00</td>
</tr>
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<td>Phenylalanine</td>
<td>4.05</td>
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<td>4.74</td>
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<tr>
<td>Tyrosine</td>
<td>2.87</td>
<td>1.75</td>
<td>2.31</td>
</tr>
<tr>
<td>β-Alanine</td>
<td>Trace</td>
<td>1.22</td>
<td>0.764</td>
</tr>
<tr>
<td>γ-Aminobutyric acid</td>
<td>Absent</td>
<td>7.81</td>
<td>7.85</td>
</tr>
<tr>
<td>“Compound 108”c</td>
<td>Absent</td>
<td>Present</td>
<td>Present</td>
</tr>
</tbody>
</table>

Total nitrogen (mg/g fresh weight)

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.765</td>
<td>0.976</td>
</tr>
</tbody>
</table>

a Nitrogen of each compound as percentage of total soluble nitrogen in compounds determined by ninhydrin.

b Analyzed by the paper chromatographic method.

c See p. 257 for description of this as yet unidentified γ-glutamine derivative.
stituted \( \gamma \)-hydroxyglutamine with an as yet incompletely identified C-5 residue. This easily recognizable compound was conspicuously absent from the cultured tissue in which even \( \gamma \)-aminobutyric acid was depleted, and only a trace of \( \beta \)-alanine occurred. This is another example, one of many, in which a distinctive compound, characteristic of the intact organs of the plant body, is not to be found in freely growing proliferating cultures.

Attention may be drawn here to the accumulation of unusual amino acids in tissue cultures derived from certain tumors. In these special cases, the accumulation of such compounds as lysopine, octopine, homoarginine, and substituted guanidines is favored even though similar cultures derived from normal tissue contain none, or only small amounts, of the compound in question (cf. 210, 233, 234, 270, 311).

Nevertheless, the use of cultures established from normal plant tissue to synthesize the more unusual, but still essentially simple, amino acids of plants has been, as yet, singularly unrewarding.

B. THE PROTEINS OF RESTING AND GROWING TISSUES

The constitution of proteins is now known to be genetically determined. Also these substances are presumed to be closely involved in differentiation and morphogenesis. There is, therefore, particular interest in the study of proteins in resting and cultured cells.

Cells in the resting state may be expected to exist in a nitrogen balance; breakdown of protoplasmic protein can be replaced by minimal resynthesis of protein from stored soluble nitrogenous compounds. The net effect of this on the carbon balance is negligible, and the main fate of any absorbed sugar could be its conversion to carbon dioxide and water via respiration. In the growing state, by contrast, much of the carbon of sugar supplied is directly incorporated into protein. Much work has shown that there is a more intimate relationship between the carbon of protein and that respired as carbon dioxide in the actively growing than in the quiescent tissue. When cells undergo an induction of growth there is much new protein synthesis and an enhanced role of protein turnover (cf. 34, 344).

1. Bulk and Storage Proteins

Most analyses of proteins in cultured tissues have involved study of the amino acid composition of the bulk protein (34, 48, 87-89, 337, 353, 383, 395, 396). It would, of course, be more attractive here to be able to deal with an identifiable protein characteristic of the growing cells. Viruses represent a case in point. Early in the history of this work,
attempts were made in this laboratory to culture tobacco mosaic and other viruses in growing cells. It was at first somewhat surprising that these viruses simply did not multiply in the active, dividing cells although they probably did so more easily in cells which merely enlarged (274). Based on later work (121) a possible explanation arises. It has been suggested (333) that the success of the living cell in growing the specific virus protein is in part a consequence of its organization. Hence the virus can profit by proximity to sites where special nitrogen compounds are being released by cells in active protein turnover. Thus, the “working” cell has properties not shared by the “quiescent” cell, but this is a feature of its organization, not of its intrinsic nature.

An outstanding feature of growing cultured cells is their ability to synthesize protein. In fact, the growth factors, over and above ordinary nutrients, that induce growth in otherwise quiescent cells, also stimulate both protein synthesis and protein turnover. Many rapidly growing cells, not only synthesize protein in bulk, but also produce a characteristic hydroxyproline-rich protein moiety (275, 341).

Ever since Steward, Thompson, and Pollard (353) first showed the higher content of hydroxyproline in the protein fraction of rapidly growing cells (e.g., tissue cultures as well as spontaneous tumors in tobacco species and induced tumors in Kalanchoe) than in comparable resting or nongrowing tissue, there has been no doubt that the protein metabolism of the actively growing cells is quite different from that of nongrowing cells. Weinstein, Nickell, Laurencot, and Tulecke (395) also have found a higher content of hydroxyproline in tissues of Agave grown in a medium containing coconut milk and 2,4-D. The same sort of result has been encountered in cultures of Acer induced to grow by coconut milk and 2,4-D (202–204). Following up this early work (cf. 216), Steward, Israel, and Salpeter (348) have now located this protein in the cytoplasm of carrot cultures using the combined techniques of electron microscopy and radioautography. Steward and Pollard (341) even suggested that the presence of the hydroxyproline-containing protein(s) may have significance in these rapidly growing cells in which growth induction is followed by morphogenetic developments. Analysis of the proteins of Haplopappus cell cultures also showed a protein moiety containing hydroxyproline to be present in the actively growing cultures but not in the intact plant bulk protein (cf. Table VII). Various growth regulators bring about detectable changes in the overall amino acid composition of the bulk proteins of cultured tissue (cf. Tables X and XI). This suggests that the growth regulating compounds, which do not act in substrate quantities, are nevertheless capable of altering the overall metabolism [cf. van Overbeek (261) for a review of the
TABLE X
ALCOHOL-INSOLUBLE NITROGEN OF PEANUT CELLS CULTURED IN VARIOUS MEDIA OVER A PERIOD OF 30 DAYS

<table>
<thead>
<tr>
<th>Compound</th>
<th>CM</th>
<th>CM + BTOA</th>
<th>CM + IAA</th>
<th>CM + NAA</th>
<th>CM + 2,4-D</th>
<th>CM + 2,3,6-TPAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Half-cystine and cysteic acid</td>
<td>1.2</td>
<td>Trace</td>
<td>1.1</td>
<td>Trace</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5.2</td>
<td>6.5</td>
<td>5.1</td>
<td>7.3</td>
<td>5.3</td>
<td>5.34</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>25.5</td>
<td>16.8</td>
<td>24.1</td>
<td>21.9</td>
<td>26.4</td>
<td>28.0</td>
</tr>
<tr>
<td>Serine</td>
<td>4.7</td>
<td>5.2</td>
<td>4.9</td>
<td>5.0</td>
<td>5.3</td>
<td>4.02</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.7</td>
<td>7.7</td>
<td>6.7</td>
<td>8.3</td>
<td>7.2</td>
<td>3.95</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.7</td>
<td>3.7</td>
<td>2.8</td>
<td>3.6</td>
<td>2.9</td>
<td>2.56</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.8</td>
<td>6.6</td>
<td>4.9</td>
<td>7.0</td>
<td>5.4</td>
<td>3.49</td>
</tr>
<tr>
<td>Histidine</td>
<td>Trace</td>
<td>0.6</td>
<td>Trace</td>
<td>6.2</td>
<td>6.2</td>
<td>2.33</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.8</td>
<td>7.3</td>
<td>5.0</td>
<td>7.7</td>
<td>5.8</td>
<td>2.80</td>
</tr>
<tr>
<td>Arginine</td>
<td>12.8</td>
<td>15.7</td>
<td>13.6</td>
<td>Trace</td>
<td>15.6</td>
<td>4.90</td>
</tr>
<tr>
<td>Proline</td>
<td>11.7</td>
<td>8.5</td>
<td>11.7</td>
<td>11.5</td>
<td>10.3</td>
<td>8.58</td>
</tr>
<tr>
<td>Valine</td>
<td>4.6</td>
<td>4.7</td>
<td>4.6</td>
<td>5.5</td>
<td>4.7</td>
<td>4.00</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.3</td>
<td>3.1</td>
<td>4.1</td>
<td>3.9</td>
<td>3.1</td>
<td>2.98</td>
</tr>
<tr>
<td>Leucine</td>
<td>6.4</td>
<td>5.7</td>
<td>6.1</td>
<td>7.0</td>
<td>6.6</td>
<td>6.30</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.3</td>
<td>2.0</td>
<td>3.1</td>
<td>3.2</td>
<td>3.4</td>
<td>4.12</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.5</td>
<td>Trace</td>
<td>1.4</td>
<td>1.1</td>
<td>1.4</td>
<td>1.84</td>
</tr>
</tbody>
</table>

Total nitrogen (µg/g fresh weight of tissue) 1044 1140 1061 970 1007 1146

*Nitrogen of each compound as a percentage of total insoluble nitrogen in compounds determined by ninhydrin. The basal medium consisted of White's basal medium, coconut milk 10%, and casein hydrolyzate 200 mg/liter plus one of the following: BTOA 10 mg/liter, IAA 1 mg/liter, NAA 5 mg/liter, 2,4-D 5 mg/liter, and 2,3,6-TPAA 5 mg/liter.

Role of growth regulators in protein synthesis. The interesting feature is that the amino acid composition of the protein does change with respect to that of the initial resting tissue (cf. Table II); moreover, the composition, as shown by electrophoretic separations of cultured tissues (Fig. 4) varies with the factors that stimulate their growth. This contrast between the resting and the cultured tissue is reminiscent of the changes in protein complement that accompany normal development (cf. 18, 351).

2. Enzymes

Other conveniently labeled proteins are those which have such properties as antibodies and enzymes. Enzymes which have received attention in this laboratory are papain and urease (199). Papaya fruit
A. D. Krikorian AND F. C. Steward

TABLE XI

ALCOHOL-INSOLUBLE NITROGEN OF TOBACCO PITH TISSUE CULTURES GROWN IN WHITE'S BASAL MEDIUM SUPPLEMENTED WITH THE FOLLOWING GROWTH REGULATORS: COCONUT MILK 10%, 2,4-D 1 AND 5 mg/liter, NAA 1 AND 5 mg/liter, AND BTOA 2 AND 10 mg/litera

<table>
<thead>
<tr>
<th>Compound</th>
<th>Coco-</th>
<th>2,4-D</th>
<th>2,4-D</th>
<th>NAA</th>
<th>NAA</th>
<th>BTOA</th>
<th>BTOA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>milk</td>
<td>1 mg/liter</td>
<td>5 mg/liter</td>
<td>1 mg/liter</td>
<td>5 mg/liter</td>
<td>2 mg/liter</td>
<td>10 mg/liter</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>11.9</td>
<td>8.8</td>
<td>10.6</td>
<td>13.7</td>
<td>8.7</td>
<td>15.6</td>
<td>18.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.6</td>
<td>10.8</td>
<td>9.9</td>
<td>8.1</td>
<td>13.1</td>
<td>7.3</td>
<td>6.8</td>
</tr>
<tr>
<td>Serine</td>
<td>3.0</td>
<td>4.0</td>
<td>3.6</td>
<td>6.6</td>
<td>5.5</td>
<td>4.8</td>
<td>5.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.5</td>
<td>6.7</td>
<td>5.7</td>
<td>4.9</td>
<td>6.3</td>
<td>5.2</td>
<td>5.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.3</td>
<td>3.6</td>
<td>2.3</td>
<td>4.8</td>
<td>4.4</td>
<td>5.6</td>
<td>5.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>7.7</td>
<td>8.6</td>
<td>6.8</td>
<td>9.0</td>
<td>10.1</td>
<td>8.2</td>
<td>7.5</td>
</tr>
<tr>
<td>Lysine</td>
<td>7.6</td>
<td>8.3</td>
<td>7.1</td>
<td>7.8</td>
<td>8.3</td>
<td>10.8</td>
<td>11.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>19.1</td>
<td>16.3</td>
<td>23.8</td>
<td>16.0</td>
<td>11.0</td>
<td>14.3</td>
<td>14.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.2</td>
<td>0.5</td>
<td>0.9</td>
<td>0.6</td>
<td>0.6</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Proline</td>
<td>3.8</td>
<td>5.7</td>
<td>4.1</td>
<td>4.8</td>
<td>4.5</td>
<td>4.6</td>
<td>5.0</td>
</tr>
<tr>
<td>Valine</td>
<td>5.1</td>
<td>5.5</td>
<td>4.5</td>
<td>5.0</td>
<td>6.3</td>
<td>5.7</td>
<td>6.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>12.1</td>
<td>7.3</td>
<td>10.6</td>
<td>7.3</td>
<td>11.0</td>
<td>7.4</td>
<td>9.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>4.5</td>
<td>6.3</td>
<td>5.9</td>
<td>6.6</td>
<td>4.9</td>
<td>6.6</td>
<td>7.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.4</td>
<td>4.2</td>
<td>1.9</td>
<td>2.6</td>
<td>2.1</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.9</td>
<td>2.3</td>
<td>1.3</td>
<td>1.9</td>
<td>1.9</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>0.3</td>
<td>Trace</td>
<td>Trace</td>
<td>—</td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Total</td>
<td>1575.5</td>
<td>1288.4</td>
<td>1272.4</td>
<td>1188.9</td>
<td>1278.2</td>
<td>1098.7</td>
<td>1138.6</td>
</tr>
</tbody>
</table>

aData are presented as percentage of total insoluble nitrogen in compounds determined by ninhydrin.

cultures (cf. Fig. 9A) were obtained specifically to test their biosynthetic capabilities compared with tissue of the intact fruit. The fact was however, that the cultured papaya fruit tissue contained virtually no proteolytic activity.

