THE BASES OF ANGIOSPERM PHYLOGENY: CHEMOTAXONOMY[1,2]

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Abstract

The use of the distribution patterns of plant natural products—alkaloids, terpenes, phenolics, etc.—is well established as a major tool for investigating population structures, species, and phyletic relationships of genera. Here, it is suggested that the distribution patterns of biogenetically closely related substances should be of considerable value for deducing evolutionary relationships at higher taxonomic levels. Approximately 540 plant taxa (cultivars through orders) have been included in approximately 150 systematic serological publications in the last 25 years. Research has demonstrated that extracts of seeds, pollen, leaves, tubers, and spores of vascular plants can be used if the required extraction procedures are followed. Both quantitative and qualitative immunological techniques have provided complementary data which have proven to be provocative and valuable in the classification of higher plants. The examples presented clearly indicate serology has contributed chemical data which can be—and have been—used with other data to aid in producing systems of classification such as those of Cronquist and Takhtajan. The phylogenetic relationships among 15 species belonging to 12 families of vascular plants based on a comparison of cytochrome *c* amino acid sequences agree in general outline with morphologically based phylogenetic diagrams. Amino acid sequence data on homologous plant proteins are in too limited a supply to permit other than very preliminary phylogenetic comparisons. Acquisition of more data will require considerable time and work before an impact will be realized. Published protein sequence data have not revolutionized presently accepted phylogenetic diagrams, and it is too soon to hint at the ultimate contribution of sequence data to phylogenetic schemes. The technique of nucleic acid hybridization is, in principle, applicable to chemotaxonomy at all taxonomic levels since it involves the fundamental hereditary material deoxyribonucleic acid (DNA) and its transcribed copy, ribonucleic acid (RNA). In contrast to the relative ease with which meaningful plant natural products distribution patterns are determined, are the difficulties and patience required to carry out nucleic acid hybridization experiments and to interpret the results from them. Thus, it is not surprising that few nucleic acid hybridization data for higher plants are available to meaningfully influence the interpretations of Cronquist and Takhtajan for the evolution of the angiosperms; nevertheless, the method inherently has great potential.

General Introduction

With the development of plant natural products chemistry, which deals with a myriad of alkaloids, phenolics, mustard oils, terpenoids, etc., botanists and chemists have revealed that it is possible to employ chemical constituents to help characterize, classify, and describe taxa. Attempts to correlate morphological and chemical characteristics are very old. Greene (1909) indicated that the most remote and primitive of botanical writers, of whatever country, found a botanical

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vocabulary in the colloquial speech. The reason for this was the many uses of local plants. These uses resulted from the plants' distinctive chemistry, as reflected in color, esculence, flavor, odor, or toxicity.

Petiver (1699) published about the correlations between medicinal (chemical) properties and certain morphological groupings. He used "Herbae Umbelliferae" together with the Labiatae and Cruciferae to illustrate the hypothesis that morphologically similar plants produce constituents (chemicals) with similar therapeutical effects. Hoffmann (1846) in his treatment of families of flowering plants described the chemical characteristics of each of them. He believed that phytochemistry offered the opportunity to check proposed classifications based solely on morphological characteristics. Greshoff (1893) stated several basic tenets. One stated that biochemists and phytochemists had to investigate evolutionary tendencies of metabolic pathways and groups of chemically related plant constituents much more thoroughly before they would achieve an understanding of evolution comparable to that of morphologists. McNair's (1965) book, which is a reprinting of his published papers, considered taxonomy in relation to oils, fats, waxes, oil and starch in seeds, and alkaloids. McNair's 1935 reprinted paper "Angiosperm Phylogeny on a Chemical Basis" included in his book has a "ring" very similar to the present symposium. Gibbs (1974) published a book (encyclopedic) containing four volumes in which he reported chemical information from a vast amount of literature and chemical tests on numerous flowering plants.

Although the concept of employing chemical data in systematic investigations is an old one, a genuine and intensified endeavor to understand possible correlations between plant constituents and classification has been relatively recent. Chemical characteristics were neglected for a long time because information in most plant groups was too scanty and scattered for any individual group. Interest in this type of research has increased as more data have been obtained from biochemical, immunochemical, and organic chemical research. The development of relatively quick and simple analytical techniques has hastened the "coming of age" of chemotaxonomy.

The "present age" of chemosystematics or chemotaxonomy commenced in the mid 1950's. The oldest of the "present age" plant chemotaxonomic approaches is serotaxonomy and the youngest is amino acid sequencing.

Three books (Alston & Turner, 1963; Swain, 1963; Leone, 1964) provided general information and/or reviews about the early chemotaxonomic and serotaxonomic research. Since that time, numerous comprehensive chemotaxonomic reports have been published in journals, symposia, reviews, and books which clearly indicate the mounting interest in this diversified field of research (Bendz & Santesson, 1974; Boulter et al., 1972; Boulter, 1973; Fairbrothers, 1968, 1975; Harborne, 1967, 1968, 1970; Harborne & Swain, 1969; Harborne et al., 1971; Hawkes, 1968; Hegnauer, 1962–1973; Heywood, 1971; Hunziker, 1969; Kubitzki, 1969, 1972; Mabry et al., 1968; Runeckles & Mabry, 1973; Runeckles & Tso, 1972; Runeckles & Watkins, 1972; Seikel and Runeckles, 1969; Steelink & Runeckles, 1970; Swain, 1973; Turner, 1969; Vaughan, 1968).

Most chemical approaches to systematic problems can be classified according

to the kind of molecules investigated. If the compounds are of relatively low molecular weight (free amino acids, alkaloids, phenolics, terpenes, etc.) they are designated micromolecules. If the compounds are of high molecular weight and polymeric (carbohydrates, DNA or RNA, and proteins) they are designated macromolecules.

Micromolecules—Plant Natural Products

Since the first attempt to place into any kind of perspective the potential of chemical characters for systematics—Alston & Turner's (1963) *Biochemical Systematics*—we have witnessed a deluge of chemotaxonomic reports, reviews, volumes, and symposia; much of these data have been painstakingly assembled by Hegnauer (1962–1973) and co-workers into six volumes. This burst of activity resulted in part because some sort of structurally precise chemical information can be readily obtained for every plant available for investigation. And the complex chemical structures often represent hundreds of genes.

However, despite the wealth of chemical information, only a few systematically meaningful interpretations have emerged. Nevertheless, the future for gaining new insights into angiosperm phylogeny using micromolecular data is bright as more and more future Cronquists and Takhtajans become trained in plant chemistry.

Before discussing the extent to which natural products are important for phylogeny at the higher taxonomic categories of angiosperms and the implications of the distributions of these compounds with respect to the Takhtajan and Cronquist systems, certain definitions and general remarks regarding such plant constituents are in order. The expression "plant natural products" is used here to denote the million or so alkaloids, terpenes, phenolics, quinones, etc. which have restricted distributions in plants. It is the "restricted distribution" phenomenon which permits these substances of low molecular weights, usually less than 1000, to be employed as phyletic markers.

Each plant species probably produces from about fifty to several hundred natural products for a variety of functions, including metabolism, defense, structure, and energy and material storage. For the most part, these functions have determined which compounds and which structural features within classes of compounds have been either conserved or modified by selection; however, some modifications of the natural products chemistry may have resulted secondarily as selection operated upon the early stages of pathways leading to the natural products. In any case, it is not uncommon to find that a high percentage of a plant's chemistry, sometimes more than 80–90%, has been conserved such that it occurs in a group of closely related species. Although for higher taxonomic categories the percentages become less, it is frequently possible at the level of tribe, family, and order to recognize biogenetically related compounds which reflect the plant's evolutionary history. It is these latter types of chemical patterns, which involve the distribution of biogenetically homogeneous classes of natural products, that form the basis of the present discussion.

It is not feasible to indicate taxonomically diagnostic classes of natural products for all orders and families; instead a few selected examples are evaluated with

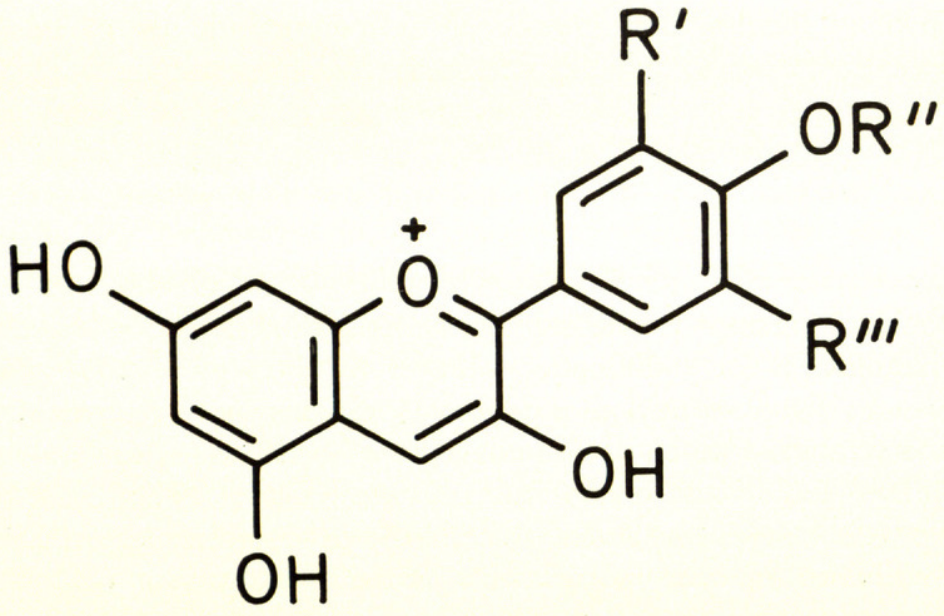


FIGURE 1. Anthocyanidins contained as the aglycone in anthocyanins. Pelargonidin: R'-''' = H; cyanidin: R''-''' = H, R' = OH; peonidin: R''-''' = H, R' = OCH_3; delphinidin: R'' = H, R'-''' = OH; petunidin: R'' = H, R''' = OH, R' = OCH_3; malvidin: R'' = H, R'-''' = OCH_3.

respect to the interpretations of Cronquist (1968) and Takhtajan (1969) for the taxa involved.

It should be emphasized that the application of the distribution of a particular class of natural products for phylogeny assumes that the same or similar structures are derived by evolutionally related sets of enzymes. Although this hypothesis is almost certainly true in most instances, especially at the generic level, it should be recognized that the independent origin of some substances apparently does occasionally occur in unrelated taxa.

PIGMENTS

Anthocyanins (anthocyanidin glycosides) are widely distributed in angiosperms and most higher plants (except for those producing betalains) contain as the aglycone one or more of six anthocyanidins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (Fig. 1).

Thus, because most plants contain anthocyanins with the same or similar anthocyanidins and glycosyl moieties, these substances are of little value for phylogenetic purposes. Even the odd distribution pattern such as the scarcity of pelargonidin in Australian plants has been attributed to the low frequence of bird pollinating mechanisms for the Australian flora rather than to phylogeny (Harborne, 1967). Anthocyanidins other than the six common ones are very rare and few in number, being found regularly in only three families, Primulaceae, Plumbaginaceae, and Gesneriaceae, all of which are treated as being phyletically unrelated by both Cronquist (1968) and Takhtajan (1969).

In contrast to the taxonomic insignificance of the distribution of the anthocyanidins, the occurrence of the red-violet and yellow betalains in several evolutionally-related families (Table 1) of what some workers refer to as the order Centrospermae (see, for example, Mabry et al., 1963) represents one of the

TABLE 1. Betalain-producing families of the order Centrospermae (Mabry, et al., 1963) or Caryophyllales (Cronquist, 1968; Takhtajan, 1969).

Aizoaceae	Didiereaceae
Amaranthaceae	Nyctaginaceae
Basellaceae	Phytolaccaceae
Cactaceae	Portulacaceae
Chenopodiaceae	

classic examples of the way a group of low molecular weight substances have been conserved at higher taxonomic categories, and thus can be employed as genetic markers at these levels (Mabry et al., 1972, and references therein).

The betalain pathway has provided a group of evolutionally-related plants (see also NUCLEIC ACID HYBRIDIZATIONS below) with substances which replace the red and yellow anthocyanin pigments common to most angiosperms. As far as is known, the two classes of pigments, anthocyanins and betalains, never occur together in the same plant or even separately in members of the same family (Kimler et al., 1970). Comparison of wavelengths of the absorption maxima for some of these pigments indicates the importance of the chromophoric group, presumably to attract pollinators (Fig. 2); nevertheless the high concentration of betalains in the stems and leaves of the plants which produce them suggest that these compounds, like many natural products, are polyfunctional.

