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Review Article

Biological and Phytochemical Screening of Plants

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INTRODUCTION

THE IMPORTANCE of plant-derived medicinals in modern medicine is often underestimated. Such useful compounds as digitoxin, rutin, papain, morphine, codeine, papaverine, atropine, scopolamine, quinine, quinidine, reserpine, ergotamine, ergonovine, cocaine, vincaleukoblastine, leurocristine, *d*-tubocurarine, protoveratrines A and B, ephedrine, sparteine, physostigmine, pilocarpine, colchicine, and caffeine—to mention a few—present a broad and representative range of pharmacologic activities. In addition, crude drugs such as *Digitalis purpurea* leaf and *Rauwolfia serpentina* root are still preferred by many physicians in their practice; whereas extracts from *Podophyllum peltatum* (podophyllin), *Rhamnus purshiana* (anthraquinones), *Cassia* species (anthraquinones), and *Plantago* species (mucilage) are widely utilized for their medicinal activity. In fact, a recent survey has pointed

out that 47% of some 300 million new prescriptions written by physicians in 1961 contained, as one or more active ingredients, a drug of natural origin. Further, between 1950 and 1960, prescriptions containing drugs of natural origin increased by 7.7% (1).

A knowledge of the biological activities and/or chemical constituents of plants is desirable, not only for the discovery of new therapeutic agents, but because such information may be of value in disclosing new sources of such economic materials as tannins (2), industrial oils (3–9), gums (10), precursors for the synthesis of complex chemical substances (11), etc. Also, a novel chemical structure, isolated from plant sources, often prompts the chemist to a successful series of modified semisynthetic compounds, *e.g.*, atropine →homatropine, reserpine→syrosingopine, morphine→*N*-allylnormorphine, which may have some medicinal or otherwise useful economic value. On the other hand, attempts at modification of model natural compounds to enhance their activity have, of course, been unsuccessful (12). A knowledge of the chemical constituents of so-called "toxic" plants could place the treatment of plant poisonings of both humans and animals on a more rational and specific basis. The possibility of discovering new research tools for use in pharmacology, *e.g.*, as has been served by the hallucinogens, should not be discounted. A knowledge of the chemical constituents of plants would further be valuable to those interested in the expanding area of chemotaxonomy (biochemical systematics), to those interested in biosynthesis, and to those interested in deciphering the actual value of folkloric remedies.

This review is intended to present the various approaches used by investigators primarily interested in the discovery of new biologically active plant principles. *Phytopharmacologic Approaches* will consider those methods which involve, as a first step, the observation or detection of biological activity induced by plant products. *Phytochemical Screening Approaches* will describe and evaluate methods used for the detection of phytochemical classes of compounds, examples of which are known to elicit some desirable biological response.

The problems of the natural product investigator interested in biologically active compounds are complex and differ distinctly from those of the organic chemist who synthesizes or manipulates molecules using structure-activity relationships as his theoretical motivation to design. Natural product investigators must initially select their plants to investigate from a total number of available species that has been estimated to be as high as 750,000, excluding the bacteria and fungi (Table I). When this selection has been made, whether it be on theoretical grounds or on the basis of preliminary experimentation, the problems of acquisition and the variability of investigational plant material become complicating factors. These problems have been discussed recently in relationship to drug plants (14). Next, the natural product investigator must enlist the aid of a cooperative pharmacologist, or make other arrangements to insure a suitable biological evaluation for his extracts and isolated compounds. The problems inherent in the biological evaluation of crude plant extracts are in themselves unique, and several will be delineated in this review. Many of these problems remain to be solved, and the personal experiences of this author cause him to believe that those who are concerned with biological evaluations have little interest in crude plant extracts. In effect, priority is usually given to the biological evaluation of crystalline, water-soluble compounds. However, it should be remembered that in natural product studies, these pure compounds are realized only after initial biological tests on crude extracts provide justification for a phytochemical investigation. This lack of interest in the biological evaluation of crude plant preparations will probably continue as the major block to progress in the study of natural products.

The selection of research plant material by the investigator interested in the discovery of new biologically active phytoconstituents is a real problem. Approaches outlined in this review are representative of those which have either been used in the past, or are currently in progress. Each has obvious advantages as well as disadvantages, and the final method or combination of methods to be used by an individual will necessarily reflect his own background, training, interests, and available research facilities.

PHYTOPHARMACOLOGIC APPROACHES

Medicinal Folklore Evaluation

From about the 11th to the 18th centuries a dogma known as the Doctrine of Signatures was almost the sole means by which man attributed medicinal value to certain plants. This dogma held that the color, shape, habitat, or other physical characteristics of a plant were indicative of its medicinal value. Thus, the worm-shaped embryo of chenopodium (wormseed) suggested it to be of value as an anthelmintic, the yellow color of saffron served to point out its value in liver disorders, the serpentine shape of rauwolfia roots (snakeroot) indicated that they should be useful in treating snakebite, etc. Obviously,

TABLE I.—ESTIMATED NUMBER OF PLANT SPECIES[a]

Thallophyta	
Bacteria	1,500
Fungi	100,000
Algae	19,000
Lichens	20,000
Bryophyta	14,000
Pteridophyta	10,000
Spermatophyta	
Gymnosperms	700
Angiosperms	
Monocots	200,000
Dicots	500,000

[a] As put forth by Schultes in 1963 (13).

no rational justification for the use of drugs selected in this manner can be made. However, as man experimented, he found by trial and error, certain plants useful for the treatment of some of his myriad illnesses. Many of the uses of these plants were, in the course of time, documented in various herbals, books on medical botany, in ethnobotanical manuscripts, and even as notes on herbarium specimens. Uses of other alleged medicinal plants remain undocumented in the literature and exist only in the lore of primitive people. Let us consider some of these sources of medicinal folklore.

Herbals.—Space will permit only a token mention of the hundreds of herbals available for scrutiny by those interested in pursuing this approach to uncover new leads to plants with potential medicinal attributes. "The Herbal," by Arber (15), tabulates many of those which are known. A further list of herbals and related works, many of which are rare, has been prepared for the holdings of the Hunt Botanical Library (16, 17). The use of such materials as sources of ideas for new drug plants has been discussed (13, 18, 19).

Medical Botany.—One could spend a lifetime surveying the available published books and periodicals describing the native flora of various regions and the medical uses ascribed for each plant. De Laszlo (20) has compiled a list of some 1500 references on books, journals, and periodicals concerning phytotherapy and Dragendorff's "Die Heilpflanzen der Verschienden Volker und Zeiten" (21) should not be overlooked as a source of new leads. Steinmetz's "Codex Vegetabilis" (22) also lists hundreds of plants together with their synonyms, constituents, and reported uses; however, references to source material are not included in this work. Perhaps because of the Indian system of Ayurvedic medicine, the plants of that country have been reported medicinally useful more often than perhaps those of any other country. At least one gains this impression from the host of available references (23–28). Other countries represented by books or review publications on medical botany are Mexico (29, 30), Poland (31), New Guinea (32), the Philippines (33), Nigeria (34), the U.S.S.R. (35, 36), China (37–39), Burma (40), Puerto Rico (41), Malaya (42), Africa (43), Greece (44), Australia (45), New Zealand (46–49), Taiwan (50, 51), and Haiti (52), as well as others (53–57). These references are to be taken only as selected examples illustrating the type of source material available to the interested investigator. Schultes has recently pointed out, in an excellent article, the opportunities for investigation through an appreciation of medical botany (13).