This may be due to the fact that proteolytic action is a very special feature of the laticifers in the young plant parts. The two parts of the plant which at all times show a high proteolytic activity are the green leaves and the rind of the green fruit. Whether or not the formation of papain is closely connected with the presence of chlorophyll is not certain (11), but there is a definite variation in proteolytic activity during the first thirteen months of growth. In a given case such activity reached its maximum on day 133 after planting of the seed, but decreased consistently thereafter (11). The pulp of the fruit has been shown by the same workers to have much lower proteolytic activity than the rind.
They also found that proteolytic activity appeared first in the leaf about 43 days after planting the seed. The peak activity in the leaf was reached at about day 133 after planting. The same variation was noticed to a lesser extent in the stem and root.

There is, therefore, a controlling mechanism during the development of the plant that regulates the production of the proteolytic enzyme papain. Hence, the inability to detect any proteolytic activity in fruit tissue cultures of papaya raises the question whether this enzyme plays any role in the plant other than that of a storage protein.

Similarly, cultured cotyledonary tissue of jack bean showed no urease activity when compared with intact seed. In this respect the
low urease activity in cultured jack bean cotyledon resembles the proteolytic activity of cultured papaya tissue. Thus, these easily demonstrable enzyme proteins may not necessarily function \textit{in situ} as enzymes, but rather as special storage proteins or other protoplasmic proteins, which do not accumulate in the rapidly growing cells. In short, the cotyledons of jack bean are characterized by very specific nitrogen compounds, both soluble and insoluble (e.g., canavanine and urease). The growing cells, cultured from cotyledons, have, however, a very different composition; i.e., they lack almost entirely both these substances. Therefore, the presence of canavanine and urease in the normal cotyledons of the seed is an aspect of its physiological and biochemical differentiation. If one could find the factors which cause these substances to return in the cultured cells, this might shed light on the factors in development which induce the cells to become cotyledons and also permit the protein metabolism of the cultured cells to be controlled.

Just as one should not be surprised to encounter free amino acids and proteins in plant cell and tissue cultures, one should expect to find the common metabolic enzymes. This topic has been reviewed (cf. 126, 360). An important point to be brought out, however, is as follows. In many instances certain metabolic enzymes are not detectable at all in cultures derived from normal tissue, whereas cultures derived from diseased plants show considerable activity (cf. 125, 127). Similarly there seems to be some strain specificity for a given enzyme complement. Early attempts to show extracellular enzymes were not convincing since the question of enzyme release into the ambient culture medium by dead or dying cells was not eliminated. None of the data we are aware of permit unequivocal conclusions to be drawn in this regard (cf. Table XII).

Intracellular enzymes are easier to demonstrate convincingly, and as expected a number of cases exist in the literature (cf. Table XII). Since the current methods of extraction and production of enzymes for commercial use are so adequate, it is predictable that the use of angiosperm cultures for specific biotransformations of physiologically active molecules (e.g., steroids, alkaloids) should be feasible (cf. 13, 137). Therefore, in this laboratory tissue cultures of carrot, tobacco, peanut, and \textit{Haplopappus} were analyzed for various digestive enzymes (199). None of the cultured tissues showed intense amounts of activity, although tobacco, peanut, carrot, and \textit{Haplopappus} showed some protease activity. Carrot and peanut tissues also showed lipase activity. None of the samples showed any detectable amylase or pectinase activities. Thus, the tissue cultures as grown did not contain commercially at-
<table>
<thead>
<tr>
<th>Enzyme detected</th>
<th>Nature and origin of cultures</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aconitases</td>
<td><em>Acer pseudoplatanus</em> (cambium)</td>
<td>(375)</td>
</tr>
<tr>
<td>δ-Aminolevulinic acid dehydratase</td>
<td><em>Kalanchoe crenata</em> (stem)</td>
<td>(355a)</td>
</tr>
<tr>
<td>α-Amylase</td>
<td><em>Rumex acetosa</em> (virus tumor on roots)</td>
<td>(49–51)</td>
</tr>
<tr>
<td></td>
<td><em>Juniperus communis</em></td>
<td>(71, 71a)</td>
</tr>
<tr>
<td></td>
<td><em>Nicotiana tabacum</em> var. ‘White Burley’ (stem crown gall tumors)</td>
<td>(165, 166)</td>
</tr>
<tr>
<td>Amylase</td>
<td><em>Daucus carota</em> (root cambial origin)</td>
<td>(171)</td>
</tr>
<tr>
<td></td>
<td><em>Rubus fruticosus</em> (crown gall)</td>
<td>(171)</td>
</tr>
<tr>
<td></td>
<td><em>Acer saccharum</em> (stem callus)</td>
<td>(227)</td>
</tr>
<tr>
<td>Arginine degrading enzyme</td>
<td><em>Ginkgo biloba</em> (pollen)</td>
<td>(378, 380)</td>
</tr>
<tr>
<td>Arginase</td>
<td><em>Opuntia vulgaris</em> (crown gall)</td>
<td>(234)</td>
</tr>
<tr>
<td></td>
<td><em>Datura stramonium</em> (seed origin)</td>
<td>(167)</td>
</tr>
<tr>
<td>Ascorbic acid oxidase</td>
<td><em>Lycopersicon esculentum</em> (crown gall)</td>
<td>(322, 323)</td>
</tr>
<tr>
<td>Catalase</td>
<td><em>Armoracia rusticana</em> (horseradish, root origin)</td>
<td>(17a)</td>
</tr>
<tr>
<td></td>
<td><em>Helianthus tuberosus</em> (tuber callus)</td>
<td>(170)</td>
</tr>
<tr>
<td></td>
<td><em>Serronera hispanica</em> (crown gall and normal)</td>
<td>(205, 206)</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td><em>Daucus carota</em> (root phloem crown gall tumor)</td>
<td>(213)</td>
</tr>
<tr>
<td></td>
<td><em>Nicotiana tabacum</em> var. ‘Xanthi’ (stem origin)</td>
<td>(82a)</td>
</tr>
<tr>
<td>Diaphorase</td>
<td><em>Daucus carota</em> (root phloem crown gall tumor)</td>
<td>(213)</td>
</tr>
<tr>
<td></td>
<td><em>Phaseolus, Lactuca, Daucus, Capsicum</em> (callus of unspecified origin)</td>
<td>(220)</td>
</tr>
<tr>
<td>β-(1 → 3) glucanase(s)</td>
<td><em>Triticum monococcum</em>; <em>T. vulgare</em> var. Thatch; <em>Hordeum vulgare</em> var. Gateway; <em>H. vulgare</em> var. Gateway (mutant) (all root origin); *Rosa var. ‘Sceptre’ (stem); <em>Reseda luteola</em> (stem); <em>Glycine max</em> (root); <em>Armoracia rusticana</em> (petiole); <em>Phaseolus aureus</em> (root, hypocotyl and cotyledon)</td>
<td>(120a)</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td><em>Trifolium repens</em> (stem origin)</td>
<td>(160a)</td>
</tr>
<tr>
<td>Hexose monophosphate shunt enzymes</td>
<td><em>Nicotiana glauca</em> (normal), <em>N. suaveolens</em> × <em>langsdorffii</em> (tumor)</td>
<td>(308)</td>
</tr>
<tr>
<td>IAA oxidase</td>
<td><em>Picea glauca</em> (tumor)</td>
<td>(286)</td>
</tr>
<tr>
<td></td>
<td><em>Parthenocissus tricuspidata</em> (crown gall)</td>
<td>(212, 412)</td>
</tr>
</tbody>
</table>
TABLE XII (Continued)

<table>
<thead>
<tr>
<th>Enzyme detected</th>
<th>Nature and origin of cultures</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAA oxidase</td>
<td>(normal and crown gall) Ephedra (unknown species) (stem) Pelargonium × hortorum var. 'Nittany Red' (stem)</td>
<td>(293)</td>
</tr>
<tr>
<td>Invertase</td>
<td>Pyrus communis var. 'Bosc' (floral tube); Rosa multiflora (stem); Nicotiana tabacum (3 strains from stem); Calocedrus decurrens (Libocedrus decurrens) (axis of staminate cone); Chamaecyparis funebris (Cupressus funebris) (axis of staminate cone); Ephedra (stem); Zea mays (endosperm)</td>
<td>(359)</td>
</tr>
<tr>
<td>Krebs' cycle dehydrogenases</td>
<td>Nicotiana tabacum var. 'Xanthi' (stem origin)</td>
<td>(82a)</td>
</tr>
<tr>
<td>Lipase</td>
<td>Daucus carota (root phloem origin); Arachis hypogaea (cotyledon)</td>
<td>(199)</td>
</tr>
<tr>
<td>Malic dehydrogenase</td>
<td>Scorzonera hispanica (crown gall) Kalanchoe crenata (green and colorless callus of stem origin)</td>
<td>(206)</td>
</tr>
<tr>
<td>NADH-cytochrome c reductase</td>
<td>Daucus carota (root phloem crown gall tumor)</td>
<td>(213)</td>
</tr>
<tr>
<td>Octopine synthetase</td>
<td>Scorzonera hispanica (crown gall)</td>
<td>(270)</td>
</tr>
<tr>
<td>Ornithine-2-oxoglutarate transaminase</td>
<td>Helianthus tuberosus (tuber callus)</td>
<td>(170)</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Parthenocissus tricuspidata (normal and gall tissue)</td>
<td>(213)</td>
</tr>
<tr>
<td>Phosphatase, peroxidase, amylase</td>
<td>Nicotiana tabacum var. 'Wisconsin 38' (stem pith)</td>
<td>(117)</td>
</tr>
<tr>
<td>Phenylalanine-2-oxoglutarate transaminase</td>
<td>Datura stramonium (callus of seed origin)</td>
<td>(82a, 82b)</td>
</tr>
<tr>
<td>(Acid) phosphatase, peroxidase, amylase</td>
<td>Rosa multiflora (stem); Pelargonium sp.; Lycopersicon esculentum; Nicotiana tabacum (3 separate strains); Cupressus funebris (axis of staminate cone)</td>
<td>(360)</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxylase</td>
<td>Nicotiana tabacum</td>
<td>(82)</td>
</tr>
<tr>
<td>Protease</td>
<td>Kalanchoe crenata (green callus of stem origin; not detected in colorless callus)</td>
<td>(229)</td>
</tr>
<tr>
<td>(endopeptidase, esterase and leucine aminopeptidase)</td>
<td>Nicotiana tabacum (including teratoma and crown gall, habituated, and albino strains); Euphorbia sp.; Parthenocissus (normal and crown gall)</td>
<td>(113–115)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(113)</td>
</tr>
</tbody>
</table>
## 7. Biosynthetic Potentialities of Tissue

### TABLE XII (Continued)

<table>
<thead>
<tr>
<th>Enzyme detected</th>
<th>Nature and origin of cultures</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease a</td>
<td><em>Nicotiana tabacum</em> var. 'Turkish' (stem pith); <em>Haplopappus gracilis</em> (stem); <em>Daucus carota</em> (root phloem); <em>Arachis hypogaea</em> (cotyledon)</td>
<td>(199)</td>
</tr>
<tr>
<td>Shikimate pathway enzymes</td>
<td><em>Solanum tuberosum</em> var. 'Norland' (tuber); *Rosa var. 'Sceptre' (stem); <em>Reseda luteola</em> (stem); <em>Glycine max</em> (root); <em>Phaseolus vulgaris</em> var. 'Bountiful' (root); <em>Armoracia rusticana</em> (<em>A. lapathifolia</em>) (petiole); <em>Fagopyrum tataricum</em> (hypocotyl); <em>Phaseolus aureus</em> (root, hypocotyl and cotyledon.)</td>
<td>(118, 119)</td>
</tr>
<tr>
<td></td>
<td><em>Scorzonera hispanica</em> (crown gall origin)</td>
<td>(206)</td>
</tr>
<tr>
<td>Succinic dehydrogenase a</td>
<td><em>Nicotiana tabacum</em> var. 'Xanthi' (stem)</td>
<td>(82a)</td>
</tr>
<tr>
<td>Tryptophan synthetase a</td>
<td><em>Nicotiana tabacum</em> var. 'Wisconsin 38' (stem pith)</td>
<td>(82c)</td>
</tr>
<tr>
<td>Tyrosinase a</td>
<td><em>Helianthus tuberosus</em> (tuber callus)</td>
<td>(170, 214)</td>
</tr>
</tbody>
</table>

*a* Intracellular enzyme.

*b* Extracellular enzyme.

*c* The term diaphorase has been applied indiscriminately to any enzyme catalyzing the oxidation of either NADH or NADPH by any one of the artificial electron acceptors, such as dyes, ferricyanide, and quinones. Diaphorase has no physiological meaning, and the term is falling into disuse.

*d* However, all preparations tested from cultures of both mono- and dicotyledons showed no activity toward β-(1 → 4) mixed linkage glucan.

Productive amounts of the digestive enzymes although they may well contain metabolic enzymes in quantity.