Unlike Mabry et al. (1963), both Takhtajan and Cronquist have included anthocyanin-producing families in the same order with the betalain-producing families. However, both Takhtajan and Cronquist, unlike most earlier systematists, did include all betalain-producing families in their order Caryophyllales. In this connection, the presence of betalains in the Cactaceae and Didiereaceae was probably a decisive factor in placing these families in this order. Cronquist aligns only two anthocyanin-producing families, the Molluginaceae and the Caryophyllaceae, in his Caryophyllales while Takhtajan also includes the Bataceae, which apparently does not produce either type of pigment (Mabry & Turner, 1964). Support for the relatively close relationship of the anthocyanin-producing Molluginaceae and Caryophyllaceae with those betalain-producing families is available from other sources, including DNA-RNA studies (for the Caryophyllaceae only; see NUCLEIC ACID HYBRIDIZATIONS below) and ultrastructural research on sieve-element plastids. Sieve-element plastids of the betalain-producing families are characterized by ring like inclusions composed of proteinaceous filaments (Behnke & Turner, 1971; Behnke, 1972, this symposium). These structures have not been observed in most other dicot families (most sieve-element plastids in dicots contain starch but no filaments). Fifty-five species belonging to the following 12 families were found to contain these unique inclusions: Phytolaccaceae, Nyctaginaceae, Didiereaceae, Amaranthaceae, Chenopodiaceae, Aizoaceae, Molluginaceae, Cactaceae, Portulacaceae, Basellaceae, Caryophyllaceae, and Dysphaniaceae (this latter taxon has just been investigated for its pigments and found to contain betalains; Mabry & co-workers, unpublished).

It is also interesting that the Polygonaceae (anthocyanin-containing)—which is treated as an order closely related to the Caryophyllales by both Cronquist

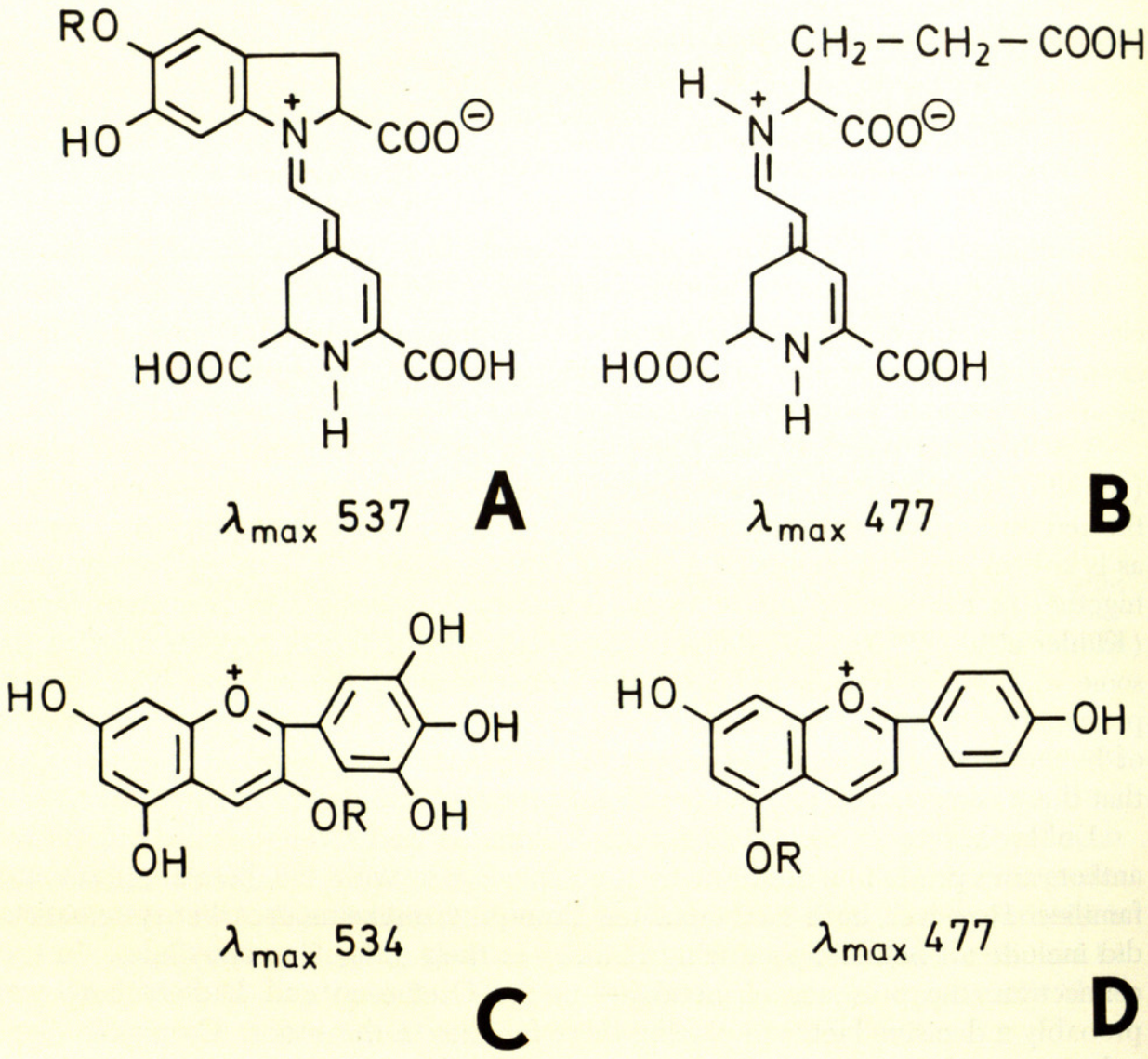


FIGURE 2. The visible absorption maxima of typical red (A) and yellow (B) betalains, which are found in nine phyletically-related plant families, are similar to those for some red (C) and yellow (D) anthocyanins, a class of pigments which occur in most flowering plant families other than those producing betalains.

and Takhtajan—as well as the Bataceae, did not contain the proteinaceous inclusions in their sieve-element plastids. As already pointed out, Takhtajan includes the Bataceae in his order Caryophyllales; however DNA-RNA studies (see below) as well as the ultrastructural data would suggest that this family is distinct from those which produce betalains (see also section GLUCOSINOLATES below). Cronquist placed the Bataceae in an order directly following his Caryophyllales but noted that it "may or may not be closely allied to (them)." The presence of anthocyanins and the absence of betalains in the Illecebraceae (see Mabry et al., 1963), treated by both Takhtajan and Cronquist as in or near the Caryophyllaceae, would suggest that they might be aligned with the Caryophyllaceae, close to but separate from the order containing the betalain families.

Among the taxa which have been traditionally treated as related to the betalain families is the Theligonaceae (Cynocrambaceae) which Cronquist and Takhtajan exclude from their Caryophyllales; Takhtajan erected an order Theligonales near

TABLE 2. Centrospermae-like taxa whose pigment content is unknown.[a]

Family	Disposition with respect to their order Caryophyllales	
	Cronquist	Takhtajan
Achatocarpaceae	Included in Phytolaccaceae	Included in Phytolaccaceae
Gyrostemonaceae	Included in Phytolaccaceae	Included in Phytolaccaceae
Barbeuiaceae	Included in Phytolaccaceae	Included in Phytolaccaceae
Sphenocleaceae	Excluded	Excluded
Hectorellaceae	Not treated	Included as a distinct family

[a] Interested collaborators are asked to send 1 gram of air-dried red-pigmented parts (flowers, leaves, or stems) of members of any of these taxa to T. J. Mabry for chemical analysis.

the Caryophyllales for this single family, whereas Cronquist placed it in the order Haloragales. More recently, Wunderlich (1971), on the basis of anatomical, morphological, and embryological evidence, placed *Theligonum* firmly in the family Rubiaceae. Strong support for this latter decision is provided by Kooiman's (1971) recent discovery that species of *Theligonum* contain terpene-derived iridoid compounds which are typical of members of the Rubiales (see Figure 10), but are not reported from the Caryophyllales. In addition, *Theligonum* does not have the proteinaceous sieve-tube plastids typical of members of the Caryophyllales (Behnke, 1972). Recently, Mabry et al. (1975) detected anthocyanins in *Theligonum cynocrambe* supporting the exclusion of *Theligonum* from the order Centrospermae.

The recent report (Hunziker et al., 1974) of betalains and P-type sieve-element plastids in *Halophytum*, a genus which has been treated as a member of the Chenopodiaceae or as a distinct family, confirms the alignment of the genus to the Centrospermae. Similarly, the discovery of betalains and P-type sieve-element plastids in *Petiveris* and *Adgestis* (Phytolaccaceae; Behnke et al., 1974) and betalains in *Giskia* (Mabry & co-workers, unpublished; not analyzed yet for sieve-element plastids) supports the assignment of these genera to the Centrospermae.

The pigments of the taxa listed in Table 2 remain to be determined. These taxa have been placed at one time or another in or near those taxa which produce betalains.

GLUCOSINOLATES (MUSTARD OIL GLUCOSIDES)

The glucosinolates are widely distributed in the families which both Cronquist (1968) and Takhtajan (1969) place in their order Capparales (Table 3). In erecting the order Capparales, Cronquist noted that "nearly all of the Cruciferae and many of the Capparaceae have specialized myrosin cells, which are chiefly though not entirely restricted to this order. . . . Myrosin is an enzyme involved in the formation of mustard oil" [from glucosinolates] (Figs. 3–5). Although there are a few reports of glucosinolates occurring outside the order Capparales, the widespread distribution of them in the five families in the first column of Table 3 supports the similar interpretations of both Cronquist and Takhtajan for this order. The partly discontinuous occurrence of glucosinolates in species of the Euphorbiaceae, Caricaceae, Gyrostemonaceae, Salvadoraceae, Limnanthaceae, and Tropaeolaceae suggest an independent origin of these substances in these taxa; it is of

TABLE 3. Order Capparales and the distribution of glucosinolates.

Cronquist	Takhtajan	Glucosinolates[a]
Capparaceae (including Koeberliniaceae, Pentadiplandraceae)	Capparaceae (including Cleomaceae, *Oceanopapaver*)	Present; *Oceanopapaver* not investigated
	Koeberliniaceae (including Canotiaceae)	Not detected in *Koeberlinia*
	Pentadiplandraceae	Not investigated
Cruciferae	Brassicaceae (Cruciferae)	Present
Moringaceae	Moringaceae	Present
Resedaceae	Resedaceae	Present
Tovariaceae	Tovariaceae	Present
	Emblingiaceae	Not investigated

[a] Distribution according to Ettlinger & Kjaer (1968) and Kjaer (private communication, 1973); see also Bataceae (in text).

interest, however, that the latter two glucosinolate-producing families are considered to be related and are placed together by both Cronquist and Takhtajan in the order Geraniales.

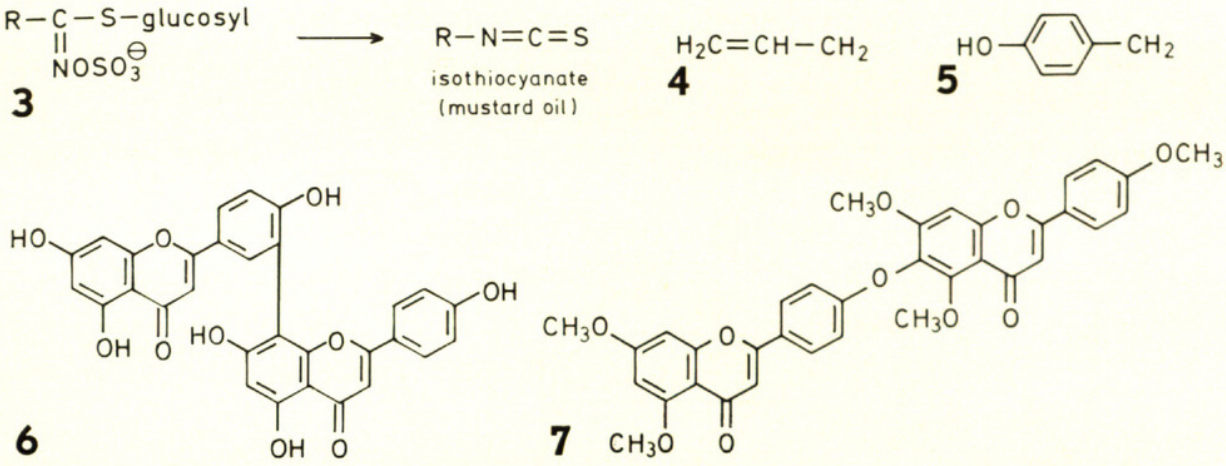
Especially interesting in relation to what has been said regarding the phyletic position of the Bataceae is the recent discovery of myrosin in this family (Schraudolf et al., 1972). But it should be noted that the Gyrostemonaceae (placed by both Cronquist and Takhtajan in the Caryophyllales) also contains myrosin, and until the pigments[6] and sieve-element plastids of this critical family are resolved it would seem premature to give undue weight to the myrosin, in spite of the morphological data which also suggests a relationship of the Bataceae with the Capparales (Eckhardt, 1959).