Of course, simply reading through these works will not automatically assure one of success in his selection of plants for investigation. A great deal of common sense, a broad background in the medical sciences, and some knowledge of plant constituents and of chemotaxonomic relationships are all necessary for one to select the most promising plants for study.

Ethnobotany.—An evaluation of those publications by persons who have studied plants in their relationship to human affairs could uncover many species with potential biological activity. The papers by Train *et al.* (58–61), which describe plants used by the Nevada Indians, are certainly worth examining for new leads. Any person interested in the discovery of new biologically active compounds in plants would do well to become acquainted initially with the ethnobotanical writings on narcotic drug plants by Schultes and others (62–76), since they contain a wealth of information found in few other references.

An interesting ethnobotanical report has described 3 plants, all members of the *Araceae*, that are used by natives in Colombia as oral contraceptives (77). Several years ago, we had occasion to investigate *Dieffenbachia picta* (*Araceae*) because reports forwarded to our laboratory indicated that natives in South America were known to utilize this plant as an oral contraceptive. Our animal experiments failed to confirm this use for *D. picta*, but subsequently an excellent review article on the toxicity of *Dieffenbachia* species was located which described in detail many cases wherein these species had been utilized for their antifertility effects (78). Extracts of *D. seguine* were shown to produce sterility in both male and female rats after 30 to 90 days of either oral or subcutaneous administration. On histological examination it was revealed that complete or partial atrophy of the entire male or female genital apparatus had been induced in the treated animals, thus rendering them sterile (79). This obviously is an undesir-

able effect for an oral contraceptive drug and interest in *Dieffenbachia* species for this purpose appears to have been curtailed. In retrospect, however, it is unusual that natives in South America and in certain of the Caribbean islands chew the leaves of *D. seguine* to impart a 24- to 48-hr. period of sterility, whereas in laboratory animals, extracts of this plant must be administered for several weeks in order to obtain the same effect (78).

Perhaps a specific example, pointing out the value of ethnobotanical studies in the search for new biologically active compounds, would be in order. During early 1956 this author had occasion to review an ethnological manuscript, representing the doctoral dissertation of Philips (80), for possible publication in the form of a book by the University of Pittsburgh Press. The manuscript was concerned, in part, with Lebanese folk cures, the idea for the dissertation arising from contacts with people in Lebanon as well as Lebanese immigrants in Brooklyn. The author found, as her investigation progressed, that the customs of the Lebanese people were greatly influenced by the use of plant drugs as a facet of their culture. Information was derived from personal interviews with 843 people and resulted in a rather extensive list of plants used as drugs. Many of the native uses for these plants could be accounted for on rational grounds through a knowledge of chemical compounds existing in the plants which had known biological activity. Others could not be explained on these bases and seemed worthy of future investigation. As I read the manuscript, a list was prepared of those plants which appeared to be used by the greatest number of people for specific, well defined, disease conditions. The completed list included 15 plant names, the alleged uses for which I could not explain at the time. Several months later an article was noted concerning the isolation and pharmacology of an alkaloid named petaline chloride, in addition to others, from *Leontice leontopetalum* (*Berberidaceae*) (81). Ironically, this plant headed my list taken from the ethnobotanical manuscript as being most likely to yield interesting and biologically active entities. *Leontice leontopetalum* had been used as a folk medicine in Lebanon for years as an aid to the treatment of epilepsy. Petaline chloride was shown to be a central nervous system depressant in mice and rabbits, and the investigators claimed antiacetylcholine activity on isolated frog skeletal muscle. Additional studies to indicate more clearly antiepileptic activity, *i.e.*, effect on chemically or electrically induced convulsions in animals, were not performed, presumably due to an inadequate supply of petaline. To my knowledge, additional pharmacologic evaluations of this alkaloid have not been reported.

Other ethnobotanical manuscripts that offer similar possibilities, or give interesting background material on this approach to the discovery of new drug plants, have been written by Tantaquidgeon (82), Gunther (83), Grover (84), and Schultes (85, 86).

Herbaria.—A rather unusual approach to the search for new leads to biological activity in plants is being pursued at Harvard University. This involves a search of individual herbarium specimens for field notes entered by the botanist making each particular collection. In many instances, as pointed out by von Reis (87), information appears on some specimens that indicates a particular medicinal use for a plant as observed by the collector. The project consists of a sheet by sheet study of the entire collection of flowering plants in the Harvard University Herbarium, which includes some 2,200,000 specimens from the collections of the Arnold Arboretum and the Gray Herbarium. Advantages to this type of study have been summarized by von Reis (87). In the first 6 months of the study, 6 families had been searched, *i.e.*, *Apocynaceae*, *Asclepiadaceae*, *Convolvulaceae*, *Lythraceae*, *Myristicaceae*, and *Rubiaceae*. About 400 species of interest were recorded and separated into several categories, *i.e.*, medicinal uses (40%), suggested medicinal applications or possible physiological activity (33%), substances eaten, but not as normal staples of the diet (13%), poisons (8%), plants collected expressly for the purpose of chemical analysis and possible drug use (3%), and plants which animals were said to be attracted to or that they especially avoided as food or contact substances (1%). It has been conservatively estimated that the study, when completed, should yield some 360 notes concerning new potential medicinal applications for plants.

The only other study of this type which has been publicized is that being conducted by Perry involving a compilation of medicinal flora of southeastern Asia. Included as a part of this effort is a study of field notes on herbarium specimens (88).

Field Exploration.—Throughout history, botanical collecting expeditions have been organized for the specific task of discovering new drugs. In 1716, Peter the Great requested the botanist Breynius, in Danzig, to organize an expedition to explore the botanical resources of Russia for new drugs. The botanist Messer-

schmidt from 1720–1727 made vast collections in Siberia of plants reputed to have medicinal value (89). In 1602 Bartholomew Gosnold, in a ship chartered by Sir Walter Raleigh, sailed the coast of New England specifically to obtain the bark of *Sassafras albidum* for export to England (89). Parke Davis and Co. sponsored expeditions into South America in 1885 which led to the discovery of *Rhamnus purshiana* (cascara) and *Guarea rusbyi* (cocillana), 2 drugs still in use today (89). In 1942, when the quinine source of the world was cut off by the invasion of the Dutch East Indies by the Japanese, American botanists Steere, Fosberg, Camp, and others relocated and identified several quinine-yielding species in South America (89). More recently, the *Strophanthus* hunt in Africa, as well as the search for new steroidal sapogenin and alkaloid-bearing plants of the world, testify to the value of field exploration for new plant products of medicinal and/or economic value. These, along with accounts of the most striking plant explorations of the past, have recently been documented by Kreig (90).

There is controversy regarding the optimal approach to plant exploration if one is to uncover information on drug plants of potential value. Some believe that a semirandom collection of plants which contain specific types of chemicals, *e.g.*, alkaloids, will be most rewarding (91–94). Others consider that a random selection of plants in combination with subsequent broad pharmacological screening will lead to new therapeutic agents (95–98). Still others feel that recording the names of plants used in native medicine by witch doctors, medicine men, etc., directly from the individuals who use the products, will give a greater insight to the selection of plants for subsequent biological evaluation (99, 100).