Although the examples discussed suggest that certain enzyme proteins are not as readily formed in cultured as in normal cells, there is a further consideration. In one instance, i.e; peroxidase in horseradish, *Armoracia rusticana* (*A. lapathifolia*), root cultures, the complement of soluble protein was different in cultured and normal tissue and at least one enzymatically active protein did occur in quantity in the actively growing cultures, although another normally present in the root did not appear (cf. Fig. 5). Lack of enzymatic activity, however, may not be due here solely to failure to synthesize the necessary protein. Certain enzymes like phosphorylase a and b, as well as the digestive enzymes of starchy seeds like barley (*Hordeum vulgare*) have long been known to occur first as zymogens which may later be activated (cf. 143, 259, 391). Thus, the growing cells may well manufacture the zymogen without necessarily causing its activation. Furthermore, examples are
Fig. 5. The separation by acrylamide gel electrophoresis of the soluble proteins and peroxidases of normal and culture horseradish root (*Armoracia rusticana*). Amido black shows the complement of soluble protein present. Benzidine-H$_2$O$_2$ shows the peroxidases present. The cultured tissue had many fewer bands revealed by amido black than the normal, and these were confined to three main areas of the gel. From work of Barber and Krikorian (17a).

now known in which given reactions are carried out by organ-specific isozymes (18, 117) and the cultured tissue may exploit these as selectively as the organs in the intact plant (165, 166). This is a comparatively new field which needs further investigation. However, one should at present reserve judgment on attempts to synthesize special enzyme proteins in quantity by the use of cultured angiosperm tissue unless one first solves the problem of controlling their differentiation as, in fact, occurs in the intact plant. Indeed it is hardly necessary for actively growing cells to accumulate the enzymes that mediate their normal metabolism, and virtually all cells must have these enzymes, although they need not accumulate.

C. ALKALOIDS

A comparatively recent potential application of plant tissue and organ culture has been the production and biosynthesis of pharmaceutically useful plant constituents. There are many plant alkaloids that are of sufficient interest both academically and practically to warrant studies on
their biosynthetic pathways and on methods of increasing their yields (222, 245).

1. Belladonna Alkaloids

*Atropa belladonna* alkaloids are synthesized by excised root organ cultures (107, 287, 397), and excised roots of various *Datura* species have similarly been used to study the incorporation of various compounds into the principal alkaloids (cf. 129). West and Mika (397) appear to be the first to have obtained callus cultures of *Atropa belladonna* root. In a basal medium, satisfactory growth was obtained using coconut milk. After 8–10 weeks on the complete medium, a number of stemlike organs formed on the nonsubmerged side of the calluses, and, after 4 months macroscopic stems and leaves had appeared. The stems were excised from the root callus and cultured separately, so that their ability to synthesize atropine could be determined. These stems developed roots at the basal end; only those stem tissues which had not formed macroscopic roots were used for alkaloid analyses. West and Mika (397) showed that only isolated roots and root callus could synthesize atropine—stem and leaf cultures could not unless macroscopic adventitious roots had been formed. The amount of alkaloid found was 0.047–0.053% of the dry weight in the root callus tissue. Excised roots grown in organ culture made slightly less alkaloid (0.042% of the dry weight); roots of intact plants, however, are known to accumulate many times more atropine (*dl*-hyoscyamine).

Other solanaceous alkaloid-yielding plants have been grown in aseptic culture (292a, 302a). Telle and Gautheret (370) grew root tissue of *Hyoscyamus niger* and crown gall tissue of *Datura stramonium* has been cultured by Nickell (cf. 123). Rerábek (288, 289) reported the culture of root callus of *Solanum laciniatum* and alkaloid production by *Datura* callus and suspension cultures have been attempted by Chan and Staba (68). The growth of *Hyoscyamus niger* ovaries in culture has been described by Singh Bajaj (314), and various cell strains of *Solanum melongena* (eggplant) have been seen to differentiate (cf. 417). It is interesting here to state parenthetically that the early use of coconut milk in tissue cultures was in the growing of *Datura* embryos (262). Hindsight reveals interesting suggestions in this work. It was reported (263) that extra cotyledons formed on embryos grown in coconut milk-supplemented media, whereas in some cases there was callusing of the embryo with no organized development. This was, in fact, a first indication of the growth-promoting qualities and morphogenetic influence of coconut milk. McLean (230), using interspecific crosses involving *Datura ceratocaula*, depended upon embryo culture to
test the viability of *Datura hybrids*. In certain ones, small embryos grown on media with malt extract yielded calluses upon which buds developed. These buds developed further into mature plants. Pollen grains of *D. innoxia* are now known to be totipotent for embryoids have been obtained from pollen (141).

Cultures of solanaceous plants have been established and maintained in this laboratory for the specific purpose of testing their abilities to synthesize alkaloids (cf. Table XIII). To date, these cultures have not accumulated tropane alkaloids either in the tissues or the culture medium. Thin-layer chromatographic examination reveals some weak alkaloid-positive spots (Dragendorff’s reagent; cf. 251) but no hyoscine or *dl*-hyoscyamine (atropine) were detectable (cf. 325a for similar results). Although the cultured cells examined do not accumulate pharmacologically interesting compounds, it has nevertheless been shown that cells of *Datura tatula* and *D. metel* are able to absorb added atropine from the medium and to accumulate it (94). Cell-free enzyme preparations from *Datura innoxia* are able to reduce hyoscine to hyoscyamine (13). The feeding of various intermediates in the biosynthetic pathway of the tropane alkaloids has yet to be explored. The point to be stressed here is that wherever appreciable biosynthetic potentialities have been noted, this has been accompanied, or is preceded by, a greater degree of organization than is commonly recognized. In fact, such solanaceous cultures organize readily both on agar and in liquid (cf. Fig. 6).

### 2. *Nicotiana* Alkaloids

Alkaloids have been found in excised root organ cultures of *Nicotiana* species (78, 80, 303, 319, 320), but Dawson (79) found that the quantities of nicotine alkaloid produced by cultured tobacco (*N. glutinosa*) decreased rapidly during the initial stages of tissue culture.

<table>
<thead>
<tr>
<th>Solanaceous Plants Grown in Callus and Free Cell Suspension Culture&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atropa belladonna</em> var. <em>lutea</em></td>
<td><em>S. miniatum</em></td>
</tr>
<tr>
<td><em>Hyoscyamus pusillus</em></td>
<td><em>S. gila</em></td>
</tr>
<tr>
<td><em>H. reticulatus</em></td>
<td><em>Datura stramonium</em></td>
</tr>
<tr>
<td><em>H. albus</em></td>
<td><em>D. stramonium var. tatula</em> (<em>D. tatula</em>)</td>
</tr>
<tr>
<td><em>H. niger</em></td>
<td><em>D. ferox</em></td>
</tr>
<tr>
<td><em>Solanum dulcamara</em></td>
<td><em>D. meteloides</em></td>
</tr>
<tr>
<td><em>S. ariculare</em></td>
<td><em>D. innoxia</em></td>
</tr>
<tr>
<td><em>S. indicum</em></td>
<td><em>D. metel</em></td>
</tr>
</tbody>
</table>

<sup>a</sup> Bleichert and Krikorian (38b).
so that none was detectable when a typical callus state was achieved. Speake et al. (321), on the other hand, could still identify nicotine in free cell and callus cultures of tobacco root, stem, or leaf (N. tabacum var. 'Virginica') after 23 transfers. This suggests that there may be no inherent difference between the cultured cells of different parts of the plant as far as their synthetic potential ability, although the overwhelming dominance of the root as a site of nicotine biosynthesis in the intact plant may be a result of its organization. The general level of
nicotine produced by the intact plant root is 29 µg/mg dry weight of
tissue, while cell and callus cultures produce only 0.1–1.0 µg/mg dry
weight. The maximum ever observed was on the order of 7 µg/gm dry
weight of cells at the end of the total growth period (1–3 months).
Furuya et al. (111, 112) have also identified small amounts of nicotine
and anatabine in tobacco (N. tabacum var. 'Bright Yellow') callus tissue,
but Benveniste et al. (29) were unable to detect nicotine in cultures
of N. tabacum var. 'p-19'.

As a preliminary to a general study of alkaloids in this laboratory,
an appraisal was made of cell and tissue cultures of tobacco (199).
These were Nicotiana tabacum var. ‘Turkish’, N. tabacum var. ‘Maryland
Mammoth’, N. tabacum var. ‘Turkish Samsun NN’, N. suaveolens,
and N. rustica.

In only one instance did the assay procedure give positive results.
Cultured tissue of embryo origin from N. rustica showed 0.85% of its
dry weight as total tobacco alkaloids. The tissues derived from pith of N.
tabacum var. ‘Turkish’ or var. ‘Turkish Samsun NN’ gave no detectable
alkaloid. N. tabacum var. ‘Maryland Mammoth’ pith tissues gave only
a slight positive test for nicotine.

Since intact tobacco plants dried in an oven at 50°C and extracted
by the same procedures gave varying quantities of total alkaloid ranging
from 2.46 to 8.07% of the dry weight, the amount synthesized by
the N. rustica cell cultures of embryo origin was, at best, only one-tenth
as much as that present in the roots of intact plants.

Explanations why the cell cultures of N. rustica produced nicotine
are as follows. Steward et al. (334) found that certain cells of embryo
origin are morphologically totipotent. It then follows that these should
also be “totipotent” biochemically. An analysis of N. rustica cell suspen-
sions derived from embryos showed that these cells were indeed more
able to synthesize alkaloids than cells derived from the pith of stems.
However, analysis of cells cultured from embryos of N. tabacum var.
‘Turkish’ failed to produce alkaloids in detectable quantity. Thus, it
appears that the synthesis of small amounts of alkaloid by N. rustica is
connected with some elusive factor. Since it is well appreciated that
total tobacco alkaloid analysis is only a rough estimate of the actual
amount of any given substance present, N. rustica cell cultures were
extracted for nicotine. The method used was similar to that of Speake
et al. (321), and is a standard procedure for alkaloid extraction. The
ultraviolet absorption data and the infrared absorption spectrum of the
purified compound extracted from the cultured tissue matched those of

Hence, some species and/or strains of tobacco are able to synthesize
some Nicotiana alkaloids (especially nicotine) when grown in cell or tis-
sue culture. There is then here a potentially useful system for further
tests (391a). However, since the amount produced by the cell suspensions is so small in comparison to the intact plant, it seems that nicotine or alkaloid production in bulk must be fostered by some events peculiar to the normal development of the plant. Dawson (78) showed that nicotine production is limited very largely to the root and that it is from the roots that the nicotine is translocated to the leaves and the rest of
the plant (80).

3. Catharanthus Alkaloids

To date, a total of 66 alkaloids have been found in Catharanthus roseus (Vinca rosea). Four of the alkaloids, leurosine, vincaleukoblastine, leurosidine, and leurocristine have demonstrated varying degrees of antitumour activity (255). A controlled system which synthesizes only these alkaloids would be of both theoretical interest and practical value.

Tissue cultures of Catharanthus roseus were first started by White (401), who isolated them from crown galls rendered bacteria-free by heat therapy. These showed a very rapid growth, and when grafted back into healthy host plants they produced typical tumors which regularly exceeded in size those that were normally produced by multiple needle puncture inoculation of tumefacient bacteria.

Implications of the increase in growth capacity of bacteria-free over bacteria-containing tumors has been the subject of a great deal of study since that time (cf. 83, 413), and the general ease of culturing this sort of Catharanthus tissue has been well established over the years (cf. 52, 153–155).

Although work with tissue cultures of Catharanthus roseus derived from normal tissue has been less extensive, normal tissues of this plant have been in culture routinely in the laboratories of Dr. Armin C. Braun of the Rockefeller University (55, 414, 415) and has also been grown in free cell culture using the method of Steward, Caplin, and Millar (345) by the Russian worker Mme. Butenko of the Timiriazeff Institute in Moscow (60). Babcock and Carew (12) have also reported successful initiation of normal stem callus cultures on an agar medium; although they encountered difficulty in establishing actively growing cultures of other apocynaceous genera (e.g., Rauwolfia, Apocynum, Nerium, Alstonia). Reserpine has been isolated from cultures of Alstonia constricta (62) and Rauwolfia (235a) in small amounts.

We, too, have maintained both callus and suspension cultures of a number of cultivars of Catharanthus roseus (cf. 199). Others have followed (63, 207). Both root, stem, leaf, and petiole cultures can be ob-
FIG. 7. Growth of *Catharanthus roseus* (*Vinca rosea*) stem callus cultured on (from left to right) the basal medium of Braun and Wood (cf. 55) without or with NAA 1 mg/liter; a high-salt basal medium (cf. 252) supplemented with coconut milk 10% and 2,4-D 1 mg/liter; and the same basal medium supplemented with coconut milk 10% and NAA 1 mg/liter. Culture period 24 days in continuous light.