PHENOLICS

Phenolics in higher plants are derived almost without exception by two biogenetic pathways, the shikimate and acetate routes. Moreover, one or more classes of phenolics have been found in every plant family examined and certain structural types usually suggest evolutionary relationships at various taxonomic levels. The significance of the distribution of a few phenolics as they bear upon the views of Cronquist and Takhtajan are discussed.

Biflavonoids.—Biflavonoids, which contain two flavonoid aglycones linked by a carbon-carbon bond as in amentoflavone, or an oxygen atom as in hinokiflavone permethyl ether (Figs. 6–7), are considered to be among the more primitive phenolic substances elaborated by plants since they are reported from most gymnosperm families and from the pteridophytes. In contrast, only four mostly woody genera all from different families of angiosperms are currently known to

[6] According to B. L. Turner, personal communication, in the living state *Gyrostemon* has a very distinctive reddish-brown pigment; as yet this has not been investigated.



FIGURES 3–7. Glucosinolates and biflavonoids.—3. Glucosinolates (mustard oil glucosides), which are widely distributed in the order Capparales, are readily converted by acid or an appropriate enzyme into isothiocyanates. R can be a variety of aliphatic or aryl groups including, notably, the groups shown in Figs. 4–5.—4. Propene.—5. 4-hydroxy toluene.—6–N. Biflavonoids, which contain two monomer flavonoid aglycone skeletons linked by either a carbon-carbon bond or an oxygen atom, are considered to be primitive phenolics. 6. Amentoflavone.—7. Hinokiflavone permethyl ether.

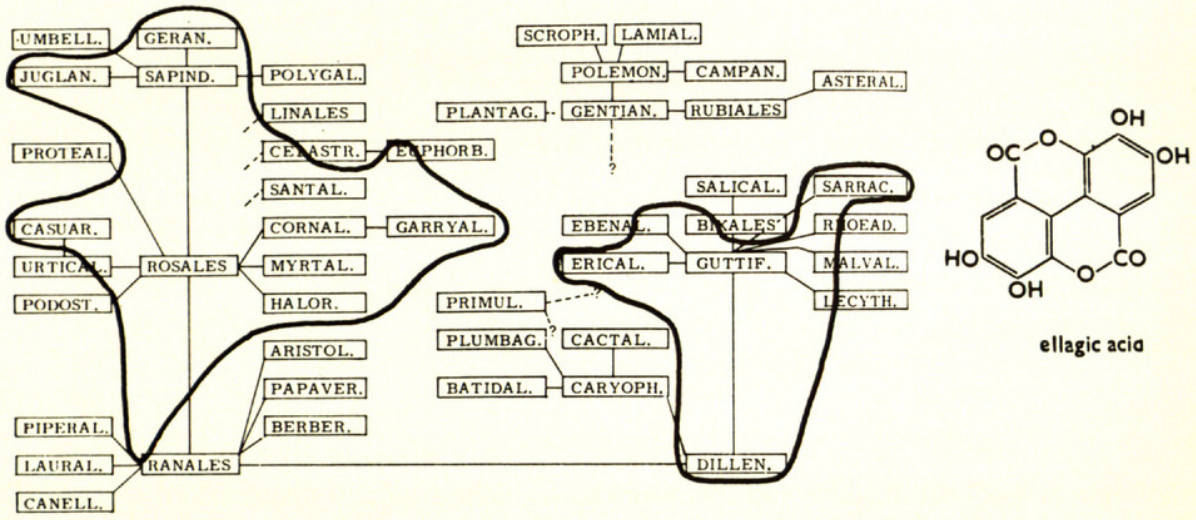
contain biflavonoids. These include *Viburnum* (Caprifoliaceae; Hörhammer et al., 1965; Glennie, 1969), *Garcinia* (Guttiferae; Herbin et al., 1970; and others) and *Hevea* (Euphorbiaceae; Madhav, 1969). However, the most interesting reports of biflavonoids in the angiosperms are those for the Casuarinaceae, a family which has been considered by at least a few previous workers (e.g. Engler and Wettstein) to be among the more primitive angiosperms. The presence of biflavonoids in *Casuarina* (see Harborne, 1967) supports these earlier views rather than those of Cronquist and Takhtajan, both of whom consider *Casuarina* to be a reduced rather than a primitive type. However, Hegnauer has privately mentioned that the polyphenols (e.g., ellagic acid) of *Casuarina* point to the Hamamelidae rather than to gymnosperms.

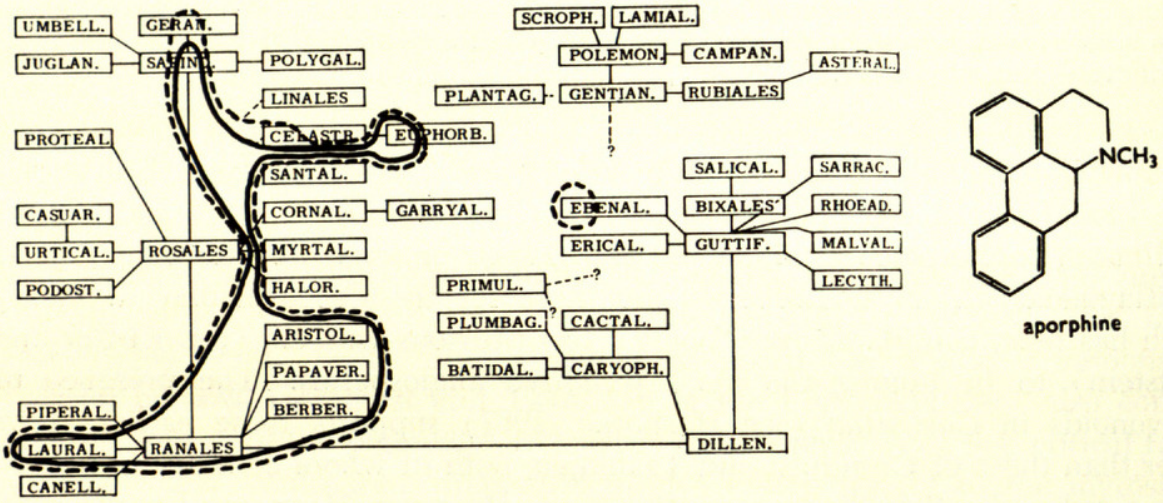
Vic-Trihydroxyphenolics and Leucoanthocyanidins.—The presence or absence of such phenolics as the widespread flavonols kaempferol and quercetin and their derivatives is of little use in relating plant families. On the other hand, Bate-Smith (1962, 1966, 1969) as well as others have suggested that the presence or absence of phenolics which contain vic-trihydroxy systems and leucoanthocyanidins is highly significant and that the presence of either or both is indicative of primitiveness.[7]

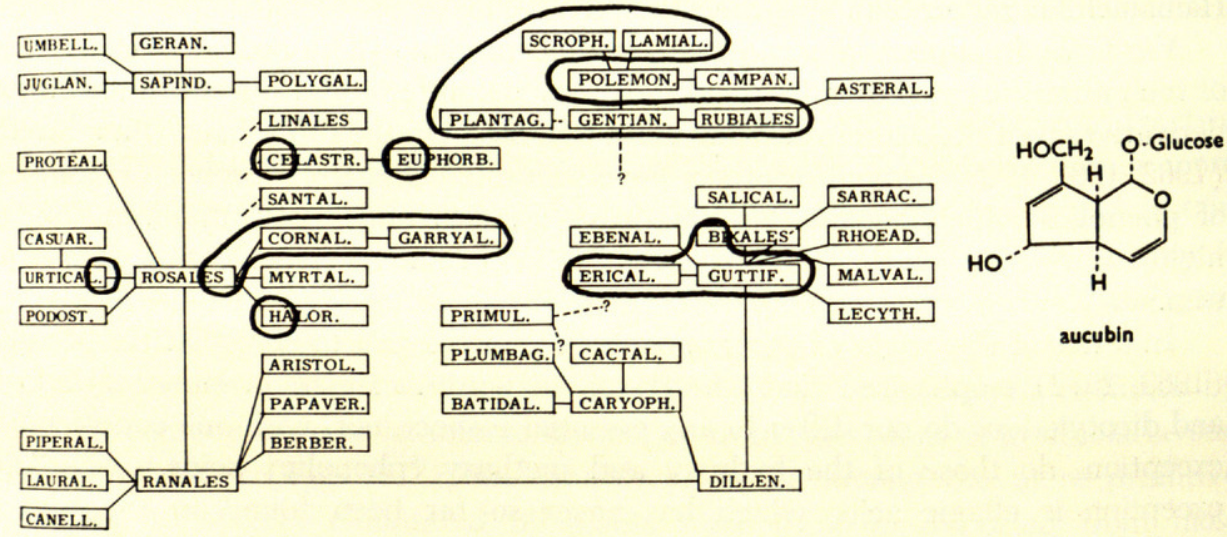
In terms of the origin of the monocots, it is interesting to note that Bate-Smith (1969, 1972) emphasized that "the flavonoid patterns found in monocotyledons and dicotyledons do not differ in any essential respect, nor, with one conspicuous exception, do those of the hydroxy and methoxy [phenolic] acids. . . . The exception is ellagic acid, which has never so far been found in the monocotyledons."[8] The absence of ellagic acid in the monocots and its presence in the

[7] It should be noted that the presence of leucoanthocyanidins can also be correlated to some extent with woodiness.

[8] It may, however, be significant that there are relatively few woody monocotyledons (see footnote 7 above).







FIGURES 8–10. The distribution of three different compounds in dicotyledons (adapted from Kubitzki, 1969).—8. Distribution of ellagic acid, which contains the elements of a vic-trihydroxyphenolic system. The presence of ellagic acid, which is not known from the monocots, is considered to be indicative of primitiveness (see Bate-Smith, 1962, 1966, 1969;

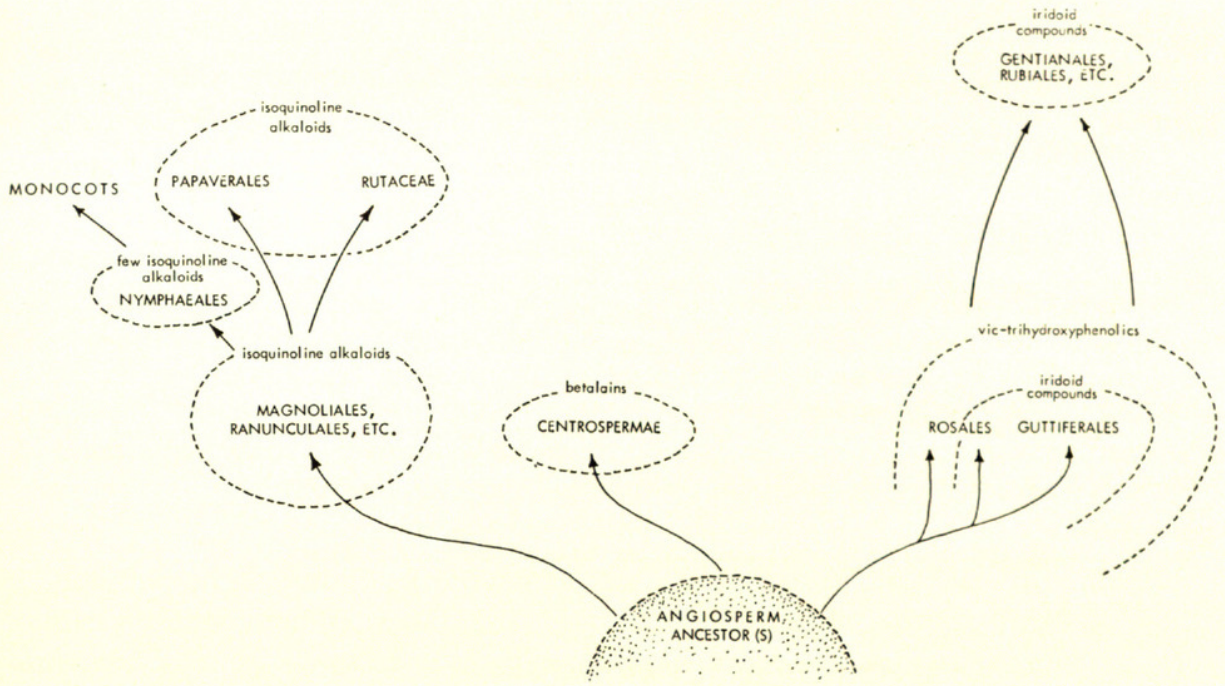


FIGURE 11. A scheme for the origin of major lines of dicotyledons based in part upon the distribution of selected natural products (adapted from Kubitzki, 1969; see also Figs. 8–10).

Nymphaeales (Bate-Smith, 1968) does not support the similar views of Cronquist and Takhtajan that a dicotyledon group such as the Nymphaeales could have given rise to the monocots. Hegnauer has privately expressed his view with regard to this matter as follows: "If we look for chemical resemblances between monocots and dicots, we find most of them if we look at Liliiflorae, Magnoliales and Ranunculales. . . . I expect that a connection between the two classes of angiosperms will ultimately be found here."