Schultes, who spent some 12 years in the Amazon, feels that natives are more apt to reveal their secrets (drug plants) only after acquaintance with an invading investigator for a long period of time (13). He has cited several examples from personal experiences that bear out this point (13). Furthermore, Schultes is of the opinion that the most successful explorations for new plant drugs will not be those set up with the express purpose of looking for medicinals and nothing else, but rather those primarily designed to acquire new botanic and ethnobotanic knowledge (13). In the course of such investigations, information regarding new drug plants and related materials should be uncovered during the normal course of events.

Recognizing that the latter approach has merit and could well be the most effective method presented, the need for an inception of such a program is immediate. That is because, as civilization spreads into primitive areas, the first aspect of primitive culture to be lost is knowledge on the use of plants as medicine (13). Schultes, with respect to this matter, has stated, "The rapidity of this disintegration (knowledge of medicinal plants) is frightening" (13). Unfortunately, trained manpower to carry out this type of field exploration program is not available for an extensive undertaking at the present time (13).

Poisonous or Toxic Plants

A documentation of toxicity for any plant material is usually evidence for the presence of biologically active material in the plant cited. The terms "poison" and "toxic" are, of course, often relative where biological activity is concerned. If one discounts reports concerning contact poisons and considers mainly plants reported to cause systemic toxicity, it is possible that proper dose administration of extracts from the particular plant in question will uncover knowledge of pharmacologic activity worthy of further study. A number of books and other periodicals are available which point out plants that have been reported to cause toxicity. Interesting historical background information on this subject has been published by Kingsbury (101) and an excellent review of the toxic plants of the United States and Canada, which is thoroughly referenced, is found in Kingsbury's recent book (102). Other references to toxic plants and fungi of the U. S. are those of Muenscher (103), Duncan *et al.*, (104, 105), Morton (106, 107), and O'Leary (108). African (43), Australian (45), Indian (109, 110), British (111), Venezuelan (112), and the Pacific region (113) toxic plants, have been similarly documented. Again, the references cited above are not meant to constitute a complete listing of all available references to toxic plants. Rather, they are offered as an introduction to the person interested in studying toxic plant constituents and the relationship of these constituents to possible therapeutic applications.

Phytopharmacological Surveys

A number of interesting surveys have been conducted in which plant extracts have been evaluated for various biological activities. Although the data presented in certain of these surveys must be considered as negative, *i.e.*, those regarding antineoplastic evaluations published by the Cancer Chemotherapy National Service Center (114–118) as well as by others (119), these reports are quite helpful to investigators

engaged in similar studies who might not want to duplicate negative efforts. Biological evaluation of plant material presents certain inherent and apparently uncontrollable problems. If one considers the well known fact that phytoconstituents can vary based on climate, habitat, soil nutrients, and time of harvest, in addition to other factors (120–122), and that distinct chemical races of plants are known to exist (123–127), variation in results from one lot of plant material to the next must be expected. Furthermore, the selection of an appropriate extraction solvent, when the chemical nature of the potentially active phytoconstituents is unknown, presents a problem. Also, the conditions to be used in preparing the extracts of plant material could be the difference between demonstration of biological activity and obtaining essentially negative results. The presence of antagonistic substances, as regards to biological activity, could result in a failure to detect either of the 2 or more individually active materials. Since many plants are known to accumulate rather large quantities of toxic inorganic constituents, *i.e.*, selenium, nitrates, copper, etc. (102), the predominant action of any one of these in a plant extract containing organic compounds with potentially interesting biological activity could lead to the dismissal of further interest in the plant. It is interesting to note that we recently isolated leurosine, an alkaloid with a high order of activity against the P-1534 leukemia in DBA/2 mice, from a crude fraction of *Catharanthus lanceus* alkaloids that by itself was shown to be devoid of activity (128). Svoboda (129) also has pointed out that leurocristine and leurosidine, both highly active against the P-1534 leukemia, have also been isolated from crude alkaloid fractions that were devoid of activity against the same neoplasm.

There appears to be a great race to determine the biological activity of plant extracts, but little is being done to investigate the problems mentioned above. When, and if these problems are solved, all negative data obtained with present methods may have to be re-examined, and perhaps duplication of similar studies will be warranted.

Antineoplastic Activity.—The plant kingdom should be a prospective and fruitful hunting ground for new tumor inhibitors. This has been illustrated by the isolation, characterization, and structure elucidation (130) of vincaleukoblastine (vinblastine) (131, 132) and leurocristine (vincristine) (129) from the apocynaceous shrub *Catharanthus roseus* (*Vinca rosea, Lochnera rosea*). Vincaleukoblastine is used clinically for the treatment of Hodgkin's disease (133) and for choriocarcinoma (133), whereas leurocristine is effective in the treatment of acute leukemia in children (133).[1] Noble *et al.* (131) were the first to publish on the antineoplastic and leukopenic activity of *C. roseus* and the isolation of crude vincaleukoblastine. Their studies were initiated by reports that *C. roseus* was used extensively as an oral hypoglycemic agent in folk medicine. Furthermore, the discovery of anticancer activity was made during their blood sugar work, only on the basis of followup studies on a toxicity observed in *C. roseus*-treated animals. Antitumor activity of *C. roseus* extracts also was noted by Svoboda in his laboratory at about the same time that Noble and co-workers made their observations of this activity. Svoboda's observations came about through a routine screen for antitumor activity, as his initial interest in this plant was a potential source of hypoglycemic compounds (134). Subsequent studies by Svoboda (129) led to the isolation of leurocristine, as well as leurosine (132) and leurosidine (129), 3 additional *C. roseus* dimeric antineoplastic alkaloids (133). However, in another laboratory, an alcoholic extract of *C. roseus* (*Lochnera rosea*) whole plant provided negative results when screened against the sarcoma 180 and the L-1210 leukemia (114). Further negative results have been reported for an aqueous extract of *C. roseus* (*Vinca rosea*) seeds on testing against the sarcoma 180, the adenocarcinoma 755, and the L-1210 leukemia (114).

Hartwell (135) has discussed the role of the Cancer Chemotherapy National Service Center (CCNSC) in screening plant extracts, as well as other compounds, for antineoplastic activity. A comprehensive plant collection program by the U. S. Department of Agriculture (USDA) as well as by others, serves to supply plant material to the CCNSC for evaluation. Most plants are semirandomly selected for testing, but home remedies alleged to be useful for the treatment of cancer are not discounted (135). Since its inception, the CCNSC has screened about 26,000 plant extracts. No figures are available to indicate the number of species represented by this number, but it has been estimated that an average of 2 plant parts for each species are tested, and for most plant materials, 2 extracts (*i.e.*, water, ethanol) are prepared. Therefore, one could estimate that about 6500 species have been evaluated to date by the CCNSC. About

[1] Dr. Gordon Zubrod, Director of Intramural Research, National Cancer Institute, Bethesda, Md., told a Fountain Subcommittee hearing on April 23, 1964, that leurocristine is the . . . "Most effective antileukemia agent that has been discovered in the last 5 or 6 or 10 years." [*F-D-C Reports—The Pink Sheet*, **26** (17) (April 27, 1964).]