**TABLE XIV**

**Effect of Various Growth Substances in Combination with Coconut Milk (10%) on the Growth of Explanted Stem Tissue of *Catharanthus roseus* (cultivar 'Twinkles')**

<table>
<thead>
<tr>
<th>Additions to the basal mediumb (mg/liter)</th>
<th>Mean final fresh weightc (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA, 1</td>
<td>1615</td>
</tr>
<tr>
<td>NAA, 5</td>
<td>1725</td>
</tr>
<tr>
<td>NAA, 10</td>
<td>425</td>
</tr>
<tr>
<td>2,4-D, 0.1</td>
<td>3780</td>
</tr>
<tr>
<td>2,4-D, 1</td>
<td>3181</td>
</tr>
<tr>
<td>2,4-D, 5</td>
<td>3170</td>
</tr>
<tr>
<td>2,4-D, 10</td>
<td>195</td>
</tr>
<tr>
<td>BTOA, 5</td>
<td>1758</td>
</tr>
<tr>
<td>BTOA, 10</td>
<td>1840</td>
</tr>
<tr>
<td>IAA, 0.1</td>
<td>1506</td>
</tr>
<tr>
<td>IAA, 1</td>
<td>1608</td>
</tr>
<tr>
<td>IAA, 5</td>
<td>538</td>
</tr>
</tbody>
</table>

a Initial explant was about 40 mg.

b Basal medium of Murashige and Skoog (252).

c Mean of twelve replicates.
tained and maintained in a number of media, both solid and liquid (cf. Table XIV, Fig. 7). A number of cultures showed some ceric ammonium sulfate (CAS)-positive spots (cf. 97) upon thin layer chromatograms. Four alkaloids were recognizable in a two-directional system; these differed from those with antitumor (oncolytic) activity. Krikorian and Steward (200) also found that a completely different alkaloid complement was obtained by a change in the medium (Table XV A and B). Ajmalicine, a principal alkaloid of the stem, was detected. As expected, there are also major differences in the amino acid composition (Fig. 8). Richter et al. (290) have identified vindoline and vindolinine in stem and leaf callus cultures; root callus did not contain either substance (cf. also 247).

### TABLE XV

#### A. \( R_f \) Values for Ceric Ammonium Sulfate (CAS)-Positive Substances in *Catharanthus* Tissue Cultures. (Solvent Path, 100 mm, One-Dimensional.) Tissues Were Grown in a Basal Medium (252) Containing CM 10%, and 2,4-D 5 mg/liter in Liquid Culture

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Color following CAS reagent (97)</th>
<th>( n )-Butanol-acetic acid–water (4:1:1)</th>
<th>Methanol</th>
<th>Ethyl acetate–absolute alcohol (3:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c</td>
<td>Yellow</td>
<td>0.41</td>
<td>0.65(^a)</td>
<td>0.75(^b)</td>
</tr>
<tr>
<td>b</td>
<td>Yellow</td>
<td>0.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>Ajmalicine</td>
<td>0.51</td>
<td>0.65</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
\(^a\) The three spots were not separated but appeared as one spot.

\(^b\) Two spots were separated in this solvent system; one remained at the origin. As shown, spot b matched an ajmalicine standard chromatographically.

#### B. \( R_f \) Values for Ceric Ammonium Sulfate Positive Substances in *Catharanthus* Tissue Cultures. (Solvent Path, 100 mm.) Tissues Were Grown in a Basal Medium Containing CM 10%, plus NAA 5 mg/liter in Liquid Culture

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Color following CAS reagent</th>
<th>( n )-Butanol–acetic acid–water (9:1:2.9)</th>
<th>( n )-Butanol–pyridine–water (9:1:2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Violet</td>
<td>0.25</td>
<td>0.27</td>
<td>0.05</td>
</tr>
<tr>
<td>Turquoise</td>
<td>0.40</td>
<td>0.45</td>
<td>0.35</td>
</tr>
<tr>
<td>Blue-green</td>
<td>0.46</td>
<td>0.47</td>
<td>0.65</td>
</tr>
<tr>
<td>Yellow</td>
<td>0.85</td>
<td>0.80</td>
<td>0.80</td>
</tr>
</tbody>
</table>
Babcock and Carew (12) and Harris et al. (149) have also reported CAS-positive substances in callus cultures of Catharanthus roseus as have Boder et al. (42) in crown gall tissue of Catharanthus. Since manipulation of the medium can alter the overall pattern of the alkaloids produced, then it is probable that the metabolism of these cells can be so manipulated that they will produce specific compounds.

![Diagram of alcohol-soluble amino acid composition of normal and cultured tissues of Catharanthus roseus (Vinca rosea). Scale gives nitrogen in each compound as a percent of total soluble nitrogen (per gram fresh weight) in the compounds as determined by ninhydrin.]

**4. Other Alkaloids**

There have been a few dubious references in the literature to the synthesis of alkaloids by miscellaneous plant tissue cultures. Steinhart (329) has suggested that stem cultures of the cactus Trichocereus spachianus have retained the enzymes involved in the methylation
reactions leading to candidine synthesis. Kovacs et al. (198) reported on the principle of an antihistaminic resembling tomatine from tomato crown gall tumors. This work has not escaped criticism (403). Staba and Laursen (327) have examined tissue cultures of three varieties of Ipomoea violacea (morning glory) and Rivea corymbosa, known to contain hallucinogenic indole compounds (84). Only trace amounts of alkaloids were detected in some callus; no alkaloids were detectable in others.

trans-Cinnamic acid-3-C\(^{14}\) has been shown to be converted to various amaryllidaceous alkaloids when supplied to excised primorial tissue from buds of Hippeastrum vittatum. Germinated seeds form some callus tissue but the alkaloid content of the callus tissue is markedly different from that of the seeds. Hippeastrine and lycorine are not present in the callus tissue, whereas these two alkaloids are present in the seeds (365).

Callus of Papaver somniferum (opium poppy) has been cultured by Koblitz et al. and Ranganathan et al., (192, 281). Alkaloid-free suspension systems when incubated with thebaine are known to metabolize this alkaloid (139). Skythanthine has been isolated from callus cultures of Skythanthus acutus (215). The steroidal alkaloid solasonine has been isolated from callus cultures derived from shoot cultures of Solanum xanthocarpum (150a). Reinhard et al. (286a) have identified harmine in tissue cultures derived from stems of Peganum harmala. It is noteworthy that the callus cultures contained only harmine whereas intact plant stems contained mainly vasicine as well as harmine and an unidentified alkaloid.

In short, some cultured material from plants known to synthesize alkaloids may form usually minute amounts of these substances. Often the substances that are synthesized in culture may be very different from those that accumulate normally. All this is, therefore, suggestive of synthetic potentialities that may ultimately be exploited but is is certainly not evidence that this has yet been fully achieved.

D. GLYCOSIDES

When a sugar is combined through its glycosidic hydroxyl with an alcohol, a glycoside is formed. The nonsugar portion of the molecule is known as the aglycone. Glycosides are often responsible for the taste and aroma of some foods and are also often used medicinally. The anthocyanins which are the coloring materials of flowers and fruits are glycosides, in which the sugar is combined with a colored aglycone of the anthocyanidin group.
I. Nicotiana Glycosides

Sargent and Skoog (297, 298) isolated and characterized scopolin and three other glycosides of scopoletin from tobacco. These correspond exactly to the glycosides found in the root cortex of the intact plant species (N. tabacum var. 'Havana Wisconsin No. 38').

The isolation and identification came about as the result of examining the fluorescent material which was released by tobacco pith cultures into the agar medium on which they were grown. Free scopoletin found in the callus tissue was of the order of 5 μg/g of fresh growing tissue (a relatively low concentration). Most of the scopoletin in the tissue is present in a bound form which is apparently retained entirely within the cells; at least it could not be detected in the medium. Skoog and Montaldi (315) found that the amount of scopoletin released varies with the concentration of auxin in the medium and increases markedly at high, toxic levels of auxin (either IAA or NAA). However, corresponding rise and gradual fall of the scopoletin levels with 2,4-D were not found. Kinetin added to the medium prevented the release of scopoletin by indoleacetic acid (IAA) and permitted the maintenance of high glycoside levels even in the presence of high, but nontoxic levels of IAA. It was suggested that the auxin-kinetin levels regulate the scopoletin-scopolin glycoside equilibria in the cells and the conversion of these substances into cell-wall materials. It is of interest that extracts of plant materials which give kinetin-like effects on growth of callus (e.g., liquid endosperm of coconut) also exert similar effects to that of kinetin by lowering the scopoletin content of the culture medium. Phenylalanine seemed to be highly active in releasing scopoletin into the medium. Fritig et al. (109) have shown C14-labeled phenylalanine and tyrosine to be incorporated into scopolin and scopoletin. Tryon (377) found that the capacities of various strains of tobacco callus to form buds is strikingly correlated with their scopoletin content and that scopoletin is associated with the presence of structures in which differentiation occurs.

Benveniste et al. (28–30) have identified several phytosterols in cultures of N. tabacum. They suggested that this system is a very suitable one for the study of steroid metabolism since the cultures contain very little chlorophyll or carotenoids (cf. 28a). Cultures of Agave toumeyana, Dioscorea composita, a bramble (Rubus fruticosus), endive (Cichorium endivia), and carrot incubated with acetate-1-C14 do not incorporate label into lanosterol, lanostadiene, and lanostadiene; however, cycloartenol was so labeled (cf. 91).
2. Steroidal Glycosides

The rapid advances in medical sciences of hormone therapy have increased the need for adequate supplies of certain cortisone derivatives, sex hormones, and other steroid compounds. Precursors of steroids are often present in plants. Hecogenin, a steroidal sapogenin with an oxygen in the 12 position is a cortisone precursor that may be isolated from *Agave*. *Agave toumeyana* can be grown in semisolid culture as well as in submerged growth (cf. 257, 382) but it does not contain hecogenin (395). Similarly, *Dioscorea composita*, which contains diosgenin, a steroidal sapogenin with a ring structure susceptible to microbial introduction of oxygen at the 11 position can be grown in culture (257, 382). Kaul and Staba (174) have reported the production and isolation of a compound tentatively identified as diosgenin from root callus suspension cultures of *Dioscorea deltoidea*. If this is confirmed, it is of interest since Bennett *et al.* (26) have shown that the biosynthetic activity of the shoot system of *Dioscorea* sp. is not only higher, but qualitatively different from that of the roots and tubers. Indeed, actively growing aerial tissues such as leader shoots appear to be the sites of formation from which diosgenin is translocated to the tubers (19).

*Digitalis glycosides.* Slow growing cultures of *Digitalis lanata* and *D. purpurea* have been initiated by Staba (324) following Hildebrandt and Riker (cf. 123) with the intent of studying the glycosidic components of these cultures. Although Kedde-positive (302) substances were detected (57, 58), there are no chemical data to show that cardiac glycosides are in fact synthesized. On the contrary, it has been found that root suspension cultures of *D. lanata* and callus cultures of *D. purpurea* and *D. mertonensis* are unable to convert digitoxigenin or a number of other precursors to glycosides (356). After 16 days, traces of conversion products were detected. This work has proved to be quite unrepeatable however (cf. 356). More recent work by Medora, Tsao, and Albert (231) on *D. mertonensis* using thin-layer chromatography, has revealed Baljet-positive spots when various steroidal “precursors” (e.g. sterol A, a multistereol surfactant, cholesterol, and polyethylene glycol 1000) were added to the culture medium. Medora *et al.* (232) also reported the biological activity of extracts of callus on guinea pigs [cf. Kaul *et al.* (176) for similar results using extracts of cultures of *Ammi visnaga*, *Cheiranthus cheiri*, and *Urginea maritima*]. There is in all these instances considerable variation in the biological development of the cultures, for it is well known that
*Digitalis* callus forms organized structures (326, 328). It may be, therefore, that it is these organized “plantlets” which, in fact, synthesize the small amounts of substances that have been detected (cf. 176, 201).

### 3. Miscellaneous Glycosides

Examination of cultured tissues of *Iberis sempervirens* for the mustard oil glucosides by the methods of Schultz and Gmelin (304, 305), Schultz and Wagner (306), or Kjaer, Conti, and Larsen (184) gave negative results. Simultaneous examination of either seeds or green parts of entire plants gave positive results for the three glucoside components of *I. sempervirens* (glucoibervirin, glucoerucin, and glucoiberin) (cf. 181, 199).

It appears then that cultures of *I. sempervirens* do not accumulate these compounds whereas they are formed during normal development of this plant. This is another example in which the cultured cells fail to express the synthetic capacities that are evident *in situ* in the plant body.

In this laboratory, actively growing cultures of *Taxus cuspidata* (*T. baccata* subsp. *cuspidata*) of leaf origin have been grown and examined for the cyanogenetic glycoside taxiphyllin (38a). Although intact leaf tissue gives a strongly positive test (cf. 374), cultured tissue does not. The same has been found for tissues of *Trifolium repens* which although genetically competent to produce cyanogenetic glucosides are unable to effect their synthesis in culture (160a).

Visnagin, a furanochromone, has been isolated from and identified in suspension cultures of *Ammi visnaga* (173, 175). A large amount of l-maackiain and a small amount of l-pterocarpin have been isolated and characterized from callus tissue of *Sophora angustifolia* (111a). Netien *et al.* (254a) noted a tremendous decline in cardolenide content (calculated as strophanthin K) during culture of *Periploca graeca*. Similarly, coumarin (1,2-benzopyrone) has been detected in cultures of *Melilotus* (293), although *Trifolium* cultures do not synthesize it. Goris (136) found that carrot root cultures were capable of converting vanillin to glucovanillin. This conversion is thought to be a sort of detoxification mechanism of vanillin under the action of a β-glucosidase.