Kubitzki (1969, 1972), who superimposed upon Cronquist's interpretation of the evolutionary relationships of the dicotyledons the distribution of ellagic acid (Fig. 8), isoquinoline alkaloids (Fig. 9), and iridoid compounds (Fig. 10), suggested that the chemical data indicated that the Rosales and Theales both have primitive characters and evolved parallel to the ranalian (s.l.) and centrospermoid lines (Fig. 11).

The presence of ellagic acid and iridoid[9] compounds in many families related to the Rosales and Guttiferales and their absence from the isoquinoline alkaloid-containing ranalian group indicates that the former were almost certainly not derived from the ranalian complex as suggested by Takhtajan and Cronquist but instead represent independent lines. Further evidence that the ability to synthe-

[9] The name iridoid is derived from *Irodomyrmex*, a genus of ants in which these terpenoid-derived plant products also occur.

←

Mues & Zinsmeister, 1973).—9. Distribution of isoquinoline alkaloids. Aporphine is a typical isoquinoline alkaloid. Solid line = quaternary bases magnoflorin, berberin, and menisperin; broken line = protoaporphine and aporphine.—10. Distribution of iridoid compounds (see Wieffering, 1966). Aucubin is a typical and well known iridoid.

TABLE 4. Genera of Compositae reported to contain sesquiterpene lactone; arranged by tribes.

Vernonieae	*Elephantopus, Vernonia*
Eupatorieae	*Eupatorium, Mikania, Stevia*
Inuleae	*Carpesium, Geigeria, Inula, Telekia*
Heliantheae	*Ambrosia, Balduina, Cosmos, Encelia, Helianthus, Hymenoclea, Iva, Parthenium, Polymnia, Xanthium, Zaluzania, Zexmenia*
Helenieae	*Bahia, Baileya, Eriophyllum, Gaillardia, Helenium, Hymenoxys, Psilostrophe*
Anthemideae	*Achillea, Anthemis, Artemisia, Chrysanthemum, Matricaria*
Senecioneae	*Petasites*
Calenduleae	*Cnicus, Jurinea*
Cynareae	*Amberboa, Arctium, Centaurea, Cynara, Onopordon, Saussurea*
Cichoreae	*Cichorium, Hyenanche, Lactuca, Sonchus, Urospermum*

size ellagic acid developed early in the course of evolution is the interesting discovery of this substance in liverworts (Mues & Zinsmeister, 1973).

CYANOGENIC GLUCOSIDES

Cyanogenic glucosides are a group of natural products which are derived from amino acids; they have the ability to release hydrogen cyanide (cyanogenesis) upon treatment with acids or appropriate enzymes.

The pattern of distribution of cyanogenesis, which has been reported from over 800 species in 70 different plant families (see Eyjolfsson, 1970), indicates frequent independent origin of cyanogenic glucosides. Nevertheless, in a few instances, the data appear to bear upon the interpretations of Cronquist and Takhtajan for certain groups.

For example, Cronquist places *Sambucus* in the family Caprifoliaceae while Takhtajan does so questioningly. The available chemical evidence favors Takhtajan and others who have considered the group as a distinct family, Sambucaceae. *Sambucus* contains the cyanogenic glucoside sambunigrin, which represents the only report of this class of natural products in the Caprifoliaceae (see Glennie, 1969). Furthermore, *Sambucus* does not contain dicaffeoyl-quinic esters, which are widespread in other genera of the Caprifoliaceae (Glennie, 1969).

Cronquist treated two families, Passifloraceae and Flacourtiaceae (both of which contain similar cyanogenic glucosides), as being closely related and placed them together in the order Violales; in contrast, Takhtajan, while recognizing their similarities ("it is very difficult to draw a clear taxonomic boundary between

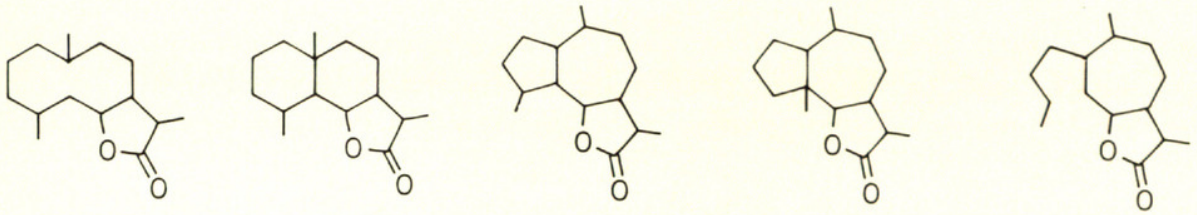


FIGURE 12. Five biogenetically-related skeletal types of sesquiterpene lactones which characterize most tribes of the family Compositae.

the Passifloraceae and the Flacourtiaceae"), nevertheless placed them in different orders, albeit closely related.

SESQUITERPENE LACTONES

Of the several hundred reports of the occurrence of sesquiterpene lactones in higher plants, more than 75% are from the Heliantheae, Helenieae, and Anthemideae, three tribes of the Compositae, Table 4 (see Yoshioka et al., 1973). In addition, virtually all other tribes in the Compositae contain one or more of the five or so biogenetically related types of sesquiterpene lactones which characterize the Heliantheae-Helenieae-Anthemideae complex (Fig. 12), indicating that the Compositae is a highly integrated natural assemblage.

Of more interest here are the accounts of sesquiterpene lactones in taxa outside the Compositae, especially in light of the recent cytochrome *c* results which indicate that this family is much older than previously thought (see Turner in GENERAL SUMMARY AND CONCLUSIONS). Both Cronquist and Takhtajan suggest similar origins for the Compositae; namely, from the Rubiales-Dipsacales[10] and Calycerales-Campanulales, respectively.

Of the 16 genera of angiosperms outside the Compositae which are known to contain sesquiterpene lactones, only those substances from the primitive families Magnoliaceae and Lauraceae appear to be biogenetically similar to those which are found in the Compositae. Indeed, *Michelia* (Magnoliaceae) and two species of the Compositae, *Ambrosia dumosa* and *A. confertiflora*, produce the very same substance (parthenolide); these data support the view that the Compositae are an older group than previously supposed[11], possessing relationships that presumably extend back to the magnolioid lines.

Two families which have similar sesquiterpene lactones, the Coriariaceae and Menispermaceae, are aligned together in the Ranunculales by Cronquist, while Takhtajan places the Coriariaceae in the Rutales with the comment that "family relationships are not clear."

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[10] It is interesting to note that one of Cronquist's comments in connection with choosing the Rubiales-Dipsacales ancestral route for the Compositae rather than the Campanulales will, I believe, prove not to be true, namely, the statement that "phenolic compounds are widespread in the Compositae and are also present in many Rubiales and Dipsacales, whereas, they are unknown in the Campanulales."

[11] In this connection, it might be noted here that C-glycosylflavones, considered by some (see Harborne, 1972) to be primitive substances because of their occurrence in mosses, liverworts, and even a green alga, are being found in an increasing number of Compositae genera.

ALKALOIDS

Perhaps 20% or more of the vascular plants contain one or more alkaloids (see Willaman & Li, 1970) and in many instances, these nitrogen-containing toxic substances have phylogenetic significance at higher taxonomic levels. Thus their distribution patterns often bear upon the interpretations of Cronquist and Takhtajan for certain families (see Hegnauer, 1963, 1967).

In applying the distribution of alkaloids for phylogeny it is essential to recognize that they are derived from amino acids by a variety of biosynthetic pathways and the mere presence or absence of "alkaloids" can not be used as a rigorous phyletic marker in the way, for example, one uses the distribution of the biogenetically homogenous leucoanthocyanidins.

The overall distribution of the isoquinoline alkaloids suggests that the major evolutionary lines of dicotyledons as proposed by both Cronquist and Takhtajan are incorrect (see Fig. 9 and the relevant discussion in the section PHENOLICS). The presence of aristolochic acid (which appears to be a structurally advanced modification of the isoquinoline alkaloids) in the Aristolochiaceae suggests that this group is an advanced member of the Magnoliidae in accord with the treatments of both Takhtajan and Cronquist.

Although Cronquist and Takhtajan differ in a number of ways in their interpretations of the Asteridae, one particularly interesting difference concerns their treatments of the Rubiaceae. The Rubiaceae is allied with the Loganiaceae and Apocynaceae in the Gentianales by Takhtajan; his view is strongly supported by the similar complex tryptophan-terpene-derived indole alkaloids which are produced in large numbers by many members of all three families. In contrast, Cronquist, while recognizing the relationship of the Loganiaceae to the Rubiaceae ("In my opinion the Loganiaceae are near-ancestral to the Rubiaceae. . . ."), placed the Rubiaceae in a distinct order.

CONCLUDING STATEMENT

During the course of the preparation of this manscript the writer's view with regard to the potential of natural products as an aid for understanding the phylogeny of major evolutionary lines of angiosperms was appreciably altered. I had generally believed that the data were insufficient to do more than hint at a meaningful arrangement of the higher categories; now, however, I am convinced that a more thorough evaluation of the presently available information for the distribution of biogenetically related natural products in conjunction with a re-interpretation of all other data will give considerable new insight into family and order relationships. Such an undertaking is presently being planned.

MACROMOLECULES—SYSTEMATIC SEROLOGY

Biologists have known for approximately 75 years that organisms may share antigenic material (substances capable of inducing the formation of antibodies and able to react with the antibodies), and when they share the same antigenic material in different proportions it is assumed that the organisms are related.

Most of the phytoserological research has encompassed what is designated

comparative serology. The basic methods for serosystematic or serotaxonomic research involve the immunization of experimental animals to induce antibodies (serum globulins) followed by the analysis of the reaction of the antibodies so produced with properly prepared antigenic material. This type of research can be conducted employing quantitative precipitation (precipitin) techniques in solutions (Boyden procedure, Moritz procedure, quantitative ring precipitation reaction), or by various qualitative precipitation techniques in gels (Oudin method, Ouchterlony method, double diffusion, and immunoelectrophoresis). Thus immunological techniques provide one kind of measurement of the protein similarities among taxa.

The precipitin reaction has a history dating from the time of Kraus (1897). The precipitin reaction has been used in taxonomic research since Nuttall (1901) published his new biological test for blood in relation to classification. Some precipitin reactions are used as an index for determining degree of serological correspondence, which is a summation of the immunological reactions throughout the antigen reaction range. The detected and measured similarities are based on the structure (amino acid sequences and molecular configurations) of the determinant groups, which are the portion of the antibody molecule that reacts or combines with a portion of the antigenic molecule. Such portions of the single antigenic molecule as are "reprinted" by the specific portion of the antibody molecule are designated "determinant groups," "determinant sites," or "determinants" (Fairbrothers, 1968, 1969, 1970). Fairbrothers (1969, 1972) reported that approximately 520 plant taxa (from cultivars through orders) have been included in approximately 150 systematic serological publications in the last 25 years. Research has demonstrated that extracts of seeds, pollen, leaves, tubers, and spores of vascular plants can be used, if the required extraction procedures are followed (Fairbrothers, 1969, 1971). Most of the vascular plant immunological studies have included extracts containing multiple antigen-antibody systems. This is why absorption techniques (removing common immunoprecipitating systems and leaving only those systems specific for each taxon compared) help reveal a measure of the relative similarity.

The terms reference antigenic material, cross-reacting antigenic material, reference reaction, and cross-reaction are frequently used in systematic serological publications (Fairbrothers, 1968). Reference antigenic material is the material used to immunize the antibody producers. Reference reaction is the reaction between an antiserum and the antigenic material used to stimulate its formation, and is the standard reference in comparative research. Cross-reacting antigenic material is material other than the reference antigenic material which will react serologically with the antibodies. Cross-reaction is the reaction between an antiserum and any antigenic material other than that used in its formation.

The following three generalizations have resulted from evaluating an array of systematic serological research: (1) The amounts of serological correspondence among proteins decrease with decreasing systematic relationships. (2) The amounts of serological correspondence are in accord with known genetic relationships. (3) Serological correspondence obtained by different antigenic material gives consistent relative systematic placements (Boyden, 1966).

While evaluating the contribution of serological data related to Cronquist's and Takhtajan's systems of classification, it became evident that I should refer to selected developing phases of each of the two systems. Thus by comparing Cronquist's (1957, 1965, 1968) and Takhtajan's (1959, 1969) publications it was possible for me to more accurately assess the role of serological data as the authors modified the two systems of classification. I did not compare the selected publications of each author to prove they changed their interpretations, but rather to determine how serological information was incorporated in their revisions.