10% of these species have been found to be "active" against one or more tumors and are considered candidates for phytochemical isolation work. The CCNSC expects that this program will be continued at about its present rate (136).

Although the CCNSC program is still in its infancy, on the basis of the number of compounds and plants that are available for evaluation, reports in the current literature are beginning to identify some of the antitumor and/or cytotoxic constituents of plant extracts that have been determined active in this screening sequence. Kupchan *et al.* (137) have isolated aristolochic acid, active against the adenocarcinoma 755, from *Aristolochia indica* (*Aristolochiaceae*). Aristolochic acid has been found to exert a high order of renal toxicity which will preclude its use in clinical studies (138). Undoubtedly, structural relatives of this compound will be prepared in an attempt to reduce toxicity and yet maintain a high order of antineoplastic activity. Monocrotaline, from *Crotalaria spectabilis* (*Leguminosae*) has also been found to be an active tumor inhibitor (AC-755) (139). Phytochemicals which are cytotoxic for the 9-KB cell culture have been isolated and found to be apocannoside and cymarin from *Apocynum cannabinum* (*Apocynaceae*) (140), podophyllotoxin from *Juniperus virginiana* (*Pinaceae*) and other *Juniperus* species (141), calotropin from *Asclepias curassavica* (*Asclepiadaceae*) (142), cissampareine from *Cissampelos pareira* (*Menispermaceae*) (143), eupatorin from *Eupatorium semiserratum* (*Compositae*) (144), and gaillardin from *Gaillardia pulchella* (*Compositae*) (145). Semipurified fractions from *Rumex hymenosepalus* (*Polygonaceae*) exert significant activity against the Walker 256 tumor and the sarcoma 180 in mice (146). The active material is thought to be a polyphenolic flavonoid tannin. A proteinaceous fraction from *Gutierrezia sarothrae* (*Compositae*) is inhibitory for sarcoma 180 in mice (147). Although many other examples concerning the isolation of active antitumor or cytotoxic plant principles could be cited, the above should serve to point out the broad distribution of antitumor activity in the plant kingdom. Table II lists the names of some 400 plant species in 315 genera and 97 families reported in the literature to be appreciably active as tumor inhibitors. A great variety of chemical classes and structure types are also represented in the known active plant principles, *i.e.*, alkaloids (vincaleukoblastine, leurocristine, tylocrebrine, lochnerinine, cissampareine, monocrotaline), cardenolides (apocannoside, caloptropin, cymarin), lignans (podophyllotoxin), flavonoids (eupatorin), tannins (*Rumex hymenosepalus*), proteins (*Gutierrezia sarothrae*), sesquiterpene lactones (gallardin), tetracyclic triterpenes (elatericin A, elatericin B, elaterin), as well as others (Fig. 1).

If one examines negative as well as positive screening data from plant extracts, it becomes apparent that active antineoplastic compounds are not usually distributed throughout related taxa. The problem of screening plant extracts is further compounded through a knowledge that active inhibitors are often localized in one plant organ and not distributed throughout the plant. Thus, the fruits or seeds of one species could well contain active material, yet evaluation of the whole plant, containing few if any fruits or seeds, would probably give negative results. Seasonal variation has been shown to affect the amount of active material present in certain species (148). Also, the selection of a proper solvent for preparation of extracts to be evaluated could be an important factor. Furthermore, most of the active compounds isolated from higher plants to date, as well as the extracts themselves, are often quite specific in their action for a given tumor or set of test conditions. Additionally, correlation of *in vitro* and *in vivo* testing results, with those of the clinical effects of antitumor agents, remains to be established.

It appears, however, in the light of present knowledge and experience, that a random selection and testing of plants selected from a broad cross section of families and genera will prove of greatest value in attempts to discover new entities for the treatment of clinical malignancies. It is interesting to note that the efforts to arrive at new antitumor agents by chemical synthesis appear to be no more productive than those of the natural product researcher. Even though the net result of these tedious procedures involving the search, collection, identification, processing, and biological evaluation of the flora of the earth does not yield additional compounds of clinical value, the new structures evolving from these efforts should prove of use to the chemist for his work involving structural modifications.

Recent findings (140, 142, 145, 149) that cytotoxicity is associated with the unsaturated lactone of cardenolides and related compounds, attached either to position 17 of the nucleus by a C—C bond or fused to ring D across the 16, 17-position, offer insight to the minimum qualifications for cytotoxicity. Medawar *et al.* (150) were the first to suggest that unsaturated lactones exert a specific inhibitory effect on the growth of tissue. Subsequent investigators confirmed this interesting relationship (151–152). Pike *et al.* (153) synthesized 150

TABLE II.—DISTRIBUTION OF ANTINEOPLASTIC ACTIVITY IN PLANTS[a]