Thus, although some publicity has been given to the claim that specific glycosides accumulate in aseptic plant cultures (328), the evidence is not strong. Wherever such substances have been detected the amounts were usually small and the compounds in question have rarely been isolated and critically identified. Again, as in the case of the alkaloids, the definitive work that will exploit the capacity of cultured cells and
tissues to synthesize these substances in quantity and at will has yet to be done.

E. VOLATILE OILS AND TERPENOIDS

The growth, development, and metabolism of peppermint has been extensively worked on by Steward and co-workers (355). Although peppermint (Mentha piperita) has been an economically important crop plant, due to its volatile oil content, little systematic work was done on its metabolism until the 1950's (cf. 73).

In 1950 Howe (160) recorded the attempted culture of peppermint. Some slow-growing tissue cultures of peppermint stem were obtained using a basal medium (400) supplemented with 2,4-D and coconut milk. Attempts to adapt the techniques then in use for tissue culture of carrot root phloem and potato tuber were not wholly successful since actively growing tissues could not be maintained continuously.

By 1960, slow growing cultures of M. piperita (‘Black Mitcham’) had been established and maintained for at least a year on a semisolid agar medium containing 15% CM and 2,4-D 1 mg/liter. Further manipulations of the media improved the callus growth dramatically (cf. Fig. 9B and Table XVI). Cultures have been examined to detect their volatile oils. All attempts to detect volatile oils in the tissue cultures of Mentha, have, as yet, been unsuccessful (199).

In 1961 Lin and Staba (209) reported the culture of peppermint (Mentha piperita) and spearmint (M. spicata). Although the data in that paper were not extensive, it appeared that a high-salt medium was necessary as well as an extraordinary amount of inositol (5000

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### TABLE XVI

THE EFFECTS OF COCONUT MILK ON THE GROWTH OF STEM EXPLANTS OF Mentha piperita IN A BASAL MEDIUM Containing BTOA or 2,4-D

<table>
<thead>
<tr>
<th>Supplement added (mg/liter)</th>
<th>Plus coconut milk (15%)</th>
<th>Minus coconut milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTOA, 2</td>
<td>905 ± 52</td>
<td>753 ± 47</td>
</tr>
<tr>
<td>BTOA, 6</td>
<td>1741 ± 315</td>
<td>438 ± 36</td>
</tr>
<tr>
<td>2,4-D, 0.5</td>
<td>1162 ± 175</td>
<td>675 ± 72</td>
</tr>
<tr>
<td>2,4-D, 2</td>
<td>1051 ± 158</td>
<td>152 ± 26</td>
</tr>
<tr>
<td>2,4-D, 6</td>
<td>997 ± 117</td>
<td>418 ± 52</td>
</tr>
<tr>
<td>2,4-D, 10</td>
<td>251 ± 43</td>
<td>226 ± 37</td>
</tr>
<tr>
<td>2,4-D and BTOA, 2 mg each</td>
<td>1587 ± 277</td>
<td>348 ± 38</td>
</tr>
</tbody>
</table>

* White’s Basal Medium, solidified with agar 0.5%.

*b Initial size of explant = 45 mg. Data presented as average fresh weight (mean weight of 3 explants) in milligrams. Length of culture = 40 days in diffuse light.
Fig. 9. Growth of otherwise recalcitrant cultures. (A) *Carica papaya* explanted fruit wall grown 90 days on a high-salt basal medium with coconut milk (5%) supplemented by (left to right), 2,4-D 5 mg/liter; 2,4-D 0.5 mg/liter; IAA 5 mg/liter; IAA 0.5 mg/liter; NAA 5 mg/liter; BTOA 5 mg/liter; BTOA 10 mg/liter. (B) *Mentha piperita* explanted stem tissue grown for 40 days on a high-salt basal medium with coconut milk 10% (far left) and supplemented (left to right) with 2,4-D at 0.5, 1.0, 2.0, 5.0, 6.0 mg/liter; and BTOA 10 mg/liter. The effect of concentration of 2,4-D above 0.5 mg/liter is slight; BTOA promoted organization.

In addition to these requirements, a complex vitamin mixture was used in the presence of either BTOA or 2,4-D. Coconut milk significantly improved callus growth of peppermint and spearmint when used in combination with 2,4-D, but proved only slightly synergistic in the presence of BTOA.

Stohs and Staba (356) noted that cell suspensions of *M. spicata* were unable to convert either digitoxigenin, digitoxin, lithocholic acid, squalene, or mevalonic acid (both in the absence and presence of certain cofactors) to any other compounds. It appears that peppermint tissue cultures (or spearmint tissue cultures for that matter (cf. 393)) are unable to synthesize oils either spontaneously or when fed appro-
appropriate precursors. Presumably, therefore, the metabolism which leads to the essential oils is linked to the environment in situ and to the development of the oil secreting glands which do not form in the cultures. On the other hand, three new sesquiterpene lactones have been isolated from *Andrographis paniculata* cultures but andrographolide, a major constituent of intact plants or related substances could not be detected (la).

In the Umbelliferae, the primary sites of volatile oil accumulation are the oil cavities known as *vittae* (seen as small openings in cross section of the fruits, cf. Fig. 10). A large number of umbellifers grown in this laboratory for the purpose of studying cultured plant cells and their significance for embryology and morphogenesis (cf. 349), have been examined for their volatile constituents. The characteristic odors are conspicuously missing from the flasks of free cultured cells. Extracts obtained from cultured cells of carrot (*Daucus carota* var. *sativus* (cultivated carrot), *D. carota* (wild carrot or Queen Anne’s lace), *Pimpinella anisum* (anise), *Coriandrum sativum* (coriander), *Anethum graveolens* (dill), and *Sium suave* (water parsnip)) have been examined by a thin-layer chromatographic technique for volatile oils with no detectable zones found using several detecting agents.

It is indeed an interesting point that plants which produce characteristic odors or essential oils both smell and taste different when they are cultured in the normal way without forming the specialized organs of shoot and root. Nevertheless, when such plants develop minute plantlets or organization, their characteristic odors return. In other words, the characteristic biochemistry of mature organs and tissues follows the lead of their growth and morphology rather than determining its course in a causal way. That is, specialized biochemistry is as much the outcome of growth and development as is the form of the organs that grow.

![Fig. 10. (A) Cross section of a fruit of fennel (*Foeniculum vulgare*) showing "vittae," i.e., sites of volatile oil formation and storage. (B) Detail of oil-bearing element (photos from a slide supplied by Dr. A. Der Marderosian, Philadelphia College of Pharmacy and Science).](image-url)
F. MISCELLANEOUS COMPOUNDS

1. Anthocyanins

Many pigmented strains have been noted in cultures [cf. Archer (6); Constabel (72a); Gautheret (123, pp. 341-343 for references); Street (363)]. Straus (357, 358) isolated several sublines of corn endosperm which varied in their ability to synthesize anthocyanin. Blakely and Steward (36, 38) described pigmentation in cultures of *Haplopappus gracilis*. These tissues may differ in pigmentation and growth form by altering the culture medium, (cf. Fig. 11).

Strain DS grown on White's basal medium containing 2% coconut milk in addition to casein hydrolyzate and 0.5 mg of NAA per liter displays a prominent red pigmentation. This culture was found to be composed predominantly of diploid cells. When grown on a medium containing a low level of naphthaleneacetic acid, with or without coconut milk, the cultures are deeply pigmented; by contrast, tissues cultured on a medium containing coconut milk and a high level of naphthalene-acetic acid do not form anthocyanin.

Under most conditions the DS strain is heavily anthocyanin-pigmented (about 0.8% of the dry weight); however, Blakely isolated three green strains after plating DS cells on an agar medium containing

Fig. 11. Photograph taken from a Kodachrome of three strains of *Haplopappus gracilis* (see text for details) grown in large flasks for biochemical analysis (199).
10% coconut milk, 0.5 mg of NAA per liter and casein hydrolyzate. One of the green strains was called G 22.

Among the colonies on plates inoculated with DS cells were some colonies of looser texture than others. Four such colonies were isolated and grown in liquid medium containing coconut milk, NAA, and casein hydrolyzate. One of these grew vigorously and was maintained as a separate strain designated F₁ (for "friable"). Not only is F₁ a highly friable strain, but is also light green in color.

This, then, is a clear-cut case of synthesis of special compounds in a culture that can be controlled nutritionally or by selection. The problem of the biosynthesis of the anthocyanin pigments [cyanidin 3-glucoside and cyanidin 3-rutinoside (148)] and their related biochemistry is presently being studied in this and other laboratories (199, 285, 366, 367). Anthocyanin precursors are also known to accumulate in cultures of other plants. Goldstein, Swain, and Tjio (131) have found leucoanthocyanins in the sycamore maple (Acer pseudoplatanus) cell suspensions derived from cambium. Synthesis of monomeric and polymeric leucoanthocyanins based on leucocyanidin (5,7,3,4-tetrahydroxyflavone-3,4-diol) appear to vary with aeration and "strain" differences. Restricted amounts of air greatly reduced the synthesis of the leucoanthocyanins.

2. Flavanoids

Kordan (193, 194), Ranga Swamy (282, 283), and Sabharwal (295, 296) cultured mature juice vesicles from lemon and other Citrus fruits and noticed that the callus masses lacked the characteristic coloration of the intact vesicles. There was an obvious inability to synthesize the necessary pigments. More recently, Kordan and Morgenstern (196) reported that the vesicle stalks from mature lemon fruits (Citrus limon var. 'Eureka') proliferating in culture frequently release a white water-insoluble material to the external medium. Examination of the alcohol-extractable substances yielded strongly fluorescent compounds. The proliferating tissue (as well as the nonproliferating) also turned bright yellow when exposed to ammonia fumes. This test is sensitive for polyphenolic carbon of compounds represented by flavones, flavanones, chalcones, and xanthones. The fluorescence patterns as seen upon chromatography differed from those patterns seen in intact tissues of young seedlings. Hesperidin, the well-known flavanone glycoside which occurs in lemon fruits was not found, however, in the cultures.

3. Lignins

Histogenesis and a measure of organized development in plant tissue cultures is well known [cf. Gautheret (122, 123, 124), Torrey, (373), and
White (402, 404) for reviews]. It is interesting, however, that the range of structural differentiation obtained in callus cultures is not as varied as that encountered during normal ontogenesis. Certain functions seem reduced or simplified in culture while others are pronounced, so that the final range of differentiation is different from what was observed in the intact plant. This is especially true, as we have seen, for the specialized cells such as glandular hairs, laticifers, and resin canals which do not usually develop in either callus or cell suspension cultures. The factors that control histogenesis in cultures seem to be very selective. On the other hand, sometimes characteristic cellular types such as sieve tubes, fibers, and vessels are differentiated, and accordingly, the metabolism peculiar to the specific cell type must occur. Młodzianowski and Szweykowska (239) have reported that a strain of carrot tissue which had been cultured for twenty-five years formed callose which was not restricted exclusively to phloem areas; the cells laid down by the cambium on the side opposite to the xylem were characterized by a special ability to synthesize this substance.

Gautheret (124) points out, however, that the lignin synthesized in cultured tissue is different from the lignin in the organs from which the cultures were derived (cf. 23, 191).

Many workers have utilized culture techniques to study the process of lignification (cf. 211) and information has been obtained from the use of labeled intermediates (cf. 86, 120, 150).

It has also been reported that kinetin may increase lignification (186, 187), and so can gibberellic acid (GA) (188). Koblitz was unable to attribute the increase in lignin to direct synthesis or to the differentiation of tracheids. This relationship was detected by Bergmann (31), who showed that a suitable supply of kinetin increased a hundredfold the number of tracheids in colonies of tobacco, and that this was accompanied by synthesis of lignin. The effect of cytokinin then is not a direct one on the lignification process (cf. 189, 190).

Bergmann (31) has interpreted his findings by suggesting that kinetin may modify the carbohydrate metabolism by shifting it toward the synthesis of shikimic acid and the accumulation of phenylpropane derivatives. Some [e.g., Wetmore and Rier (399)] have found that sugar levels greatly influence the formation of phloem and xylem and the degree of lignification (cf. 237, 238).

Thus, there is considerable evidence that lignin synthesis and differentiation into lignified cells are physiologically independent. Saussay (300) has noted that kinetin, IAA, and NAA block lignification in cambial cultures of willow (Salix cinerea) even though fibrovascular bundles form. The work of von Wacek et al. (392) supports this idea since
lignin precursors like coniferin, when supplied to carrot cultures, merely increased lignin production and deposition, but no more tracheids or vascular elements were formed.

4. Growth Factors and Antibiotics

It is not unexpected that cultures of angiosperms may produce both growth-promoting substances and antimetabolites. In the first place, if the cells are potentially totipotent they ought to be able to manufacture any natural growth regulator for which the whole plant is eventually autotrophic. A simple example of this effect is seen when cultured carrot explants are placed in petri dishes containing free cells distributed on a coconut milk agar medium. It is found that the cells in the vicinity of the previously cultured explant form more and bigger colonies than those remote (Fig. 12). This suggests that the cultured tissue, releases to the medium substances that stimulate the growth of free cells (37). This is in fact a dramatic example of a phenomenon encountered in such situations as the utility of “conditioned” culture media and in the use of the “nurse tissue technique” (248, 249). Tobacco callus cultures can be activated in light to synthesize thiamine (32a).