MAGNOLIALES AND ILLICIALES

Johnson's (1953, 1954) and Johnson & Fairbrothers's (1964, 1965) information indicated that *Liriodendron* produced serological reactions which isolate the genus as a monotypic tribe or subfamily in the Magnoliaceae, a view shared by both Cronquist and Takhtajan. Serological data (Johnson, 1953, 1954; Johnson & Fairbrothers, 1964) comparing species of *Illicium* and *Schisandra* support those classifications which transfer the two genera from the Magnoliaceae to two separate families (Cronquist, 1968), or even to a separate order Illiciales (Illiciaceae and Schisandraceae) as proposed by Takhtajan (1969). Taxa of the Illiciaceae and Schisandraceae had greater serological similarity with each other than either family had with tested genera of the Magnoliaceae.

RANUNCULALES

Hammond (1955), Jensen et al. (1964), and Jensen (1968a) all determined that the genus *Paeonia* had very little or no serological affinity with taxa of the Ranunculaceae. Hammond (1955) also indicated a serological correspondence between *Hydrastis* and taxa of the Ranunculaceae, although several botanists would exclude this genus from the family. Jensen (1966, 1967, 1968a) indicated *Hydrastis* had little serological correspondence with the Berberidaceae (including Podophyllaceae) and greater correspondence with the Ranunculaceae. Thus the serological data supports Takhtajan's (1969) and Cronquist's (1968) family Paeoniaceae and its inclusion in a separate order from the Ranunculaceae, but the data would not support Takhtajan's inclusion of *Hydrastis* in a monotypic family Hydrastidaceae, or his statement that the genus is intermediate between the Ranunculaceae and the Podophyllaceae (Berberidaceae). The genera *Nigella* and *Erianthis* both are serologically isolated in the family Ranunculaceae (Jensen, 1968a, 1968b).

Jensen (1974) conducted a serological comparison of seed proteins of 12 genera of the Berberidaceae. He reported a high degree of serological similarity between *Mahonia* and *Berberis* (indicating one genus), and also between *Podophyllum* and *Diphyleia*. His data did not support those classifications which separate the genus *Nandina* from the Berberidaceae. *Nandina*, *Berberis*, *Mahonia*, *Podophyllum*, and *Diphyleia* formed one grouping (subfamily?). *Achlys*, *Bongardia*, *Caulophyllum*, *Epimedium*, *Jeffersonia*, *Leontice*, and *Vancouveria* formed another grouping (subfamily?). However, the serological similarities among the taxa forming the second grouping were not as close as were those genera forming the first grouping (Jensen, 1974). Cronquist (1968) placed all the genera in the

one family Berberidaceae, while Takhtajan (1969) placed the genera in the three families: Podophyllaceae, Nandinaceae, and Berberidaceae. However, Takhtajan in a printed discussion following Jensen's (1974) article stated that a recently published paper written by Melikian and Takhtajan indicated that all the genera should be placed in three subfamilies within the Berberidaceae.

CAPPARALES AND PAPAVERALES

The serological investigation of the Rhoeadales (Jensen et al., 1964) demonstrated that this order was composed of two distinct groups. One group, the Papaverales (Papaveraceae and Fumariaceae), revealed serological correspondence with the Ranunculales. The other group, the Capparales (Capparaceae and Cruciferae), stands apart serologically from the Papaverales and the Ranunculales. Cronquist (1957) stated that the four families formed a natural order; however, in 1965 he indicated it might be best to divide the order. In 1968 he listed two orders, the Papaverales and Capparales, and expressed the same relationships revealed by the serological data. Takhtajan (1969) also indicated the same orders and relationships in his classification as detected by the serological data.

CARYOPHYLLALES

Jensen (1965) and Moritz (1966) reported serological data which clarified the taxonomic position of the Didiereaceae, an endemic family of the arid regions of southwest Malagasy Republic (Madagascar). The systematic position of these cactus-like, thorny shrubs and trees has been controversial for approximately 80 years. Cronquist (1957) placed the Didiereaceae in the Euphorbiales. However, in 1965 he cited the betacyanin data (Rauh & Reznik, 1961; Mabry, 1964) and indicated that the family might ultimately be included in the Caryophyllales. Takhtajan (1959) gave some indication of taxonomic affinity with the Nyctaginaceae.

Jensen (1965), using the antisera from the genus *Alluaudia* of the Didiereaceae to test 23 antigenic systems derived from taxa usually placed in the Caryophyllales (Centrospermae) and 11 antigenic systems of non-Caryophyllales, clearly demonstrated strong serological correspondence between the Didiereaceae and tested members of the Caryophyllales. Statements by Cronquist (1968) and Takhtajan (1969) clearly indicated that the serological data were important in their placement of the Didiereaceae in the order Caryophyllales, as well as in establishing the position of the family in regard to other families included in that order.

CORNALES

The order Cornales (Cornaceae, Davidiaceae, Garryaceae, and Nyssaceae) has been investigated serologically for 10 years (Fairbrothers & Johnson, 1964; Fairbrothers, 1966a, 1966b, 1966c, 1968). The data have shown that the genus *Cornus* is divisible into serological groupings. Data have also illustrated a serological correspondence among species of *Cornus*, *Davidia*, *Garrya*, and *Nyssa*. Data obtained from quantitative and qualitative immunochemical techniques indicated very little or no serological correspondence of *Corokia* (Cornaceae) with any tested taxa belonging to the Cornales.

The placement of *Nyssa* in the Nyssaceae and *Davidia* in a subfamily of the Nyssaceae or as a separate family Davidiaceae, *Cornus* in the Cornaceae, *Garrya* in the Garryaceae, and *Corokia* removed from the Cornales, best expressed the serological data. Serologically *Davidia* is most similar to *Nyssa*, *Nyssa* is most similar to *Cornus*, and the Garryaceae is the most isolated of the four families.

The serological data support Cronquist (1968) who did not include the Araliaceae and Umbelliferae in the Cornales as did Takhtajan (1969). Takhtajan excluded the genus *Corokia* from the Cornales and placed it in the Escalloniaceae (Saxifragales). Cronquist discussed the status of *Corokia* as a possible non-missing link between the Cornaceae and Escalloniaceae, Grossulariaceae, or Saxifragaceae sensu lato. Both treatments reflect the serological data which indicated the distinctiveness of *Corokia* from members of the Cornales.

Rodriguez (1971) compared the data from diverse disciplines (including serological) and discussed the indicated relationships for members of the Cornales.

UMBELLALES

The analyses of data obtained from extracted seed proteins of 13 genera of the Umbelliferae employing the Boyden procedure and Ouchterlony technique (double diffusion) revealed three distinct serological groupings. These groupings essentially correspond to the three subfamilies. The data also indicated that one grouping (Apioideae) was more similar to the Saniculoideae than to the Hydrocotyloideae (Pickering & Fairbrothers, 1970). Seventeen taxa of the subfamily Apioideae were investigated serologically and five major groupings were detected which correspond to five tribes. Other serological groupings revealed a relationship suggested by some of the designated taxonomic subtribes of the Apioideae (Pickering & Fairbrothers, 1971).

Varying degrees of protein similarity have been obtained among members of the Araliaceae, Cornaceae, Garryaceae, Nyssaceae, and Umbelliferae. These preliminary data indicate a possible common ancestral complex for these five families (Hillebrand & Fairbrothers, 1970a, and unpublished data).

LAMIALES, POLEMONIALES, AND SCROPHULARIALES

The Scrophulariaceae is generally considered by taxonomists to be the family most nearly related to the Solanaceae (Hawkes & Tucker, 1968). The genus *Schizanthus* has been placed in both the Scrophulariaceae and Solanaceae. Hawkes & Tucker (1968) in their extensive serological assessment of relationship within the family Solanaceae have also made preliminary comparisons with taxa of the Scrophulariaceae, Boraginaceae, Convolvulaceae, and Leguminosae. For the inter-family comparison they used antisera prepared to *Schizanthus*, a genus serologically isolated in the Solanaceae, and *Salpiglossis*, a genus strongly reacting serologically with other genera of the Solanaceae. The Boraginaceae and Convolvulaceae taxa produced extremely faint or no serological reactions with taxa of the Solanaceae. All the Scrophulariaceae taxa produced faint cross-reactions with the Solanaceae, which Hawkes & Tucker (1968) interpreted as an indication of some relationship between the two families.

Cronquist (1968) placed the Solanaceae in the same order as the Convolvulaceae (Polemoniales), Boraginaceae in another order (Lamiales), and the Scrophulariaceae in still another order (Scrophulariales). Takhtajan (1969) placed the Solanaceae and Scrophulariaceae in the order Scrophulariales, and the Convolvulaceae and Boraginaceae in the order Polemoniales. Takhtajan's arrangement better reflects the serological data reported by Hawkes & Tucker (1968).

DIPSACALES AND RUBIALES

A serological investigation of intrageneric relationships in *Viburnum* revealed that the genus was a taxon distinct from all others tested. However, serological groupings would support the division of the genus into taxonomic subgroupings (subgenera, sections). Representatives of the most primitive taxonomic grouping of the genus displayed the least serological reactivity with the most advanced taxonomic grouping (Hillebrand & Fairbrothers, 1969).

A three-dimensional model illustrating serological correspondence among five tribes of the Caprifoliaceae and the families Cornaceae and Nyssaceae indicated the following: (1) Tribes Lonicereae and Diervilleae were very similar. (2) Linnaeae was close to the Lonicereae and Diervilleae. (3) The families Cornaceae and Nyssaceae and the tribe Sambuceae were approximately equally similar to the above three tribes. (4) Viburneae was serologically removed from the other four tribes and two families, but most similar to Sambuceae. (5) *Cornus*, *Nyssa*, and *Sambucus* were more similar to each other than to any other members tested. (6) The families Cornaceae and Nyssaceae were as similar or more similar serologically to the three tribes of the Caprifoliaceae than the three tribes were to the Viburneae and Sambuceae of the Caprifoliaceae (Hillebrand & Fairbrothers, 1970a).

The serological correspondence of the Cornaceae and Nyssaceae with most representatives of the Caprifoliaceae, especially *Sambucus*, contrasts with the very low or negative correspondence of genera of the Caprifoliaceae (including *Sambucus* and *Viburnum*) with the Rubiaceae; this indicates a closer protein similarity between the Caprifoliaceae, Nyssaceae, and Cornaceae than between the Rubiaceae and Caprifoliaceae (Hillebrand & Fairbrothers, 1970b). Cronquist (1968) placed the Rubiaceae in the monotypic order Rubiales, and the Caprifoliaceae (including *Sambucus* and *Viburnum*) in the order Dipsacales. He concluded that, depending on how the evidence is weighed, the Rubiaceae could be included in the Dipsacales, Gentianales, or placed in a monotypic order. He interpreted the serological evidence as indicating that the nearest common ancestry of the Caprifoliaceae and Cornaceae would be in the Rosales. Hillebrand & Fairbrothers (1965), based on serological data, alluded to such a possibility. Takhtajan (1969) included the Caprifoliaceae in the Dipsacales and placed *Sambucus* in the Caprifoliaceae with a question mark. He also indicated that the Dipsacales was related to the Cornales, and that the Caprifoliaceae exhibits definite links with the Cornales. He placed the Rubiaceae in the order Gentianales and indicated that this order has a common origin with the Dipsacales.

The serological data are best reflected by Takhtajan's classification. However,

both systems of classification have not adequately dealt with the chemical information available for either *Sambucus* or *Viburnum*. Both systems reflect the serological data which indicated that the Caprifoliaceae and Rubiaceae are not as taxonomically similar as has been traditionally indicated.

PRE-MONOCOTYLEDONOUS DICOTS

Cronquist (1968) and Takhtajan (1969) both indicated that the monocotyledons originated very early and that their ancestors were primitive dicotyledons most like the Nymphaeales. Takhtajan indicated that the Nymphaeales have been classified both as dicotyledons and monocotyledons. Cronquist (1968) placed the Nelumbonaceae and Nymphaeaceae in one order (Nymphaeales) and Takhtajan (1969) placed the two families in separate orders (Nelumbonales and Nymphaeales). Simon's (1970, 1971) serological data indicated that *Nuphar* and *Nymphaea* were close and *Nelumbo* was isolated from these two; thus Takhtajan's treatment best expressed the serological information. Serological affinities were also detected between Nymphaeales, Magnoliales, Laurales, and Ranunculales. The Nymphaeales antisera also produced partial identity reactions with taxa of five monocotyledon families belonging to three orders. These data support the suggested pre-monocotyledonous dicot affinity with nymphaeous-like plants.

TYPHALES

Diverse placement of the order Typhales (Sparganiaceae and Typhaceae) within the monocotyledons indicates disagreement about the origin and evolutionary history of the order. Hutchinson (1959) indicated the order originated from the primitive Liliales. Cronquist (1968) placed the Typhales in the subclass Commelinidae and postulated a generalized commelinalean ancestry. Takhtajan (1969) viewed the Typhales as having developed in a long evolutionary line from plants which preceded the Liliales, and placed it in the subclass Arecidae. He also indicated evolutionary affinities between the Typhales and Pandanales. Stone (1972) reported that his investigations of the Pandanaceae indicated little affinity with the Typhaceae, and suggested that postulated relationships between the two families resulted from superficial resemblances. Other classifiers have indicated relationships with the Palmae, Arales, Alismatales, Commelinales, etc.