Plant Name[b]	Neoplasm[c]	Ref.
Acanthaceae		
Andrographis paniculata	S-4	(176)
Jacobinia coccinea	C-1, S-4	(166, 176)
Aceraceae		
Acer negundo	C-5	(175)
Acer rubrum	C-1	(161)
Amaryllidaceae		
Agave americana	H-2, S-1	(160)
Agave expansa (A. americana)	C-1	(165)
Agave micracantha	S-1	(160)
Cooperia pedunculata	C-1, S-7	(148, 163, 167)
Galanthus nivalis	S-1	(160)
Haemanthus puniceus	S-1	(160)
Hymenocallis sp.	C-2	(376)
Hymenocallis caribaea	S-1	(160)
Hymenocallis occidentalis	S-1	(160)
Leucojum aestivum	S-1	(160)
Lycoris incarnata	S-1	(160)
Lycoris sprengeri	S-1	(160)
Lycoris squamigera	S-1	(160)
Manfreda maculata	C-1	(165)
Narcissus poetaz var. *geranium*	S-1, S-2	(160)
Narcissus tazetta	C-5, S-13	(171)
Polianthes tuberosa	S-1	(160)
Rhodophiala chilensis	S-1	(160)
Zephyranthes carinata	C-5, S-4, S-10, S-13	(171)
Zephyranthes texana	C-1, S-12	(148, 164, 167)
Anacardiaceae		
Anacardium sp.	C-1	(165)
Loxopterygium huassango	C-1	(158)
Melanorrhoea woodsiana	S-4	(176)
Pistacia chinensis	C-1	(158, 162)
Pistacia lentiscus	C-1	(158, 161, 162, 165, 166)
Pistacia vera	C-1	(161)
Rhus glabra	C-1, C-2	(164)
Rhus javanica	C-7	(171)
Rhus toxicodendron	C-1, C-2	(148, 163, 167)
Rhus typhina	C-2	(166)
Annonaceae		
Annona cherimola	C-1	(161)
Artabotrys suaveolens	S-4	(176)
Apocynaceae		
Apocynum androsaemifolium	S-1	(155)
Apocynum cannabinum	C-1, E-1	(140, 161)
Catharanthus lanceus	C-2, E-1, L-2	(128, 377)
Catharanthus pusillus	E-1	(341)
Catharanthus roseus	L-2	(133, 134)
Ervatamia dichotoma	E-1	(378)
Funtumia sp.	C-1	(158, 165)
Nerium oleander	C-1	(167)
Plumeria obtusa	S-4	(176)
Strophanthus hispidus	C-1	(376)
Trachelospermum asiaticum	C-5	(170, 173)
Aquifoliaceae		
Ilex coriacea	C-1	(161)
Araceae		
Symplocarpus foetidus	S-1	(157)
Araliaceae		
Eleutherococcus senticosus	S-9, S-10	(380)
Panax ginseng	S-9, S-10	(380)
Panax repens	C-5	(173)
Tetrapanax papyriferum	C-5	(172)
Aristolochiaceae		
Aristolochia sp.	C-1	(161)
Aristolochia indica	C-4	(137)
Asarum canadense	C-1	(161)
Asarum reflexum	C-1	(161)
Asclepiadaceae		
Asclepias curassavica	E-1	(142)
Asclepias decumbens	C-1	(148)
Marsdenia cundurango	C-1	(157)
Tylophora crebriflora	L-1	(381)
Berberidaceae		
Berberis aquifolium	C-2	(167)
Podophyllum emodi	S-1	(383)
Podophyllum peltatum	S-1	(382, 383)
Betulaceae		
Alnus serrulata	C-1	(166)
Carpinus caroliniana	C-1	(166)
Corylus americana	C-1	(161)
Ostrya virginiana	C-1	(161, 166)
Bixaceae		
Bixa orellana	C-2	(161)
Boraginaceae		
Alkanna sp.	C-5	(384)
Lithospermum arvense	C-1	(161)
Symphytum officinale	C-1, C-2, C-3	(328, 385)
Bromeliaceae		
Bromelia pinguin	C-1	(166)
Pitcairnia corallina	C-1	(166)
Tillandsia usneoides	C-2, S-2	(167)
Cactaceae		
Opuntia sp.	C-1	(167)
Opuntia maxima	CIT	(386, 387)
Cannabinaceae		
Cannabis sativa	C-1	(164)
Humulus japonicus	C-1	(158)
Capparidaceae		
Forchhammeria watsoni	C-1	(376)
Caprifoliaceae		
Sambucus simpsonii	C-1	(161)
Viburnum acerifolium	C-1	(158)
Viburnum macrocephalum	C-1	(376)
Viburnum prunifolium	C-1	(161)
Caryophyllaceae		
Arenaria caroliniana	C-2	(161)
Celastraceae		
Celastrus scandens	C-4, C-6	(388)
Chenopodiaceae		
Beta vulgaris	C-1, C-2	(167)

(*Continued on next page.*)

TABLE II.—(*Continued*)

Plant Name[b]	Neoplasm[c]	Ref.
Beta vulgaris var. *rubra*	C-5	(389)
Chenopodium ambrosioides var. *anthelminticum*	S-1	(156)
Cistaceae		
Lechea villosa	C-1	(161)
Combretaceae		
Conocarpus erectus	C-1, C-2	(158, 165, 166)
Terminalia bellerica	C-2	(166)
Terminalia chebula	C-5	(390)
Compositae		
Acroptilon picris (*Centaurea picris*)	C-5	(175)
Arnica montana	S-1	(157)
Artemisia tournefortiana	C-5	(175)
Aster pilosus var. *demotus*	C-2	(166)
Bidens pilosa	C-1	(158, 165)
Bigelowia nudata	C-1	(158, 165)
Boltonia diffusa	C-1	(158, 165)
Brauneria pallida (*Echinacea pallida*)	S-1	(157)
Cacalia atriplicifolia	C-1	(165)
Calendula officinalis	C-5	(384)
Centaurea balsamita	C-5	(175)
Chrysopsis tricophylla	C-1, C-2	(161)
Cirsium altissimum	C-1	(158)
Cirsium arvense	C-2	(166)
Coreopsis lanceolata	C-2	(166)
Echinacea pallida	C-2	(166)
Eupatorium semiserratum	E-1	(144)
Gaillardia pulchella	C-1, E-1	(145, 158)
Gutierrezia sarothrae (*G. enthamia*)	S-2	(147)
Inula helenium	C-5	(172)
Lactuca canadensis	C-1	(161)
Liatris punctata	C-1, C-2, S-12	(163, 164, 167)
Mikania scandens	C-1	(158)
Onopordon acanthium	C-5	(384)
Parthenium hysterophorus	C-1, C-2, S-12	(164)
Ratibida pinnata	C-2	(166)
Saussurea sp.	C-5	(175)
Silphium compositum	C-1	(158)
Solidago fistulosa	C-2	(166)
Solidago gigantea	C-2	(166)
Sonchus oleraceus	S-1	(154)
Spilanthes americana (*S. mutisii*)	C-1	(158, 165)
Tanacetum vulgare	C-2	(166)
Tessaria integrifolia	C-1	(161)
Trilisa paniculata	C-2	(161)
Verbesina aristata (*V. nudicaulis*)	C-1, C-2	(161)
Xanthium sp.	C-1	(161)
Convolvulaceae		
Ipomoea orizabensis	S-1	(154)
Cornaceae		
Aucuba japonica	C-5	(170, 173)
Cornus florida	C-2	(161)
Cornus officinalis	C-5	(172)
Cruciferae		
Capsella bursa-pastoris	S-1	(155)
Descurainia pinnatum (*Sisymbrium pinnatum*)	C1-12	(161)
Cucurbitaceae		
Bryonia alba (*B. dioica*)	S-1	(154)
Citrullus colocynthis	C-5, S-1, S-2, S-5	(154, 391, 392)
Cucumis melo	C-1, C-2, C-5	(163, 167, 175)
Cucumis melo var. *cantalupensis*	S-1	(157)
Cucumis myriocarpus	S-1	(157)
Cucumis sativus	C-1	(167)
Cucurbita foetidissima	C-1, C-2, S-12	(163, 164, 167)
Cucurbita pepo	C-1, C-2	(167)
Ecballium elaterium	C-4, C-5, C-6, S-1, S-2, S-5	(154, 388, 392)
Datiscaceae		
Datisca cannabina	C-5	(175)
Dioscoreaceae		
Dioscorea villosa	S-1	(155)
Dipsacaceae		
Dipsacus sylvestris	C-1	(161)
Ebenaceae		
Diospyros discolor	S-4	(176)
Diospyros kaki	C-1	(158, 165)
Elaeagnaceae		
Elaeagnus philippensis	C-1	(166)
Hippophae rhamnoides	C-5, S-2, X	(174, 175, 393)
Hippophae salicifolia	S-6	(394)
Equisetaceae		
Equisetum arvense	S-1	(155)
Equisetum hellocharis	C-5	(175)
Equisetum hyemale	S-1	(155)
Ericaceae		
Arctostaphylos uva ursi	C-5	(173)
Chaemaedaphne calyculata	C-2	(166)
Gaultheria procumbens	S-1	(157)
Lyonia mariana	C-1	(161)
Menziesia pilosa	C-2	(166)
Oxydendrum arboreum	C-1, S-1	(155, 161)
Euphorbiaceae		
Acalypha phleoides	C-1	(164, 167)
Bridelia ovata	S-4	(176)
Croton monanthogynus	C-2, S-7	(163, 167)
Emblica officinalis	S-4	(176)
Euphorbia amygdaloides	C-5, S-2	(395)
Euphorbia drummondii	S-1	(154)
Euphorbia marginata	C-1	(148)
Euphorbia pilulifera	S-1	(154)
Euphorbia resinifera	S-1	(154)
Excoecaria agallocha	S-4	(176)
Macaranga triloba	S-4	(176)
Mallotus philippensis	S-1	(156)
Piscaria sp.	S-12	(164)
Poinsettia sp.	S-2	(396)
Ricinus communis	C-1, S-12	(148, 164)
Sapium sebiferum	C-1	(158, 165)
Stillingia sylvatica	C-2	(161)