In addition, Nickell (256) has reported the detection of gibberellin-like substances in a number of cultures. Positive effects were noted

Fig. 12. Effect of cultured carrot explants on a medium containing a rigorously filtered suspension of carrot cells. The photograph was made 45 days after plating. On the plate to the left, which received no explants, 4 visible colonies appeared. On the plate to the right, which received 3 explants, there were over 300 visible colonies. From Blakely and Steward (37).
in the dwarf pea seedling growth test with extracts of both leguminous and nonleguminous dicotyledons as well as some monocotyledons. Activity was not limited by the type of tissue or by the plant part from which it originated. Stem, root, leaf, and cotyledonary tissues were represented as well as nonpathological callus tissues and tissues of virus tumor and crown gall origin. Moreover, Carr and Reid (66) also have recently shown that extracts of carrot root phloem cultures have gibberellin-like activity.

Secondly, it is a common experience that once vigorously established in growth, many cultures preserve their sterility and, this being so, they may well build up their own complements of antibiotics. It is not only comparatively easy to maintain the sterility of the plant tissue cultures but, in the case of viruses and certain organisms that grow in mixed culture, it is very hard often to inoculate them (17, 53, 69, 157, 416).

Aspen (Populus sp.) tissue cultures of cambial origin yield antimicrobial substances which produce inhibitory zones when culture plates are inoculated with a number of bacteria and fungi (225, 226). An antibiotic substance has similarly been noted to be synthesized by avocado cotyledonary cultures; this antibiotic is quite unstable, however, and is primarily active against gram-positive bacteria (cf. 257).

Section IV above has passed in review the relationships that obtain between many classes of compounds in cultured and normal tissue of angiosperms. In the final outcome one needs to recognize both the potential biochemical versatility of such plant material and, simultaneously, the evident difficulties in its full exploitation. The problems here are not genetic. This is clear whenever plants are grown from cells (see Chapter 8) for they then display the full range of genetically determined biochemistry. The challenge now is so to modify the behavior of the tissue in culture that any of its biochemical properties that emerge during normal development also appear under culture conditions. In fact, the dilemma may be that the prior development of form in the cells and the cultures is a prerequisite for the synthesis of certain compounds. Attention will be directed to some examples in Section V which deals with growth and metabolism in more organized systems.

V. Biochemical Relationships between Unorganized and Organized Tissue Cultures

One of the many problems to be faced in assessing the properties of cultured cells and tissues is that of distinguishing relevant from irrelevant change. That cells and tissues undergo changes in their metabolism as they are brought into culture seems well established. The
changes are as varied as the analytical techniques employed to detect them. There appears to be virtually no property of differentiated cells that does not change upon growth induction.

With such a mass of data to deal with, it is a problem to choose criteria that effectively measure the biosynthetic capacity of cultured cells in terms of their ability to perform according to their original cell type.

This raises further problems. What is a differentiated cell? The technique of cell and tissue culture has contributed to our understanding of differentiation, but at the same time has raised problems about the essential features of the differentiated state and calls in question its irreversibility.

One of the best examples of permanent change in tissue cultures is that of "habituation" to auxin (cf. 123). In this situation cultures that originally required an exogenous supply of auxin for growth, gradually lost this requirement. Variations in growth requirements of clones derived from single cells, taken from a common culture, is also well recognized (cf. 363). Proliferated tissue masses have also given rise to friable sublines and strains with distinctive properties (36).

Although the direct approach is to focus attention upon the biochemistry per se, it is clear that this is often predetermined by events which induce morphological changes in the cellular organelles. Thus one would expect the behavior of cultured cells with respect to chloroplast pigments to be greatly affected by the ability of plastids to develop in the cells as cultured and the same considerations should apply to the biochemistry or metabolism that is linked to any other cellular organelle. Obvious as these ideas are they have nevertheless often been ignored. Consideration will now be given to these points.

A. Compounds Associated with Organelles

1. Plastid Pigments

Carrot of course, accumulates carotenoids in the storage root. Carotenoid synthesis in tissue cultured does occur, but usually not in quantity and kind, as in the root, to form the characteristic orange-red color (cf. Table XVII). Some carrot cultures, however, do synthesize carotene as in the mature plant (cf. 253). But the point is best made by reference to chlorophyll which forms in the light in cultures of carrot phloem even as it does in mature leaves. The reason is, however, that in the appropriate medium and in the light, the plastids of cultured carrot form chloroplasts and the chlorophyll synthesis then follows upon the formation of the organelle (347). In other words, the chlorophyll syn-
thesis is preceded by the organization. The synthesis of the pigments is, in turn, determined by nutrition (cf. 39, 301, 367a, 411) and light (cf. 271).

Mahlberg and Venketeswaran (219, 390) have shown chlorophyllous tobacco calluses to possess pigmentation ratios similar to those of the normal leaves, although the total amounts (micrograms per gram fresh weight) were very much less. Hildebrandt et al. (156) reported that the greatest concentrations of chlorophyll, in several different callus cultures, occurred in the culture medium devoid of sugars or those which contained high sugar (8%) concentrations (110). Sunderland (366, 367) has described the effect of various auxins on pigmentation in cultures of Oxalis dispar, Hypochaeris radicata, Haplopappus gracilis, and Acer pseudoplatanus. The calluses contained pigments at concentrations very similar to those in young leaf primordia in which the chloroplasts were rudimentary. Calluses contained fewer chloroplasts per cell on the average than the leaf, and chloroplasts of the callus did not have the complexity of structure now known to be associated with the mature leaf. That the ratio of chlorophyll to carotenoids is lower in the calluses than in the leaves, supports the second conclusion. Carotene bodies, like chlorophyll are derived from proplastids (27), but the factors which control their development are poorly understood (27a, 132, 284).

There is some evidence that the sulfolipids ordinarily associated with chloroplasts (e.g., 6-sulfoquinovosyl diglyceride) may be found in undifferentiated cultures of rose stem ('Paul's Scarlet') which do not contain functional chloroplasts (77). In the same laboratory it has been noted that plastoquinone is always present in normal green tissue in greater concentration than ubiquinone, and at higher levels than those found in rose cultures. In nonphotosynthetic tissue, the situation is reversed, and plastoquinone is generally significantly lower than the

### Table XVII

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight (mg)</th>
<th>Carotene</th>
<th>Xanthophyll</th>
<th>Total carotenoid</th>
<th>Total culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial explant</td>
<td>4.0</td>
<td>0.40</td>
<td>0.09</td>
<td>0.49</td>
<td>0.002</td>
</tr>
<tr>
<td>Cultured tissue</td>
<td>175.0</td>
<td>0.16</td>
<td>0.30</td>
<td>0.46</td>
<td>0.081</td>
</tr>
</tbody>
</table>

*After Steward et al. (335).*
content of ubiquinone (372). These relationships are of interest because of the involvement of these substances in the photosynthetic system (74).

Williams and Goodwin (135, 407) also identified carotenoids in cultures from rose stem ('Paul's Scarlet'). The total concentration of carotenoids in the normal stem and leaf was four and thirty times greater, respectively, than in the cultures (cf. Table XVIII). These tissues contained only traces of terpenoids characteristic of functional chloroplasts (e.g., carotenoids, tocopherol, Vitamin K) and no chlorophyll. The exception was plastoquinone. The cultures did, however, contain considerable amounts of sterols which were present in plastids and did not require light for their synthesis (cf. 133).

Tulecke (381) has found that, when γ-aminolevulinic acid (a precursor of tetrapyrroles) is fed to cultures lacking chlorophyll, porphyrins do form; however, they do not contain magnesium. This suggests that chlorophyll synthesis in the systems studied (haploid albino Ginkgo biloba cultures) may be blocked by their inability to incorporate magnesium into the protoporphyrins.

Russian workers have shown the effects of a two-stage red impulse, given in the course of a series of nights, on the accumulation of pigments in carrot tissue culture (cf. 130). Table XIX, presented here in
translation, shows that the tissues subjected to the action of red light accumulated considerably more pigments—both chlorophylls and, especially, carotenoids. This suggests the involvement of phytochrome. Most spectrophotometric assays of a number of callus tissues for phytochrome have revealed only very low levels in spite of the fact that *Nicotiana tabacum* stem pith callus responds by growth to red and far-red light which is suggestive of phytochrome activity (172). Stem pith of three varieties of tobacco grew better in the presence of red light (660 nm) than the dark controls and those grown only in far-red (730 nm). Tissues irradiated with red light, followed immediately by far-red light, showed a reversal of the effect of red light. There is

<table>
<thead>
<tr>
<th>TABLE XIX</th>
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</thead>
<tbody>
<tr>
<td><strong>THE INFLUENCE OF THE RED LIGHT ON THE ACCUMULATION OF PigMENTS IN CULTURED CARROT TISSUE</strong>&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day of test</th>
<th>Variant</th>
<th>Chlorophyll</th>
<th>Carotenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>Initial tissue</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>5</td>
<td>Test</td>
<td>2.0</td>
<td>---</td>
</tr>
<tr>
<td>Control</td>
<td>1.0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>11</td>
<td>Test</td>
<td>12.7</td>
<td>8.1</td>
</tr>
<tr>
<td>Control</td>
<td>10.1</td>
<td>6.1</td>
<td>1.6</td>
</tr>
<tr>
<td>16</td>
<td>Test</td>
<td>21.6</td>
<td>10.2</td>
</tr>
<tr>
<td>Control</td>
<td>14.5</td>
<td>6.0</td>
<td>2.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> From Godnev et al. (130).

<sup>b</sup> Values given as micrograms per gram of fresh tissue.

now direct evidence that a phytochrome system was involved (172a).

Wetherell and Koukkari (398) have found high phytochrome levels in cultured cells of wild carrot grown in darkness. The amounts of phytochrome detected in the cultured cells are similar to those detected in intact etiolated oat (*Avena sativa*) seedlings. It seems, however, that organization in the cells is a prerequisite to phytochrome accumulation since undifferentiated cells grown in suspension culture do not give positive results. The ability of wild carrot cell suspensions to form embryoids is well documented (cf. 349), and it is in these differentiated, organized systems that the high phytochrome levels seem to occur (197).

In fact, the conclusion to be drawn from all this scattered literature on the variable formation of pigments and substances more or less associated with plastids is that the biosynthesis may reflect the ability of
the cultured cells to develop their organelles in the full complexity of their structure [cf. the role of growth substances and light on the development of plastids in carrot as described by Israel and Steward (162); cf. also Goodwin (134) and Kirk and Tilney-Bassett (179) for a comprehensive treatment of plastid biochemistry].

2. Starch

Mohan Ram and Steward (240) failed to see ergastic bodies like starch and oil globules in tissue cultures of banana (Musa) fruit (cf. Fig. 3, C and F). And although the cells of the potato tuber can be made to grow in culture, the conditions under which they will readily turn sugar in the medium into typical large starch grains in the cell have yet to be found (cf. Fig. 3, B and E). Most cultured cells are usually remarkably free of accumulated storage products, and, though they lack many of the other features by which the mature parenchyma are recognized, they are distinguished in culture by the visible signs of great protoplasmic activity. One exception seems to be the sugarbeet (Beta vulgaris), which normally produces practically no starch, but upon culturing is found to contain a considerable quantity. This starch-producing property is acquired by beet cells only during their growth (294).

Another conspicuous example seems to be the lemon (Citrus limon) (195). Ordinarily, citrus fruits do not accumulate a starch reserve during growth and maturation, but starch synthesis occurs in vesicle stalks from mature lemons within a short period of culture. The ability to make starch is retained thereafter. During the course of development, young lemons when only a few millimeters in diameter, do contain starch in all tissues of the fruit. As the fruit grows and approaches maturity, starch disappears except that which is contained in the seeds and a few granules in the peel. Consequently, although sugars are being supplied to lemons during normal growth and maturation, they are not converted to starch in the normal development of the fruit. However, Kordan (195) found that cultures of lemon fruit and vesicle stalks form “starch-producing parenchymatous callus growths with xylem formation and lipid containing hairs or hair-like extensions from the surface cells.” The outstanding event which occurs in vesicle stalks in culture is the formation of a nucleolar body and starch synthesis.