A serological investigation of the Typhales (Lee & Fairbrothers, 1972) indicated the following: (1) significant serological correspondence between *Typha* and *Sparganium*, thus making the placement of the two families in one order appropriate, (2) low serological correspondence between the order and several Liliales, indicating possible distant evolutionary relationship, (3) negative or no significant serological affinity with Araceae, Commelinaceae, Cyperaceae, Gramineae, Juncaceae, Palmae, and Pandanaceae.

Lee and Fairbrothers (1972) suggested as a working hypothesis that the Typhales originated in the primitive or ancestral Liliales. It would then be placed in Cronquist's and Takhtajan's subclass Liliidae. However, serological data reflected (as do all other data) an extremely isolated position for the Typhales within the monocotyledons.

CONCLUSIONS

Researchers employing both quantitative and qualitative immunological techniques have obtained valuable additional and/or complementary data for taxonomic investigations. Phytoserological research has also provided provocative and valuable chemical characteristics for use in the classification of higher plants; and in some research immunological data have transcended in significance data available from other characters. The examples presented clearly indicate that serology has contributed chemical data which have been used, in conjunction with other data, to produce the Cronquist and Takhtajan systems of classification and which could be used to refine these systems. The increased use of such data in classification only awaits the increased production of comparative phytoserological research.

Macromolecules—Amino Acid Sequences

The realization that the amino acid sequences of homologous proteins in different taxa contain phylogenetically useful information derives from advances in molecular biology during the past several decades. The appreciation that a particular nucleotide sequence in the genome (a structural gene) programs for a unique amino acid sequence in a protein led Zukerkandl & Pauling (1965) to note with characteristic insight that knowledge of the amino acid sequences of genetically homologous proteins from different species permits reconstruction of the evolutionary history of, at the least, single genes. Zoologists have rather widely used the comparison of amino acid sequences to illuminate phylogenetic relationships among animals, but only very recently have the data become available to apply this approach to the study of phylogeny within the plant kingdom. By way of introduction to this topic, selected aspects of the use of amino acid sequences in phylogenetic studies are discussed below.

Rationale.—Two phylogenetically related taxa shared a common ancestor at some point in time, earlier or later depending on their degree of relatedness. Homologous structural genes common to these taxa have descended by fixation of point mutations from a nucleotide sequence in their common ancestor. The degree of similarity of the nucleotide sequences in the genomes of the two organisms is a measure of the extent of their divergence from the common ancestor and of their phylogenetic relatedness. At present it is technically impossible to rigorously and directly assay the similarity of two entire genomes or even single homologous genes embedded in those genomes (DNA-DNA hybridization techniques and sequencing of small nucleic acids notwithstanding). Since a nucleotide sequence programs for a unique amino acid sequence, it is possible to assay the similarities of two homologous genes indirectly by comparison of their gene product, the protein. Thus the comparison of amino acid sequences among homologous proteins allows evaluation of degree of relatedness among several taxa.

Assumptions.—It is assumed that the structural genes programming the particular protein whose sequences among several taxa are being compared are evolutionally homologous. Two other sources of sequence similarity exist: (1) random chance and (2) evolutionary convergence in which constraints imposed by the biochemical function of the protein allows only certain amino acid

sequences (analogy). The former of these two sources can be detected with statistical tests (Fitch, 1970). The latter possibility cannot be rigorously excluded, but semi-rigorous statistical tests indicate that the cytochromes *c* of animals and fungi are evolutionally homologous (Fitch & Markowitz, 1970) and an analysis yielding similar results for plant cytochromes *c* has been performed (Ramshaw & Brown, unpublished).

Additional assumptions are tacit in the method used in the construction of the phylogenetic tree from sequence data. For a discussion of these the reader is referred to Boulter et al. (1972).

Weaknesses.—A phylogeny based on the comparison of amino acid sequences of a protein is the phylogeny of a structural gene, not necessarily of species. To the extent to which changes within that gene reflect the evolution of the organism possessing that gene, this approach is valid. If the rate of change in a gene is linear in time and relatively slow, sudden bursts of morphological change accompanying adaptive radiations may not be reflected in gene products. In such cases a phylogeny constructed on a single gene will yield a correct, but incomplete topology. Alternatively phrased, variation in morphology need not necessarily be reflected in all gene products. This problem was considered by Simpson (1964). He noted that the closer one gets to DNA in the process of transcription of genetic information, the farther one gets from the "cutting edge" of natural selection, namely, the phenotype. Due to the technical difficulties inherent in protein sequencing, phylogenetic trees derived from protein comparisons will be based on a relatively few structural genes for some time to come.

A practical problem arises in that each protein (i.e., gene) appears to have a characteristic evolutionary rate (about which more will be said below) determined in part by the biological role of the molecule. Therefore, the rate of change of one protein (e.g., cytochrome *c*) might make that protein very useful for familial comparisons and useless at the generic level. The converse is observed in other, more variable proteins. So the particular protein must be carefully selected for a comparative study at a given taxonomic level.

Strengths.—The construction of a phylogenetic tree based on amino acid sequences and using the ancestral sequence method permits the reconstruction of a precise, quantitative, and objective topology of relationships. At the taxonomic level for which that protein is validly used, a sequence comparison will demonstrate the order in which each group represented diverged from the common ancestor with its phylogenetic neighbors. As discussed further below, a time scale for such divergences may be appended to such a tree.

Comparative sequence data are highly amenable to increasingly sophisticated statistical analysis. Statistical techniques are being developed to detect and eliminate such potential sources of errors as back mutations, double mutations, redundancy in the genetic code, and convergent biochemical evolution (Fitch & Margoliash, 1969).

Comparison of amino acid sequences yields some insights into the actual molecular mechanism of the evolutionary process. For example, the currently controversial topic of the random fixation of selectively neutral mutations is largely a spin-off of comparative protein sequence data.

Why Use Comparative Sequence Data for Plants?—*A priori*, there is no compelling reason to place much confidence in a phylogenetic history of the higher plants based on a single structural gene within the huge genome of modern angiosperm taxa. Why then should botanists believe a phylogenetic tree based on amino acid sequences of a single (albeit homologous) protein among several plant families? The sole answer is simply that in the animal kingdom, for which a clear-cut fossil record exists for the general pattern of vertebrate evolution, a phylogenetic scheme based on the cytochrome *c* gene mimics almost perfectly such a tree constructed on the basis of the extensive fossil record. In the absence of a well preserved fossil record, angiosperm phylogenists must assume that phylogenies based on comparisons of amino acid sequences from homologous proteins in plants are fairly accurate. This acceptance is based on the zoologist's experience and we operate somewhat under the dictum attributed to Thomas Edison, who, when pressed as to the basic nature of electricity is reported to have replied: "Electricity is, use it."

METHODS

The techniques for protein purification and amino acid sequence determination are legion, lengthy, laborious and well reviewed elsewhere (Blackburn, 1970; Needleman, 1970). The sequencing techniques, per se, do not bear upon the biological arguments and no purpose would be served treating them here.

DISCUSSION

Currently Available Data.—A picture is emerging of evolution at the molecular level which suggests that each protein evolves (i.e., accepts point mutations) at a constant and characteristic rate. For cytochrome *c* the number of accepted point mutations per 100 amino acid residues each 100 million years is 3; for the much more conservative protein histone IV, 0.06; for the highly variable fibrinopeptides, 90 (Dayhoff, 1972). This characteristic rate of evolution for a particular protein defines the taxonomic category at which the comparison of amino acid sequences will be phylogenetically fruitful. The most "conservative" proteins (e.g., histones) are useless for phylogenetic studies because they are virtually invariant across entire kingdoms. "Moderately conservative" proteins such as cytochrome *c* and hemoglobin are useful at higher (ordinal and familial) levels. Highly variable proteins will be more useful at the lower levels of specific and generic comparisons.

Cytochrome *c* evolves at a rate such that comparisons of its sequences are useful at the familial level. The two congeners, *Brassica oleracea* and *B. napus*, have been shown to have identical cytochrome *c* amino acid sequences (Richardson et al., 1971; Thompson et al., 1971) and members of the same family (*Gossypium* and *Abutilon*, *Helianthus* and *Niger*) have very similar sequences (Boulter, 1973). Because of its useful rate of evolution and other reasons (size, ubiquity of occurrence, relative ease of purification, optical properties) mitochondrial cytochrome *c* is one of the most widely sequenced of all proteins.

Only two proteins, cytochrome *c* and ferredoxin, have been purified and sequenced from a sufficient number of different angiosperms to permit com-

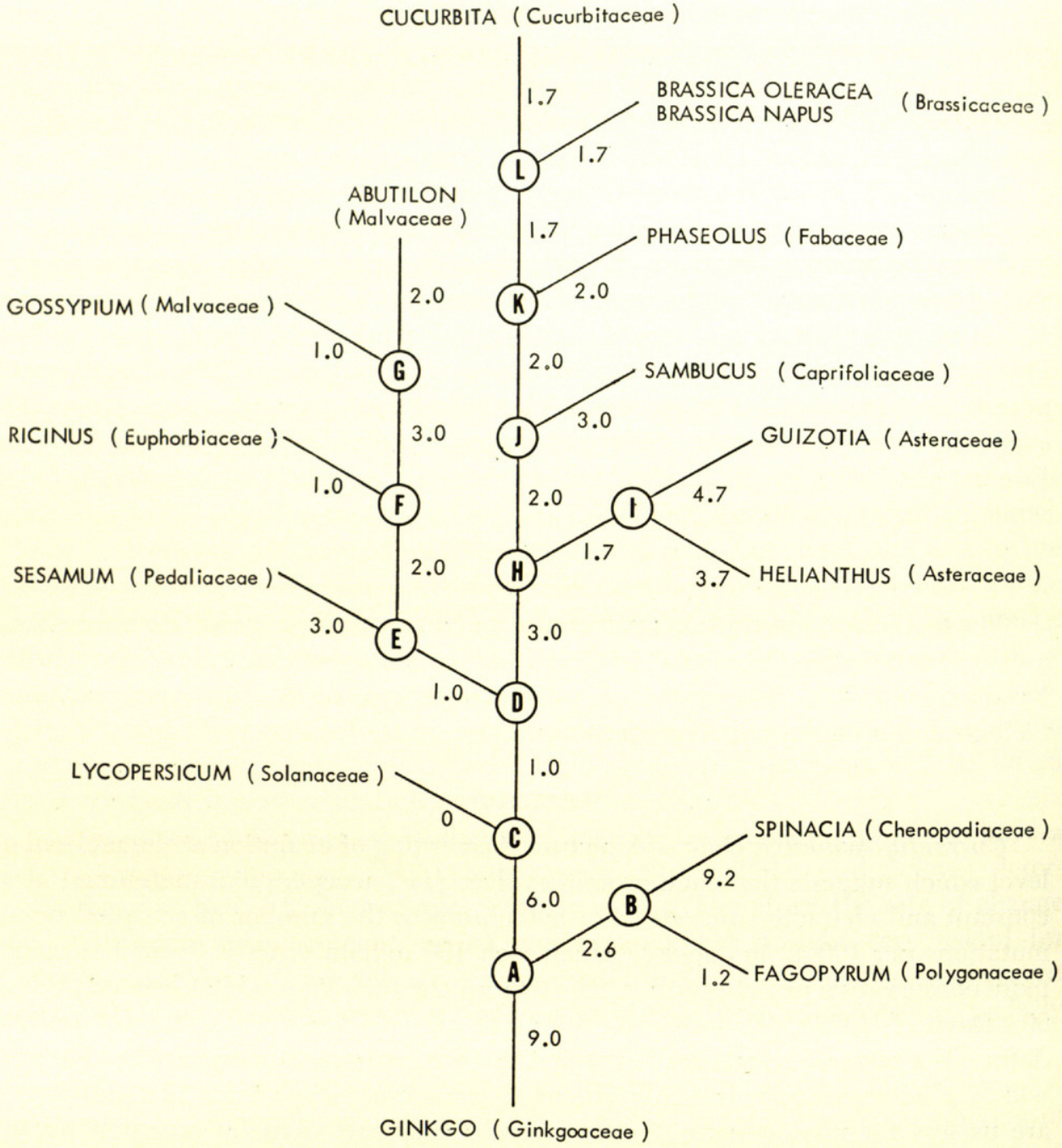

FIGURE 13. A phylogenetic tree relating fifteen plant species, constructed using the "ancestral sequence" method. Numbers refer to the "average" amino-acid difference between the nodes A–L and lineages concerned. (After Boulter et al., 1972).

parisons of sequences and construction of preliminary phylogenies. The amino acid sequence of the respiratory electron transport protein, mitochondrial cytochrome *c*, has been reported from 19 angiosperm species (Boulter, 1973; Brown et al., 1973; Brown & Boulter, 1973). As yet unpublished sequences are known from several algae (B. T. Meatyard, unpublished) and several additional monocots (D. Richardson, unpublished). These data have recently been used to construct a phylogenetic tree of the higher plants based on the cytochrome *c* gene (Boulter et al., 1972) and this tree is shown in Fig. 13. Although based on a single gene in the 15 species, this tree is remarkably similar to more traditionally derived trees based primarily on morphological considerations.