(*Continued on next page.*)

TABLE II.—(*Continued*)

Plant Name[b]	Neoplasm[c]	Ref.
Fagaceae		
Quercus virginiana	C-1	(161)
Gentianaceae		
Gentiana lutea	C-5	(173)
Gentiana scabra var. *buergeri*	S-4	(397)
Gnetaceae		
Ephedra trifurca	C-2	(161)
Gnetum latifolium	S-4	(176)
Gramineae		
Anthoxanthum odoratum	C-1	(376)
Arundo donax	C-1, C-2	(163, 167, 376)
Arundo plinii	C-1	(376)
Coix lachryma-jobi	S-11	(398)
Digitaria sanguinale	C-1	(161)
Sasa albomarginata	C-5, S-2	(399)
Spartina synosuroides	C-1	(161)
Tripsacum laxum	C-1	(376)
Uniola paniculata	C-1	(161)
Zea mays	C-2	(167)
Guttiferae		
Garcinia hanburyi	S-1	(154)
Haemodoraceae		
Haemodorus corymbosum	C-4, C-6	(388)
Hamamelidaceae		
Hamamelis virginiana	C-2	(161)
Liquidambar styraciflua	C-1	(161)
Hippocrateaceae		
Pristimera indica (*Hippocratea indica*)	C-4, C-6	(388)
Hydrocaryaceae		
Trapa natans	C-5	(173, 390)
Iridaceae		
Iris japonica	X	(171)
Julianiaceae		
Amphiteryngium adstringens	C-1	(164)
Juncaceae		
Juncus biflorus	C-1	(161)
Juncus repens	C-1	(161)
Labiatae		
Marrubium vulgare	S-1	(157)
Mentha piperita	S-7	(167)
Mentha spicata	C-1, C-2	(167)
Nepeta cataria	C-1	(161)
Plectranthus rugosus	C-5, S-2	(400)
Plectranthus trichocarpus	C-5, S-2	(400)
Salvia sp.	C-1	(164)
Salvia greggii	C-1	(164)
Thymus serpyllum	S-1	(157)
Teucrium canadense	C-1	(161)
Lauraceae		
Persea pubescens (*P. carolinensis*)	C-1	(161)
Leguminosae		
Abrus precatorius	C-5	(172)
Acacia auriculaeformis	S-4	(176)
Acacia spadicigera	C-1	(158, 165)
Adenanthera microsperma	C-1	(158)
Albizza julibrissin	C-1, C-2, S-7	(163, 167)
Albizza sassa	C-1	(161)
Andira surinamensis	S-4	(176)
Arachis hypogaea	C-1, C-2	(167)
Astragalus wootoni (*A. subcinereus*)	C-1, C-2	(164, 167)
Bauhinia japonica	S-10, S-13	(171)
Cassia alata	S-1	(154)
Crotalaria spectabilis	C-4	(139)
Delonix regia	S-4	(176)
Erythrina senegalensis	C-1	(161)
Euchresta japonica	H-1, S-4	(401)
Gleditschia triacanthos	C-1, C-5, S-2	(158, 165, 175, 402)
Indigofera hirsuta	S-4	(176)
Leucaena glauca	C-1	(158)
Lupinus texensis	C-1	(167)
Mimosa sepiaria	S-4	(176)
Parkinsonia aculeata	C-2	(164)
Peltophorum vogelianum	C-1	(376)
Piscidia mollis	C-1	(158, 165)
Prosopis glandulosa	C-1, C-2, S-12	(163, 164, 167)
Robinia nana	C-1	(161)
Robinia pseud-acacia	C-1	(161)
Sophora angustifolia	S-3	(171)
Sophora subprostrata	S-3	(403)
Tamarindus indica	C-1	(376)
Tephrosia virginiana	C-1	(161)
Trifolium pratense	S-1	(157)
Vigna sinensis	C-1	(165)
Wistaria chinensis	C-5	(390)
Liliaceae		
Aletris aurea	C-1	(161)
Allium halleri	C-2	(164, 167)
Allium sativum	C-5, S-2	(173, 404)
Aloe perryi	S-1	(154)
Asparagus chochinchinensis	C-5	(172)
Asparagus davuricus	C-1	(165)
Asparagus officinalis	S-1	(155)
Aspidistra elatior	S-3, S-10	(171)
Hosta sieboldiana	S-3, S-10, S-13	(171)
Ornithogalum umbellatum	E-1	(149)
Smilax spinosa	C-1	(376)
Trillium apetalon	C-5, S-3, S-10, S-13	(171)
Yucca arkansana	C-1, S-12	(148, 163, 164)
Yucca gloriosa	C-1	(376)
Yucca pallida	C-1	(376)
Loranthaceae		
Arceuthobium vaginatum	C-1	(148)
Phoradendron flavescens	C-1, C-2, S-12	(163, 164, 167)
Viscum album	M-1, S-2	(405–407)
Lythraceae		
Lagerstroemia speciosa	S-4	(176)
Lawsonia inermis	S-4	(176)
Magnoliaceae		
Kadsura japonica	C-5	(172)

(*Continued on next page.*)

TABLE II.—(*Continued*)