Thus, there are examples of different kinds of behavior (cf. 371a). At the one extreme, as in the potato tuber, the cells as they develop normally form starch with ease and in abundance, but in culture, the cells derived from the tuber do this with great reluctance. At the other extreme, cells which do not normally produce starch do so with ease in culture.
The examples here are those of the sugarbeet and lemon already referred
to. It is clear, however, that the cultured cells possessed the innate ability
for starch formation, for this occurs normally elsewhere in the plant
body (e.g., in the leaves of the sugarbeet and of the lemon). In other
words, the condition of culture suppressed one innate property in the
potato tuber and released it in the others. Similarly, some cultured tis­sues of artichoke tuber (*Helianthus tuberosus*) produce fructosans
(278), though not necessarily the same ones which constitute the main
product stored in the normal tuber (i.e., inulin). In fact, normal and
crown-gall callus from the tuber of the same species (*H. tuberosus*)
when examined by Kaneko (170) contained no detectable inulin. English
sycamore or sycamore maple (*Acer pseudoplatanus*) cambial cells grown
in suspension culture are known to produce extracellular polysaccharides
which can be isolated from the medium by ethanol precipitation (25).
In a given case, it was qualitatively similar to the composition of the
wall polysaccharides obtained from the cambial region of an intact tree
except that the cambial cells of the tree yielded a predominance of
xylose whereas the external and wall polysaccharides in culture pos­sessed a predominance of arabinose. All these, and no doubt other
similar examples, simply emphasize that the treatments employed to
bring the cells in question into culture intervene to determine their
use of their innate genetic potentialities. Furthermore, one may assume
that starch formation fails in the cultured potato cells because leuco­plasts are not perfected while conversely, in the sugarbeet, these organ­elles presumably form more effectively in culture than in the normal
storage root.

3. *Latex*

The literature on laticifers is extensive for they attracted much early
attention (cf. 81, 143, 241). Although in recent times the special features
of these cells have proved of less interest, several laboratories are con­cerning themselves with the problems of latex tube growth and develop­ment (cf. 243). The latex may be regarded as the cell sap of the
laticiferous element and, as such, is here appropriately considered as
the product of a highly organized cellular inclusion.

Since laticiferous systems are so complex and their mode of develop­ment in the plant body so obscure, it is to be anticipated that these
systems will present special difficulties under culture conditions.

Early attempts were made by Knudson and his group at Cornell
University (cf. 269, 317, 318) to exploit tissue cultures of laticiferous
plants and to investigate their potentialities as rubber-producing systems.
Snyder (318) failed to observe latex tube growth in cultures, nor even
further growth of the portions of the latex tubes which are already present in pith explants. The same worker (cf. 317) observed only one explant, out of many, to proliferate latex tubes in stem pith explants of *Cryptostegia grandiflora*. Neither was latex flow observed from any of the explants broken during subculturing. Snyder (317) also made attempts to grow numerous laticiferous plants [*Allamanda cathartica* var. *hendersonii*, *Apocynum androsaemifolium*, *Apocynum cannabinum*, *Euphorbia tirucalli*, and *Ficus elastica*]. Difficulty was encountered in establishing viable and continuing cultures of these plants and neither morphological nor biochemical evidence of the function of the laticiferous system was forthcoming.

Babcock and Carew (12) reported the tissue culture of certain members of the Apocynaceae (*Rauwolfia serpentina*, *Alstonia constricta*, *Nerium oleander*, *Apocynum cannabinum*)—a family known to contain medicinally active constituents. In general, these cultures were slow growing and neither growth nor chemical identification of these constituents were reported.

Later *Apocynum cannabinum* cultures more than 3 years old were found to be able to biosynthesize glycosides which were not identical with those found in normal tissue (cf. 149).

Mahlberg (217) has worked on the development of nonarticulated laticifers in proliferated embryos of *Euphorbia marginata*. A later report (218) discusses the isolation of a floating cell strain from submerged cell cultures of *E. marginata*. Mahlberg produced actively growing cell cultures by using the medium of Bonner and Devirian (45) with the addition of both 2,4-D and NAA (10⁻⁶M) to the medium. (He also used 4% sucrose rather than the more usual 2%.)

To date, Mahlberg (217, 218) has not seen laticifer formation; neither has he reported latex in cultured cells. Bouychou (47) reported that segments of articulated laticifer vessels were formed by cambial activity when excised tissues from the stem of *Hevea brasiliensis* were maintained on a medium (White's) containing auxin, and that laticifer segments in culture were capable of producing rubber. He concluded that the rubber was produced under culture conditions without the presence of an active photosynthetic mechanism. An important criticism needs to be made here, however. The synthesis of rubber was detected only by staining with Sudan III. This dye will stain many substances. Furthermore, no other report of the culture of *Hevea* has ever been made. If indeed this preliminary observation is correct, then *Hevea* would be an interesting experimental system, but much stronger evidence is needed to support the claim that some rubber was in fact synthesized under tissue culture conditions.
Arreguin and Bonner (9) have shown that although rubber is accumulated in the parenchymatous cells of root and stem in guayule (*Parthenium argentatum*), there is a very important role played by the leaves in the accumulation process. Isolated leafless stem fragments made callus growth, but despite this growth, such sections formed only minute amounts of rubber which did not increase in concentration during culture. That the tissue could accumulate rubber was shown by the fact that its formation was induced by the addition to the nutrient of a leaf extract of intact plants which were themselves rapidly synthesizing rubber. Acetate, acetone, and β-methylcrotonic acid were effective in increasing rubber synthesis. Since these cultures were slow growing (they merely doubled their dry weight in 7 weeks) and had not been subcultured, there is serious question whether they would retain their rubber synthesizing activity after continuous growth in culture.

However structures approximating to laticifers have been seen to differentiate in some cultures; for example in chicory (*Cichorium*) and *Scorzonera* (cf. 122, 123). The laticifers in question developed from large phloem cells which appeared in the middle of a cluster of dividing cells. Some cells which also resembled laticifers have been seen to form cells which arose from a cambium.

Freehand sections of papaya callus cultures made by Krikorian (199) showed no signs whatever of any elongated or laticiferous cells, or of any exuded latex upon subculture when the explants were broken apart and transferred to fresh media.

Secretory canals, if not real laticifers, have also been seen in tissue cultures of *Helianthus tuberosus* (123, 258) and *Dahlia* (75). Gautheret and his group reported frequent observations of ramifying secretory canals or differentiated secretory pockets in the midst of carrot parenchyma produced by the peripheral cells of an explant. Bartoš (24) has reported oil droplets in scattered cells but no secretory pockets. Satsangi and Mohan Ram (299) have obtained cultures of the mature endosperm of castor bean (*Ricinus communis*). The cells of the endosperm as grown on a basal medium alone were small and full of oil. In those more actively growing on a medium supplemented with the synthetic auxin 2,4-D, the cells were larger but the oil content was depleted.

Morel (244) observed the formation of mucilage cells in tissue cultures of *Malva*. However, Ball (16) noted the disappearance of tannin cells in *Sequoia sempervirens* callus cultures during the course of rapid proliferation, and Barker (21) also noted the disappearance of tannin cells in *Tilia* as growth and cell proliferation ensued [also see Constabel (72, 72b)]. Gautheret (cf. 123, p. 255) points out that the surfaces of endive (*Cichorium endivia*) cultures are sprinkled with small drops of coagulated latex. These mark the site of so-called “pseudolaticifers.”
As anticipated at the outset, remarkably little has been learned from the attempted study of laticiferous systems in culture ever since the 1920's when Bobilioff (40, 41) attempted to grow isolated laticifers in culture. This simply follows from the extreme difficulty encountered in reproducing in isolation the conditions under which the laticifers develop \textit{in situ}. This is not surprising. The interesting point is, however, that without the organization of the laticiferous system as it exists \textit{in situ} the cultures are also unable to produce the characteristic latex.

VI. Biochemical Integration in Organelles, Cells, Tissues, and Organs: Control Mechanisms

A. The Role of Organization

The general conclusion is clear and is summarized as follows. It is still difficult to cause cultured tissues and free cells of angiosperms to recapitulate in isolation the metabolism and biochemistry which they exhibit in the environment of the intact plant. This can only mean that the precise way in which one needs to imitate in culture the environment of the cells \textit{in situ} has not yet been found. In part this may be due to the complexity of the stimuli which are needed. Since it took nearly sixty years to realize Haberlandt's prediction (142) that plants could be grown from cells and that free cells could make "artificial embryos" it is not surprising that progress in the utilization of the freely cultured plant system to stimulate normal biochemistry and metabolism has proved slow. The reason may be that cell and tissue culture techniques have leaned toward the use of standardized media; they have even stressed the special virtues of defined media before one was necessarily fully aware of all the purposes for which that medium should be used. They have also tended to place cultured tissue under as constant conditions as possible; constant in the composition of the ambient fluids to which it is exposed, and also constant in respect of the principal environmental variables that regulate its growth. In point of fact, however, the metabolism of intact plants, like their morphological responses, is the consequence not so much of a response to uniformity of external conditions but frequently is a response to a periodically fluctuating environment. This has become particularly evident in the metabolic consequences of length of day, fluctuating day and night temperature, and their interactions with nutrition; but a further principle needs now to be considered.

Differentiation and growth represent an orderly, perhaps programmed, sequence of changes which occur in time. The sequence of these changes is as important as the events themselves. Therefore, to take
the resting or mature tissue and place it, not only in the actively growing state, but in a state in which it grows (not merely by proliferation) but in ways that recapitulate embryology and ontogeny, may well require a sequence of stimuli that are applied in the correct order. This indeed has proved to be the case. Many mature organs now respond to complex synergistic mixtures of growth-regulating substances. Coconut milk itself is such a complex synergistic mixture in which the hexitols of the so-called "neutral fraction" work with, and supplement, the active cell division components of the "active fraction" (276, 312). And, in the role of such substituted adenines as those which Skoog et al. (316) termed cytokinins, interaction with indoleacetic acid or synthetic auxins is a cardinal feature of their activity (cf. 208). But to stimulate tissue into active proliferative growth is one thing, to furnish the conditions which enable it to perform in the free cell state like a zygote and to grow in an orderly fashion with the production of roots and shoots and the formation of minute plantlets requires still further consideration (see Chapter 8). A frequent factor in this type of response has proved to be the application of what has been called the "sequential effect" (223, 350). This effect occurs, and may be utilized, when it is seen that cells are induced to grow rapidly in such a combination as a basal medium supplemented with coconut milk and one or other of many synergists (like NAA or 2,4-D). Having got the system into the most active state of growth, which may not necessarily be compatible with morphogenesis, the cells or cell colonies can be removed to a medium more nearly resembling that which surrounds an immature zygote, and this has been best achieved by the use of the basal medium supplemented only with the coconut milk, or the liquid endosperm of the coconut. In this way many examples have been found in which the free cells have been able to grow in a more organized embryonic way. When this was tried with a strain of carrot cells, very rich in green pigments and found to be capable of profusely forming chloroplasts, a large number of minute plantlets developed which, although composed of a relatively few cells, showed the biochemical diversification characteristic of whole carrot plants (347). The shootlike portion was deep green; a rootlike portion with normal root tips developed proplastids; and along a gradient between the two extremes, where cambial activity and secondary thickening began, one could see the very dense bright red pigmentation characteristic of the formation of carotenoid in mature carrot roots and quite distinct from the creamy yellow carotenoids that normally occur when carrot root tissue is explanted and cultured. In order to achieve the kind of visible biochemical specification, one had to induce in this
system a sufficient degree of morphological specialization or morphogenetic development to furnish the environment in which the cells could respond biochemically in the manner indicated. In this connection, however, it is important to stress that mutants can be selected. Eichenberger (92) isolated a yellow-orange subline from a normally unpigmented carrot culture, and Naef and Turian (253) isolated an orange mutant strain from carrot root cultures that contained as high as 1.6% total carotene and 0.17% total xanthophylls.

By contrast, cultured potato cells of tuber origin, have not as yet been seen to turn green or to differentiate chloroplasts.

B. INTEGRATION OF SYSTEMS IN CULTURE: DIVISION OF LABOR

While it has been, and still is, a laudable ambition to recapitulate in simpler systems the attributes of the more complex, one should nevertheless be prepared for the fact that this mode of attack may have its limitations. Although roots have long been cultured from root tips in certain well known cases, nevertheless the isolated culture of roots is unexpectedly difficult, even today, for many plants (cf. Chapter 6). If one removes leaf primordia at an earlier and earlier state from the shoot apex, the prospect of their normal development in isolation becomes increasingly difficult to achieve. If entire shoot apices are removed, the smaller the amount of subjacent tissue, the harder they are to culture, and the fewer leaf primordia that exist on the apical portion so removed, the greater the difficulty in its culture. In angiosperms, when the attempt is made to remove the central apical dome alone and to show what external requirements control its growth very severe difficulties have been encountered. So much so that to the present no one has really succeeded in achieving this [see Nougarede (260) for a review of this field]. In fact, embryos isolated from ovules are easy enough to grow if cotyledons have already formed upon them, but globular embryos are even harder to grow, if isolated from the ovule, than if they originate in the growth of proembryo-like colonies from free cells (cf. 254). In other words, at the level of cell clusters and organs there is obvious interplay between the organs in question and the stimuli and nutrients it normally receives from the rest of the plant body.

At the cellular level the same principle obviously applies. Intact cells are still far more versatile than isolated particulate preparations, or preparations of cellular organelles. Surviving chloroplasts have been known since the late 1880's, and even had been shown to be able to carry out at least for brief periods the essential steps of photosynthesis
(cf. 95, 96). But when one compares the behavior of the chloroplast in isolation under the best conditions to which it can at present be exposed and the behavior of chloroplasts in situ in cells, one is impressed not only with the similarities but with the disparities that are encountered (8, 128, 163, 169). In the area of protein synthesis, another subject area in which molecular biology seems to have shown how nature performs this all-important process, it is again encountered that the isolated preparation can only perform to an infinitesimal extent and for a very short period any synthesis that remotely resembles the process of protein synthesis in vivo (cf. 76, 98, 145, 146).