In comparing the relationships derived from comparison of cytochrome *c* sequences with existing classification schemes it is noted that the clustering of related families is virtually identical. If the superorder Malvanae of the Takhtajan scheme were transferred from the subclass Dilleniidae to the subclass Asteridae, virtual congruence with the cytochrome *c* tree would be achieved.

At present too few sequences from too few families are known to resolve any but the most general outlines of angiosperm phylogeny. As more sequences are accumulated, the confidence in, resolving power of, and contribution of phylogenetic trees based on amino acid sequences should greatly increase.

To the extent to which molecular evolution in proteins is constant and linear in time, trees based on amino acid sequences permit the attachment of a time scale for the divergence of major groups from their ancestral stocks. Such a time scale has recently been examined (Ramshaw et al., 1972) based upon the known cytochrome *c* sequences.

The only other plant protein of possible phyletic significance in the near future is the photosynthetic electron carrier, ferredoxin. The amino acid sequence of ferredoxin has been determined from four angiosperms (Dayhoff, 1972). Comparison of these known sequences allows no phyletic deductions among these taxa at present. The number of amino acid substitutions between two members of the same family (*Medicago sativa* and *Leucaena glauca* with 24 residues different) is greater than the differences between a dicot and a monocot (e.g., *L. glauca* and *Colocasia esculenta* with 22 residues different). Ferredoxin is obviously a less conservative protein than cytochrome *c* and it remains to be determined at which taxonomic level comparison of ferredoxin sequences will be useful.

Prospects for the Future.—The prospects for the use of amino acid sequence data in plant phylogeny in the near future are limited. This derives largely from the fact that the real bottleneck in plant protein sequence determination is the isolation and purification of suitable plant proteins for sequencing. Several limiting factors are operative in the selection of a protein for sequencing: size (less than about 15,000 daltons for practical comparisons among numerous taxa), relative ease of purification in milligram quantities, wide taxonomic occurrence, and high cellular concentration. Technical requirements of purification often exclude the taxonomically most interesting species. For example, purification of sufficient cytochrome *c* for a sequence determination requires several hundred pounds of highly viable, rapidly germinating seed, which effectively limits the plants examined to horticultural crops. Automated devices (sequenators) are now commercially available to automatically determine the amino acid sequence of peptides up to 40–50 residues long (subject to certain conditions). These devices will greatly facilitate the determination of sequences, per se, but higher plants are generally poor sources of protein for both biological (low metabolic activity) and technical (resistant cell walls) reasons. Therefore, purification is likely to remain the bottleneck in the use of plant protein sequences in the near future.

CONCLUSION

Before the full impact of utilizing amino acid sequence data in phylogenetic studies among the angiosperms is felt, it will be necessary to have available a much

greater number of sequences from numerous and diverse taxa. Acquisition of these data will require considerable time, work, and a modicum of good luck in identifying and sequencing suitable proteins. Protein sequence data have not and are unlikely to revolutionize presently accepted phylogenetic proposals. The value of protein sequence data in botany, as in zoology, is to provide an independent, objective source of data against which to compare traditional phylogenetic schemes. It is too early to assess the ultimate impact of sequence data on phylogenetic schemes. Indeed, if the discipline of biochemical systematics in general could be said to be approaching puberty (and certainly not yet maturity), the use of plant protein sequences for phylogenetic studies remains in very early infancy.

MACROMOLECULES—NUCLEIC ACID HYBRIDIZATIONS

The technique of nucleic acid hybridization is, in principle, applicable to chemotaxonomy at all taxonomic levels since it involves the fundamental hereditary material deoxyribonucleic acid (DNA) and its transcribed copy, ribonucleic acid (RNA). Following development of techniques for denaturing (or unwinding) the helical DNA of viruses and bacteria and subsequent "hybridizations" of the derived single strands, similar techniques were developed for use with the DNA's of animals and plants. The methods involve the extraction of denatured DNA strands and, most commonly, trapping these single-stranded DNA's on nitrocellulose filters. RNA or fragmented DNA from the same or another organism is used as a test against the long-strand DNA already present in the test system. The low molecular weight nucleic acid (RNA or sheared DNA) has a tendency to pair with the original DNA; the affinity (or extent of pairing) reflects similarity between the two interacting nucleic acids.

In contrast to the relative ease with which meaningful plant natural products distribution patterns are determined are the difficulties and patience required to carry out nucleic acid hybridization experiments and to interpret their results. Thus, it is not surprising that too few nucleic acid hybridization data (see, for example, Bendich & Bolton, 1967; Bendich & McCarthy, 1970) are available for higher plants that bear upon the interpretations of Cronquist and Takhtajan for the evolutionary relationships of angiosperms; nevertheless, the method inherently has great potential. One investigation involving DNA-DNA and DNA-RNA hybridization studies with plants belonging to Centrospermae (Caryophyllales) and related families (Mabry et al., 1972; Chang, 1971 and references therein) can serve to illustrate the technique and its potential. DNA-DNA and DNA-RNA hybridizations were employed for determining the extent of genetic homology among species which belong to various betalain-producing plant families relative to species which are members of anthocyanin-producing families, especially the Caryophyllaceae.

The DNA-DNA hybridization results were somewhat surprising in that only between varieties of the same species (*Beta vulgaris*, the red and sugar beets) was competition detected; that is, no differentiation between genera, let alone higher taxonomic categories was observed.

Next, ribosomal RNA (r-RNA) was used for hybridization with DNA since it is well known that the cistrons for r-RNA are relatively conserved (few

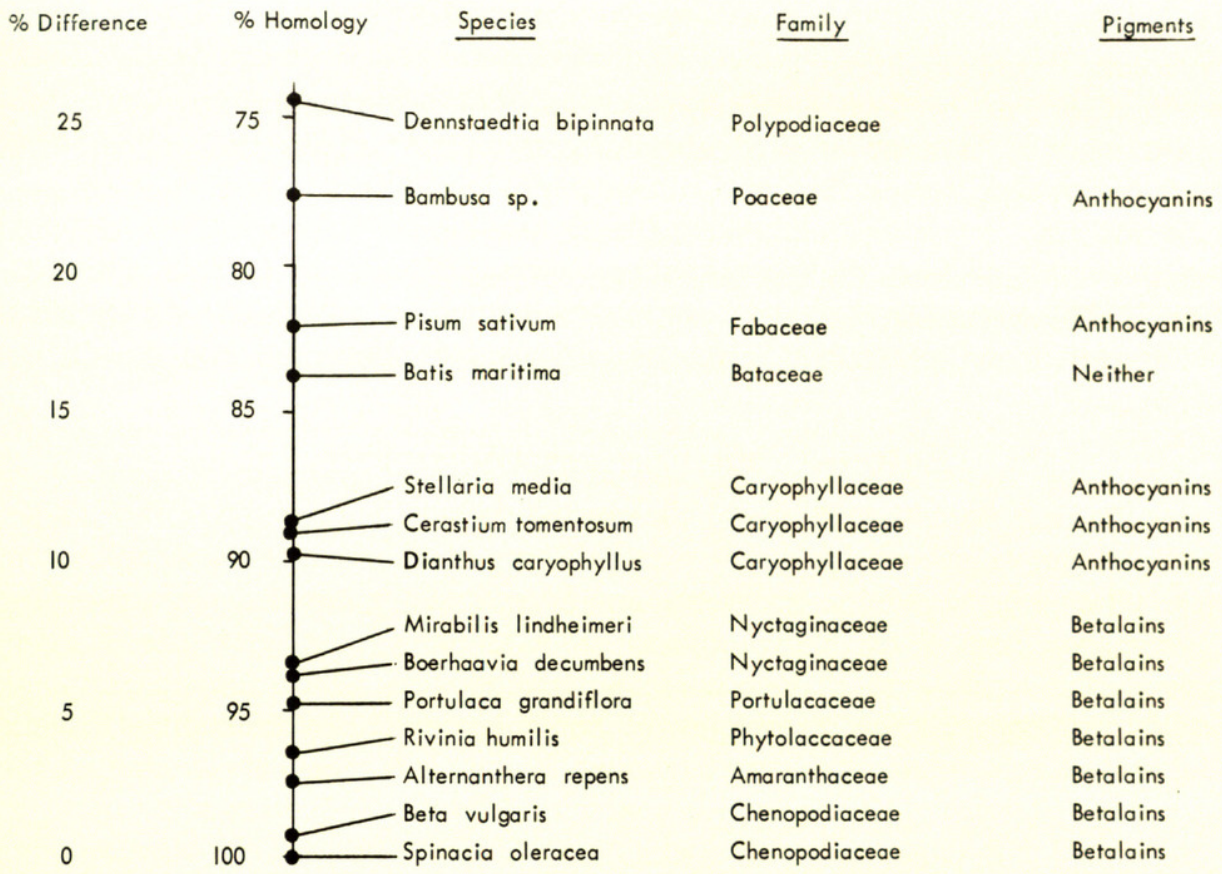

FIGURE 14. 16S r-RNA homologies in plants. In all experiments, 0.6 μg of ^{3}H-spinach 16S r-RNA (4000 cpm/μg) were incubated with 12 μg of spinach DNA bound on a nitrocellulose filter in 0.1 ml of foramide: 4 SSC (1:1) at 40°, in the presence of increasing amounts of r-RNA from other taxa. After 38 hours, each filter was washed with 2 ml of foramide SSC solution for 5 minutes at 40°, then with 2 more ml SSC for another 5 minutes. Each value in this figure represents the average obtained from three determinations. The ratio of labeled r-RNA to DNA in the hybrid in the absence of competitor r-RNA was 0.33%, representing 7% binding of the input labeled r-RNA. All values have been corrected for background binding using calf thymus DNA filters (Chang, 1971).

mutations) compared with the average DNA cistrons. The results obtained by DNA-RNA hybridizations indicated that excess r-RNA from a distantly related yeast (see Chang, 1971) could not inhibit the labeled 16S r-RNA from the betalain-producing spinach (*Spinacia oleracea*, Chenopodiaceae) from hybridizing with the filter-bound DNA from the same plant; on the other hand, excess r-RNA from the still somewhat distantly related pea, *Pisum sativum*, a member of the anthocyanin-producing Fabaceae, did reduce the homologous spinach-r-RNA/spinach-DNA hybridization to about 18% (Fig. 14).

The crucial experiments involved r-RNA from the Caryophyllaceae and from the betalain-producing families. Excess r-RNA from either of three genera (*Dianthus*, *Cerastium*, and *Stellaria*) from the anthocyanin-producing Caryophyllaceae and one species from the Bataceae (neither anthocyanins nor betalains) reduced the spinach r-RNA/spinach-DNA interaction to 10–15% and 17%, respectively. Significantly, however, excess r-RNA from several betalain-producing families reduced the spinach-r-RNA/spinach-DNA hybridization in every case to less than 7% (see Fig. 14). That is, the r-RNA from betalain-producing plants

showed 93.5% or more homology with the r-RNA from the test system, *Spinacia oleraceae* (Chenopodiaceae).

All of the data available (see earlier section II-A on pigments) support a close evolutionary relationship of all the betalain-producing families and indicate that the Caryophyllaceae and Bataceae, although phylogenetically close to, are nevertheless distinct from the betalain-producing families.[12] Although these data do not necessarily discount the interpretations of either Cronquist or Takhtajan with regard to these families, the results are in close agreement with Mabry et al. (1963) and suggest that Cronquist is correct in excluding the Bataceae from his Caryophyllales.

General Summary and Conclusions

As has been stated repeatedly by the present author (Turner, 1967, 1969, 1972), the most convenient way to present and discuss biochemical data as related to systematics is to treat these under the broad headings, macro- or micromolecular approaches. Each of the above authors has presented, in at least brief fashion, *macro*molecular data bearing on angiosperm phylogeny (Fairbrothers, serology; Scogin, primary structure of proteins; Mabry, nucleic acid hybridizations), while Mabry has attempted the almost impossible task of making meaningful the *micro*molecular data (Hegnauer was unable to do this in six volumes!).

MICROMOLECULAR APPROACHES

Over the years more effort in man hours, albeit mostly by chemists, has gone into the accumulation of micromolecular data than in the assemblage of macromolecular data. This has resulted in a large mass of information, a kind of flotsum from the bench of the organic chemist which was swept into the literature following his particular structural interests. Most of the early reports of such molecules (and even many today) were largely undocumented as to plant source, and consequently, many identification errors were incorporated, thus compounding the effort of systematists to organize and "make sense" of these data[13] (cf. the excellent discussion by Ettlinger & Kjaer, 1968).