Plant Name[b]	Neoplasm[c]	Ref.
Liriodendron tulipifera	S-1	(157)
Malpighiaceae		
Galphimia glauca	S-4	(176)
Malvaceae		
Hibiscus cannabinus	C-1	(165)
Gossypium sp.	C-2	(164)
Melastomataceae		
Tetrazygia bicolor	C-1	(166)
Meliaceae		
Carapa guianensis	S-4	(176)
Melia azedarach	C-2, S-12	(164)
Melia azedarach var. *japonica*	C-5, C-7, S-13	(171)
Swietenia macrophylla	S-4	(176)
Trichilia hirta	C-1	(166)
Menispermaceae		
Cissampelos pareira variegata	E-1	(143)
Fibraurea chloroleuca	S-4	(176)
Moraceae		
Ficus aurea	C-1	(166)
Ficus elastica	S-2	(396)
Maclura pomifera	C-1, C-2	(167, 376)
Myristicaceae		
Knema hookeriana (*Myristica hookeriana*)	S-4	(176)
Myrsinaceae		
Ardisia elliptica	S-4	(176)
Maesa ramentacea	S-4	(176)
Myrsine capitellata	S-4	(176)
Myrtaceae		
Eucalyptus triantha	C-1	(165)
Eugenia jambos	C-2	(166)
Eugenia javanica	S-4	(176)
Nepenthaceae		
Nepenthes arbomarginata	S-4	(176)
Nymphaeaceae		
Nuphar luteum	C-5	(175)
Oleaceae		
Chionanthus retusa	C-1, C-2	(376)
Forsythia sp.	C-1	(158, 165)
Fraxinus oregona	C-1	(376)
Palmae		
Acoelorrhaphe arborescens	C-2	(166)
Aeria attenuata	C-2	(166)
Butia nehrlingiana	C-1	(165)
Latania commersonii	C-1	(165)
Phoenix pusilla	C-1	(165)
Phoenix roebelenii (*P. humilis*)	C-1	(158)
Pseudophoenix vinifera	C-1	(158, 165)
Seaforthia elegans	C-1	(165)
Papaveraceae		
Chelidonium majus	C-3	(408)
Papaver orientale	C-5, S-4, S-8	(409)
Papaver rhoeas	C-5, S-4, S-8	(409)
Passifloraceae		
Passiflora incarnata	C-2	(167)
Phytolaccaceae		
Limeum aethiopicum	C-1	(165)
Pinaceae		
Callitris quadrivalvis	C-1, C-2	(158, 165, 166)
Juniperus chinensis	C-5, C-7, S-3, S-10, S-13	(171)
Juniperus communis	S-1	(155)
Juniperus lucayana	S-2	(159)
Juniperus sabina	S-2	(159)
Juniperus sabina var. *tamariscifolia*	S-2	(159)
Juniperus scopulorum	S-2	(159)
Juniperus silicicola	S-2	(159)
Juniperus virginiana	E-1, S-2	(141, 159)
Polygalaceae		
Polygala senega	S-1	(157)
Polygonaceae		
Rheum sp.	C-2	(164)
Rheum japonicum	C-5	(172)
Rheum officinale	S-1	(154)
Rheum palmatum	C-5	(172)
Rumex crispus	S-1	(154)
Rumex hymenosepalus	S-2, S-10	(146)
Polypodiaceae		
Dryopteris marginalis	S-1	(156)
Dryopteris filix-mas	S-1	(156)
Polyporaceae		
Fomes igniarius (*Polyporus igniarius*)	S-3	(410)
Primulaceae		
Anagallis sp.	C-5	(175)
Pyrolaceae		
Chimaphila maculata	C-1	(376)
Ranunculaceae		
Aconitum napellus	S-1	(157)
Actaea spicata	C-5	(411)
Anemone decapetala	C-1, C-2	(163, 164, 167)
Clematis chinensis	C-5	(172)
Coptis japonica	C-5	(173)
Delphinium sp.	C-2	(164)
Delphinium ajacis	C-2	(167)
Delphinium staphisagria	S-1	(156)
Helleborus odorus	S-6	(384)
Paeonia albiflora	C-5	(173)
Paeonia suffruticosa	C-5	(172, 173)
Ranunculus glaber (*R. ternatus* var. *glaber*)	C-5	(170, 173)
Rhamnaceae		
Rhamnus cathartica	S-1	(154)
Rhamnus japonica	C-5, C-7, S-4, C-13	(171)
Rhamnus purshiana	C-7	(167)
Rosaceae		
Agrimonia gryposepala	C-1	(158, 165)
Brayera anthelmintica	S-1	(156)
Crataegus cuneata	C-5	(172)
Duchesnea indica (*Fagaria indica*)	C-1	(158)
Geum aleppicum var. *strictum*	C-2	(166)
Gillenia stipulata	S-1	(157)
Padus racemosus	C-5	(175)
Physocarpus opulifolius	C-2	(166)

(*Continued on next page.*)

TABLE II.—(*Continued*)

Plant Name[b]	Neoplasm[c]	Ref.
Prunus americana	S-1	(157)
Spiraea ulmaria	S-1	(155)
Rubiaceae		
Cephaelis acuminata	S-1	(157)
Cinchona succirubra	C-5	(173)
Gardenia jasminoides	C-5	(173)
Genipa americana	S-4	(176)
Mussaenda glabra	S-4	(176)
Randia densiflora	S-4	(176)
Randia spinosa	S-4	(176)
Timonius wallichianus	S-4	(176)
Uncaria longifolia	S-4	(176)
Uncaria pteropoda	S-4	(176)
Uncaria roxburghiana	S-4	(176)
Rutaceae		
Atalantia citroides	C-1	(158, 165)
Citrus amblycarpa	C-2	(166)
Evodia roxburghiana	S-4	(176)
Evodia rutaecarpa	C-5, S-10, S-13	(171)
Phellodendron sp.	C-1	(165)
Phellodendron amurense	C-5, II-1, S-4	(173, 412)
Sapindaceae		
Alectryon subcinereum	C-1	(165)
Dodonaea viscosa	C-1	(165)
Nephelium longana	C-5	(172)
Sapindus senegalensis	C-2	(166)
Sapindus utilis	C-1	(158, 165)
Sapotaceae		
Achras sapota	S-4	(176)
Madhuca indica (*Bassia latifolia*)	C-1	(165)
Saxifragaceae		
Dichroa febrifuga	X	(413)
Hydrangea arborescens	S-1	(155)
Scrophulariaceae		
Veronica virginica	S-1	(154)
Simaroubaceae		
Castela nicholsoni texana	S-1	(156)
Simaruba amara	S-1	(156)
Solanaceae		
Cestrum parqui	C-1, C-2	(158, 166)
Datura sp.	C-1	(167)
Datura stramonium	C-1	(167)
Dunalia campanulata	C-2	(166)
Hyoscyamus niger	C-1, C-2	(167)
Lycium halimifolium (*L. vulgare*)	C-2	(166)
Solanum rostratum	C-1	(148, 161, 167)
Solanum torvum	S-4	(176)
Sterculiaceae		
Cola nitida (*C. acuminata*)	S-4	(176)
Thymelaeaceae		
Daphne mezereum	S-1	(157)
Umbelliferae		
Aletes acaulis	C-2	(163)
Angelica brevicaulis	C-5	(175)
Anthriscus neglecta (*A. vulgaris*)	L-1	(414)
Conium maculatum	C-1, C-2	(163, 164, 167)
Pimpinella saxifraga (*P. magna*)	S-1	(157)
Urticaceae		
Parietaria officinalis	S-1	(155)
Vacciniaceae		
Polycodium floridanum (*Vaccinium floridanum*)	C-2	(166)
Vaccinium bracteatum	C-5	(169)
Verbenaceae		
Verbena bipinnatifida	C-2	(167)
Vitaceae		
Vitis candicans	C-2, S-12	(164)
Zingiberaceae		
Zingiber officinale	C-5	(173)
Zygophyllaceae		
Peganum harmala	C-5	(175)