The reason for all this seems obvious, even though it may not be widely accepted. The reason why there is a general failure to make isolated organelles to work in vitro simply is that in evolution they were never selected to work in isolation. The complex cellular organization in fact depends for its efficiency on the very subtle way in which, in that organization, one organelle can supplement the effect of, and work with, all the others. Therefore, the time has come when, in the understanding of cellular biochemistry, the analysis of individual reaction steps and sequences may have already told us most of what it may show. What is now needed, however, is an understanding of how the cells function in vivo as intact organizations and how the various parts of the cell can operate in an integrated and controlled way by isolating reaction steps in sequences and in separate compartments.

The study of contrasted metabolism in growing and nongrowing cells of tissue explants, or isolated free cell cultures, has done much to point the way in this direction and to consolidate the ideas expressed above. This has been so in the study of the interactions between carbohydrate metabolism, as this moves toward carbon dioxide as the end product of respiration, and as it moves toward the formation of nitrogen compounds via nitrogen donors which contribute their nitrogenous groups to carbohydrate “ports of entry,” such as the keto acids. From this point on, the metabolism of carbon moves in the direction of protein synthesis, but it is a cardinal feature of cells that the processes of anabolism and catabolism occur simultaneously, side by side in the same cell, though not necessarily in the same compartment or organelle. Therefore, in various papers from this laboratory (34, 337, and references there cited) it has been emphasized that one now expects the intermediary biochemistry to show the feasible steps in vitro, but this may not, necessarily, prescribe what occurs in vivo. This has proved to be the case, because the conventional carbohydrate biochemistry of respiration emphasizes the reaction steps that occur with but little
recourse to, or connection with, the concomitant nitrogen metabolism of the cells. It seems, therefore, that carbon from carbohydrate moves through the Krebs' cycle (in one or other of its modifications) and culminates in the output of carbon dioxide in predictable ways. But, in plant cells, the course of carbohydrate metabolism is not as remote from the course of nitrogen metabolism as these views seem to indicate. Much of the carbohydrate that passes over the metabolic cycle, with the production of the intermediates of the Krebs' cycle, may furnish carbohydrate for protein, but that protein may also enter into metabolism as it is degraded or turned over. So much so, that the amino acids stored in the vacuoles of many cells should not be regarded as the immediate intermediates of protein synthesis, for they are either storage pools of reserve substances or they should be seen as the reworked or converted products of protein breakdown stored in nitrogen-rich forms that conserve the nitrogen for its reuse. Meanwhile the carbon framework from protein breakdown may be fed into the oxidative cycle and so emerge as carbon dioxide.

On this sort of plan, which has been documented elsewhere (cf. 338) much of the carbon that actually emerged from cells—especially growing cells—in the form of carbon dioxide has recently been embodied in protein molecules. The nitrogen that is reduced, and combined with carbon in the form of organic compounds, is recirculated, for rarely is it excreted and rarely is it lost in substantial amounts after it has once been fixed. The point of mentioning this here is that it testifies to the complexity and importance of biochemical integration. Even in such apparently well understood features of metabolism as carbohydrate metabolism and respiration, on the one hand, and amino acid metabolism and protein synthesis, on the other, we need to make many concessions to the complexity of cells. We should also recognize that frequently the conditions that obtain in one organelle or compartment cause a given substrate (e.g., glutamic acid) to behave in a quite different fashion from its counterpart in other areas or organelles (147). Thus, it is clear that we have still a long way to go before we can with confidence prescribe what cells, cultures or resting, will actually do.

The conventional metabolic charts which stipulate the steps of intermediary metabolism and specify the enzymes and the coenzymes by which they are mediated and the current tenets of molecular biology which stipulate how genetic information is transmitted into biochemical reactions merely furnish the component parts of the system (108). But the subtle and complex biological engineering of cells takes these feasible steps and makes them work.

Much is still to be learned both about the way in which the cellular
compartments and organelles operate, in their discrete ways and how they, nevertheless, form part of the integrated whole. Still more, we need to know how the entire system falls under the spell of the factors that control its growth and development. When all this is understood, it may be possible to grow the cells of jack bean and tell them when and how to make urease, but as of now, this seems feasible only if the cells of the jack bean form jack bean plants, for these, in turn, will make urease in the cotyledons of developing jack bean seeds.

C. CONTROL MECHANISMS

It is a basic tenet of modern biology that genes determine metabolism through their control of protein and enzyme synthesis. It is an equally obvious fact that gene expression is programmed and regulated during the course of growth and development. The terms "epigenesis" and "epigenetic control" have been, and are being, used to denote that "something else" which dictates how the innate genetic potentiality is, at any given point along a developmental sequence, expressed. This is the biological problem of differentiation seen in biochemical rather than morphological terms. This may, however, be not two problems but one, for the conditions that restrict the cellular organization into the pattern of a particular differentiated form may automatically prescribe the way it functions as a biosynthetic system or vice versa. The important point is, however, that these are problems which can be understood only in terms of cells, tissues, or organs as organized systems. Therefore, the lessons that can be derived from the behavior of isolated particulate cell free systems may be strictly limited.

This principle can be illustrated even in such apparently simpler systems as the fungi. Thus, biochemical performance is related to the complexity of the system in which it works, as shown by the following selected examples.

The spores of many fungi contain unique substances which are in themselves particular metabolites even as the metabolites released by mycelia into the media. This is obviously so in those species which form colored spores from colorless mycelium; it is equally true of such spore constituents as the antibiotic peptide fungisporin (236) (cf. 385). Even in the more stringent cell economy of bacteria, similar relationships are known, as in the case of bacitracin synthesis. This peptide antibiotic is produced by Bacillus subtilis in a post-log phase during which the onset of sporulation is determined (33).

Nearly all strains of ergot (Claviceps purpurea) are capable of pro-
ducing alkaloids when grown on rye (*Secale cereale*) (cf. 69a), but only a few biochemical races are able to produce these compounds under saprophytic conditions (369, 372a). The indole nature of the ergot alkaloids has suggested to some that either auxin of the host plant, or similar substances originating from tryptophan metabolism of the fungus, may play a role in the biosynthesis of these alkaloids. Although auxins are known to increase yields of alkaloids significantly, the effect of these additives is still small in comparison with high alkaloid production in the normal case in which the fungus is in contact with its host (371). Clearly, strain specificity is a problem in alkaloid production, as in antibiotic production. The efficiency of antibiotic-producing microorganisms, can be increased by selection and by the use of mutations. Abundant alkaloid production is correlated with restricted growth brought about by the composition of the medium and also with reduced growth rate in the culture; it is also correlated with the morphological form of the constituent hyphae (222a, 372a).

Muscarine has been isolated in a yield of 0.013% from mycelia of *Clitocybe rivulosa* grown on a medium supplemented with beer wort. This suggests that the fruiting body (carpophore) of the fungus is not essential (368).

The phenomenon of spore formation, and its relationship to secondary metabolic processes, merits some further comment. In the first place, the conditions for sporulation are very similar to those which determine the onset of a specific metabolism. Both are usually responses which occur when some limiting factor becomes operative. The two are not indissolubly linked, however. Either may occur independently of the other, but both are responses to similar determining conditions.

Thus, even in the field of biochemistry of microorganisms, which has been so greatly exploited for specific syntheses, relations to the organization of the system in question are still apparent.

Perhaps as much is known about the structure and properties of certain viruses as of any similarly complex biologically important materials. But their synthesis is still far more feasible in the milieu provided by living cells than in any test tube and, without the system with which they interact, the viruses are merely interesting, complex, large organic molecules.

A study of tobacco mosaic virus synthesis in leaves (121) attributed its success *in vivo* in part to the fact that much of the carbon that enters into the virus molecule was recently in chloroplast protein, and it becomes available in the vicinity of the plastid, after protein turnover, for virus synthesis. In other words, it would seem a contradiction in terms and a failure in understanding to interpret virus synthesis and
action without recognizing it as part of the organized cell system which itself has a developmental history, and in which the processes of synthesis and turnover proceed at a controlled and compatible pace.

In fact, there may well be a limit to our ability to understand even photosynthesis and protein synthesis by removing chloroplasts or ribosomes, respectively, from the smoothly working integrated cellular system of which they form a part. And certainly no interpretation of these problems that fails to recognize that it is cells that furnish the environment in which these organelles can multiply and grow can have much real meaning.

A widely publicized approach to this general problem is the "molecular biology of development" as interpreted by Bonner (44). The general thesis here is that the abundant histones in nuclei and surrounding chromosomes constitute the gene-control system. Genes are activated, or suppressed, inasmuch as they are covered or uncovered by the "histone coat." (What in turn controls the histones may be, even so, shrouded in mystery.) An activated gene is one that is free to release its mRNA into the cytoplasm and so promote the synthesis of its corresponding protein at the ribosome template. Along these lines, Bonner has studied globulin of pea cotyledons, for which serological methods of detection are available. He develops evidence to show that the specific ability to form the protein is not dictated by the ribosomes or by their origin, but rather by the message they receive. But, at best, the amounts of "pea globulin" made in this way are infinitesimal; if one really needed to make such protein, it would still be best to use pea globulin cells and preferably to grow them in pea plants into cotyledons!

In fact, there seem to be no well-authenticated data that can claim the synthesis in cell-free systems of protein in quantity (cf. 76, 145, 146). Experiments in this laboratory with bacteria-free, cell-free systems, drawn from actively growing cultured cells, and which have simulated the conditions from which most claims for protein synthesis have been made, have emphasized the great gap between the biosynthetic potential of the best cell-free systems and the cells from which they were derived (346).

Work on higher plants, which incidentally are responsible for most of the world's protein synthesis, seems still to have much to add to the conventional "dogma" of molecular biology. The very idea of a control system based on transient mRNA's may need to be supplemented when it is considered how highly organized plant cells are, how relatively great are the distances between their organelles, and how discrete their functions, and how complex are their interrelationships. The molecules
Fig. 13. Regulators of metabolism and synthesis and their relation to protein and nucleic acid. This chart emphasizes the ways in which various specific inhibitors (antibiotics) have been used (cf. 313). The various plant growth regulators may, therefore, intervene at similarly specific points. There is a general school of thought that now implicates specific mRNA's induced by auxin (IAA) as mediators of the auxin effect (116, 177), but, to the present, specific mRNA's for the wide range of synthetic auxins (NAA, 2,4-D, etc.) have not been reported. If the gibberellins stimulate de novo enzyme synthesis, e.g., α-amylase (386), they should also induce the corresponding mRNA. If all the gibberellins [now about 28 in number; cf. Brian (56)] act through specific enzymes, then their respective antimetabolites [e.g., CCC, B-nine, Amo-1618, Phosphon-D; Cathey (67)] might act at the same sites (14, 265). Other growth retardants (e.g., abscisic acid, maleic hydrazide, 6-methylpurine) could act at selected points along the DNA-RNA sequence as shown (cf. 93, 264). Since abscisic acid reverses the effect of gibberellin in the α-amylase test (70), this implicates the same site in both actions. The complexes which stimulate cell division (cytokinins and their synergists) ultimately set in motion all aspects of protein and nucleic acid metabolism with many possible points of contact with the schemata as shown. In particular the presence of zeatin derivatives in transfer RNA's (106, 144) may link these compounds to amino acid recognition and protein metabolism. Bonner and his associates envision histones as a “protein coat” which regulates DNA-mediated mRNA synthesis (46, 59). Modified, with permission, from a map by Dr. Y. Miura, Chiba University, Chiba, Japan (cf. Calbiochem Biologies 44, October, 1965).
that control this organization and regulate biosynthesis in cells of higher plants should, therefore, be more stable even as they need to be responsive to environmental stimuli which are extrinsic to the cells within which they act. All the evidence of plant responses to length of day and to night temperature, to seasonal periodicity, etc., conform to this view; there is, therefore, every reason to believe that the various classes of stable growth-regulating substances (auxins, cytokinins and the gibberellins with their respective inhibitors, as well as those substances that mediate the effect of various morphogenetic stimuli) make their impact on cells through their ability to control metabolism and biosynthesis. Whether these exogenous, nonnuclear agents, which are certainly not nucleic acids per se, must act in the cells through their ability to promote the production of appropriate mRNA’s may still be a possible, but still largely unproven, hypothesis [see Galston (116) and Heslop-Harrison (152) for a discussion of regulation of differentiation]. The possible points of contact between current hypotheses of protein and enzyme synthesis and their regulation on the one hand, and biochemical differentiation mediated by exogenous growth substances on the other, could be visualized in the terms of Fig. 13. Only further work can show how meaningful these relationships might be.

However, it seems inconceivable that all the varied molecules now known to act as growth regulators could do so by entering into covalent linkages with nucleic acids (cf. 58a).

Whatever the final outcome, one needs to recognize the profound metabolic consequences of the external environment of cells and how much metabolic change may be mediated by exogenously supplied chemical growth-regulating compounds. One also needs to concede that staple, continuing processes of biosynthesis are still dependent upon the organization of the cells in which they occur. Moreover, the widely accepted doctrines of molecular biology (cf. Fig. 13) may now need to be balanced by a renewed respect for the organization of cells that grow and for the extent to which, as they grow and develop, their metabolism and biosynthesis conforms to a prescribed pattern.

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