The only really good recent account of the distribution of micromolecules among flowering plants generally is the six-volume compendium of Hegnauer, and unfortunately for the average American doctorate (including myself), its contents are not easily deciphered, either as to translation or phyletic meaning.

MACROMOLECULAR APPROACHES

Because of its early development, inexpensive and easy application, and relatively comprehensible form of data presentation, the serological approach

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[12] In this connection, it is especially noteworthy that neither the Bataceae, Caryophyllaceae, nor the Molluginaceae contain alkaloids in contrast to most of the betalain-producing families (Raffauf, 1970).

[13] I am reminded especially of Cronquist's (1968: 178) one paragraph digression on a reputed "exception to the mutual antipathy of betalains and anthocyanins" in the Aizoaceae, appropriately referred to by Mabry. The literature is replete with such errors (in this case an artifact of chemical procedure, not identification) and one could keep the hounds at bay a very long time by just digging up this or that erroneous exception to defend a point of view.

has heretofore had the most to offer systematists interested in classification at the familial level or higher. But even here the data are fragmentary and, except for an occasional genus or family, information is mostly missing for the more critical groups.

More perplexing for this reviewer is the fact that at least some serological data are available for the hypothetically "more primitive," woody magnolian lines, but other kinds of macromolecular data (either protein or nucleic acid) are absent for these groups. This is unfortunate since, taken alone, serology has little to offer in the way of evidence bearing on the relative age of a group. Still, as indicated by Fairbrothers, in the case of *Illicium*, *Paeonia*, and numerous other genera among several orders, serological data have been useful in suggesting, in a relative way, cladistic distances among selected families. It would seem unnecessarily repetitive to reiterate here what he has so nicely sketched out for us. I will, therefore, confine any additional "serological comments" to those few instances where the presentations of either Mabry or Scogin seem to warrant such considerations.

Unquestionably, the most remarkable new data which have become available to plant systematists for use at the familial level or higher has been that of cytochrome *c*, mostly coming out of Professor Donald Boulter's laboratory in Durham, England. This work has been placed in proper perspective by Scogin, who recently worked in Dr. Boulter's laboratory as a post-doctoral fellow, helping with the amino acid sequence of cytochrome *c* from tomato.

Because of the *potential* significance of this work in determining the more or less "primitive" groups among angiosperms generally, I think the taxonomic community owes Professor Boulter (who, after all, is a plant physiologist by training) a special accolade. And I can't help but add here a vignette of my own. During 1966–1967 I also worked as a post-doctoral fellow in Dr. Boulter's laboratory (at that time associated with the University of Liverpool), mostly "mucking" around with protein bands and isozymes. I soon became disillusioned with the potential of these data as taxonomic guidelines above the generic level and, being familiar with Margoliash's comparative work on cytochrome *c* among animal groups, strongly urged Don to turn his botanical efforts in this direction. At first he made light of my suggestions, setting up instead, *for my use*, an appropriate column for cytochrome *c* isolation. I failed miserably, but he must have been sufficiently impressed with my dedication and interest (or perhaps partial success?) to take on the task himself. In this vain but humble way, I too feel part of the "cutting edge" (as current terminology would have it) of the phyletic art being practiced today. Now let's look at the data, as sparse as it is.

If, as has been suggested, cytochrome *c* has evolved at a relatively uniform rate over time, then one has a kind of clock with which to calculate the likely divergence of cladistic assemblages. As noted by Scogin, this clock has kept relatively good time in the animal kingdom; at least it jibes with what is known of the fossil record.[14]

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[14] The clock might occasionally run fast (Carlson & Brosemer, 1973), but the exceptions seem open to other interpretations, or at least seem confined to short-term specialized lines.

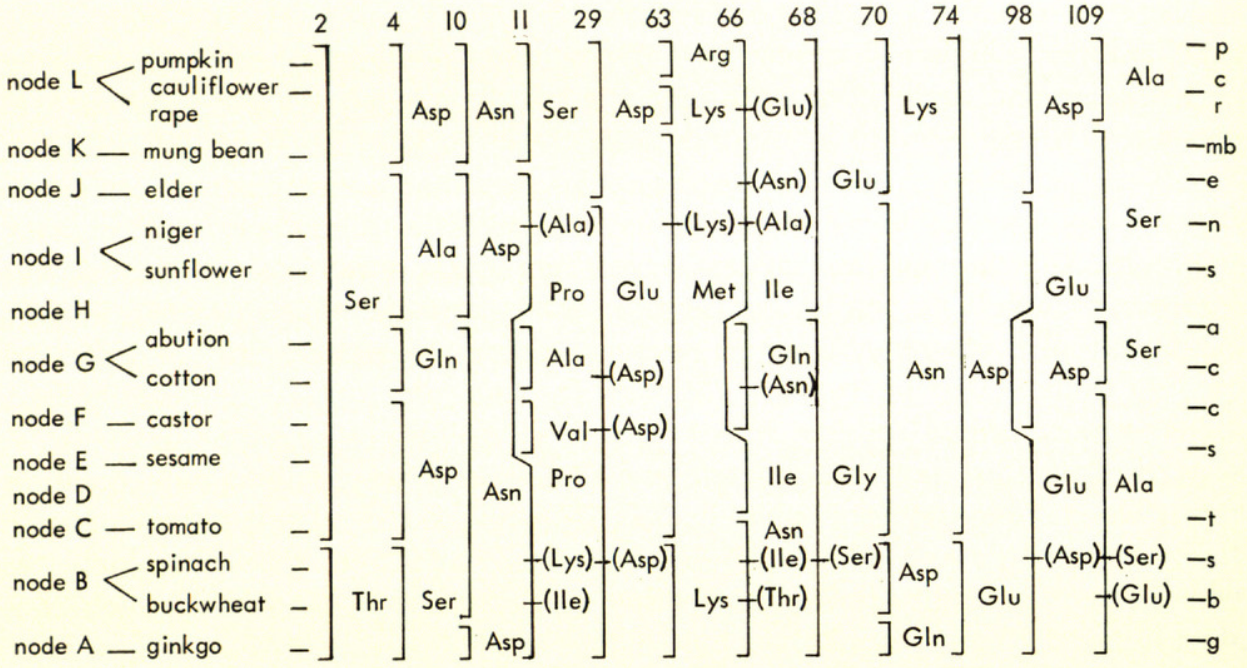

FIGURE 15. Differences between plant cytochromes *c*. Positions where differences in the sequences occur, which lead to differences along the nodal line of descent. The nodal residue for each group of nodes is shown. In cases where a sequence differs from the adjacent node, the amino acid is indicated in brackets. The lettering and topology of the nodes is as given in Fig. 13, and the numbers refer to the positions in the complete sequence (after Boulter et al., 1972).

Has the clock also kept reasonably good time for the plant kingdom, especially among angiosperms? This, of course, we do not know, for there is that abominable fossil gap between the angiosperms and other plant groups somewhere below the Cretaceous. But let's assume that the amino acid ticker for plants runs at about one substitution every 25 million years or so, much as it apparently has for most animals. What can we say then about the relative time of divergence of various plant groups, especially angiosperms, based on the data tabulated to date?

This, in fact, has been done by Ramshaw et al. (1972), using data from approximately 20 species of plants, including 14 dicots distributed among 12 families (Fig. 15) and several monocots. Assuming a monophyletic origin, these data suggest that the angiosperms arose somewhere before the Jurassic, between 400 and 520 million years ago. Further, the data suggest that the monocots were probably derived from the dicots around 230 million years ago, and that of those angiosperms examined to date, the Chenopodiaceae-Polygonaceae line was among the first (about 300–400 million years ago) to diverge from that line leading out of *Ginkgo*, the only truly primitive vascular plant examined by Boulter's group.

Figure 13 in Scogin's paper summarizes much of what is believed to be a "best fit" phyletic tree of the dicots, constructed from one of several, highly sophisticated, computer-programmed approaches (some, if not all, of which have been soundly criticized by Crowson (1972). However scrappy the data, several interesting suggestions emerge:

1. The Chenopodiaceae-Polygonaceae line seems to have branched quite early from the ancestral plexus which gave rise to the angiosperms generally, supporting the contention of Mabry et al. (1963), and perhaps others, that the betalain-

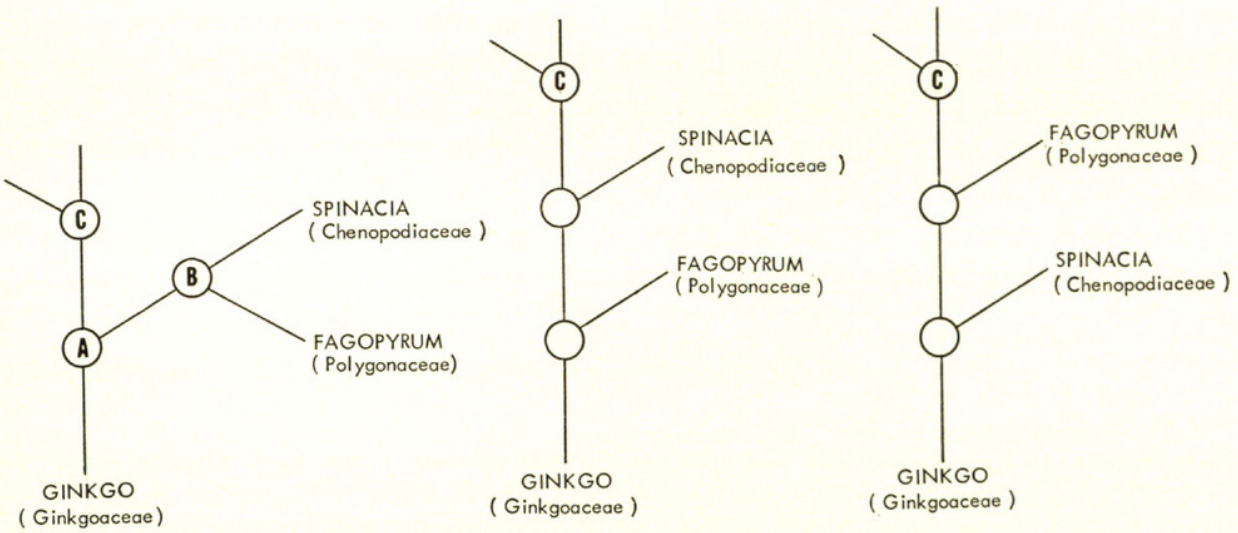

FIGURE 16. A summary of the three minimum amino-acid substitution phylogenetic trees relating 15 plant species constructed using the "ancestral sequence" method. Node C and the remaining unshown topology is common to all three trees, and is shown in Fig. 13.

containing families largely developed before anthocyanins were developed in the angiosperms generally. And I would add that the eight (!) amino acids separating *Spinicia* from *Fagopyrom* (Fig. 15) suggest that the Polygonaceae is an old line arising out of relatives close to, *but not in*, the betalain-containing complex. In this, I would subscribe to Boulter et al.'s (1972) phyletic arrangement shown in Fig. 16, this being one of several possible, computer-derived, branches of the tree at this particular level. Unfortunately, as already indicated, data of this type are unavailable for the woody, presumably ancient, groups belonging to the Magnoliatae, but it would not be surprising to find that this subclass arose close to, *or even after*, the betalain-containing groups, or Node A on the phyletic tree shown in Fig. 13.

2. Only two amino acids separate the Cucurbitaceae from the Brassicaceae. Ridiculous? Morphologically speaking, of course, for who among us would reckon these two families to be less closely related, either phenetically or cladistically, than the two genera of Asteraceae discussed below? Too little is known at present, but hopefully new sequences among these groups will make us better believers, or disbelievers.

3. Cytochrome *c* from *Helianthus* and *Guizotia*, both members of the tribe Heliantheae of the Asteraceae, differs by three amino acids, *suggesting* that the two genera parted ways perhaps 60 or more million years ago, *suggesting* that the subtribe Coreopsidinae (to which *Guizotia* belongs) is quite remote from the subtribe Heliantheae, *suggesting* that, perhaps, the large family Asteraceae is very old, indeed, with lineal branches back to rather remote ancestral groups, such as the Magnoliatae, which, as Mabry points out, contains some of the same kinds of sesquiterpene lactones as the Asteraceae.

The nucleic acid hybridization data presented by Dr. Mabry is interesting, but clearly the results hardly justify the difficult and complex methodology which one must master before meaningful data can be obtained. The results to date suggest that the betalain families are more closely related one to another

than they are to yet other groups. In fact, the results are so tenuously expressed (Chang, 1971) that one must wince at the prospect of adding yet additional families to the list of families already examined *if* the percent homology shown in Mabry's Fig. 14 is reasonably correct. Unfortunately, the DNA hybridization technique, which held such promise in the beginning, has simply failed to live up to expectations, at least for the moment. Like an iceberg, most of its body must lie somewhere below the surface.

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