[a] All plants listed were considered active in the original reference against the tumor(s) indicated. Criteria for activity vary and undoubtedly many of the plants listed in this table exert only marginal, if any, real activity. For plant parts used and type extract evaluated, see original reference. [b] Names of plants are entered as found in the original reference except for corrections in spelling. Classification is according to the "Index Kewensis" and its supplements (375). Plant names in parentheses are preferred according to the "Index Kewensis." [c] Key for neoplasms (see reference for host): C-1, carcinoma, C_3H, mammary; C-2, carcinoma, RC, mammary; C-3, carcinoma, mammary, spontaneous; C-4, adenocarcinoma 755; C-5, carcinoma, Ehrlich; C-6, carcinoma, renal; C-7, carcinoma, Bashford, 63; E-1, 9 KB cell culture; L-1, leukemia, L-1210; L-2, leukemia, P-1534; S-1, sarcoma 37; S-2, sarcoma 180; S-3, sarcoma, Crocker; S-4, sarcoma, Yoshida; S-5, sarcoma, Black, SBL-1; S-6, fibrosarcoma; S-7, lymphosarcoma, unspecified; S-8, sarcoma, genital; S-9, sarcoma, DBA; S-10, sarcoma, Walker; S-11, sarcoma, Ehrlich; S-12, sarcoma, DBA; S-13, sarcoma, NF; H-1, hepatoma, ascitic; H-2, lymphoma No. 1; M-1, mastocytoma, P-815; CIT, chemically induced tumor; X, active against several neoplasms (see reference).

steroids and found that 25 were cytotoxic. All of the 25 cytotoxic steroids contained an α-β-unsaturated lactone ring, whereas this moiety was absent in the steroids devoid of cytotoxicity. An inspection of the heterogeneous group of structures making up some of the known antineoplastic or cytotoxic plant constituents suggests that factors other than the α-β-unsaturated lactone ring are involved with cytotoxicity (Fig. 1).

Several extensive surveys for antineoplastic activity in plant extracts have been published. A majority of the studies have been carried out with plants collected in the U. S. (154–167), Japan (168–173), the U.S.S.R. (174, 175), and most recently in Malaya (176). Correlation of data derived from these surveys is difficult because of inconsistencies in the processing of plant extracts, in the test procedures, in the tumor systems utilized, and in the validity of data interpretation.

Antimicrobial Activity.— References concerning the biological screening of plant extracts are encountered most frequently in connection with the determination of antimicrobial activity.

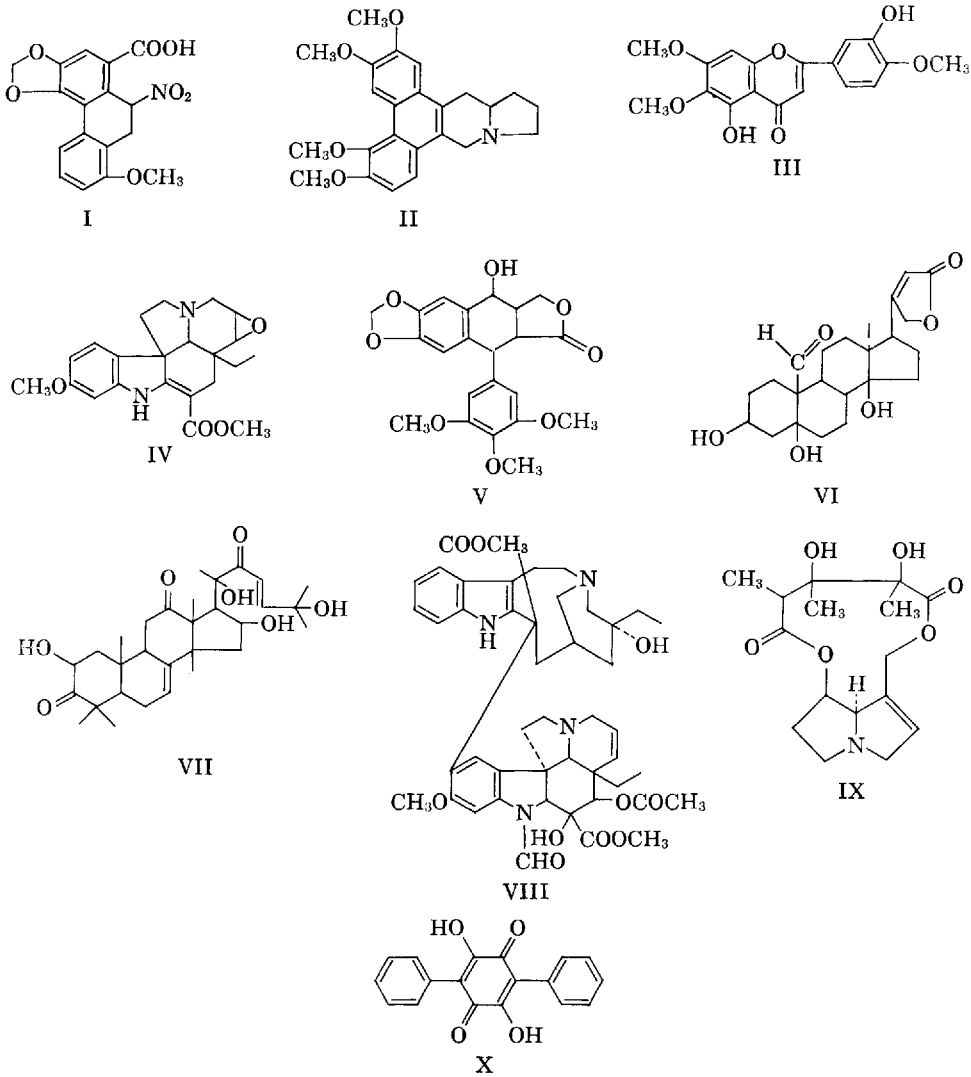
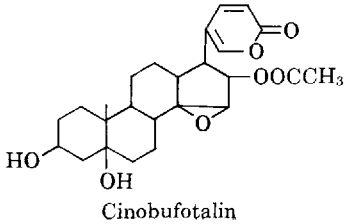
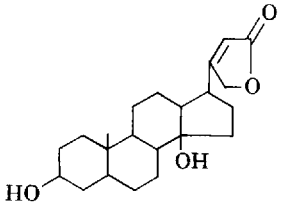


Fig. 1.—Structures of natural products known to have either antineoplastic or cytotoxic properties. Key: I, aristolochic acid (adenocarcinoma 755) (137); II, tylocrebrine (L-1210 leukemia) (381); III, eupatorin (9 KB cell culture) (144); IV, lochnerinine (9 KB cell culture) (341); V, podophyllotoxin (sarcoma 37, 9 KB cell culture) (141, 383); VI, strophanthidin (9 KB cell culture) (149); VII, elatericin A (sarcoma 180) (392); VIII, leurocristine (P-1534 leukemia) (133); IX, monocrotaline (adenocarcinoma 755) (139); X, polyporic acid (L-1210 leukemia) (379).

These evaluations are usually carried out by means of standard *in vitro* assays (disk, cup, cylinder, diffusion) utilizing a broad selection of pathogenic as well as nonpathogenic microbes. In most cases, a minimum of 1 Gram-positive and 1 Gram-negative organism, usually *Staphylo-*



Cinobufotalin



Digitoxigenin

Cytotoxic Unsaturated Lactones

coccus aureus and *Escherichia coli*, are included for initial screening. However, filamentous fungi, yeasts, and acid-fast organisms are often included. The studies of Lucas, Gottshall, Frisbey *et al.* are typical surveys of plants for antimicrobial activity (177–184). These investigators screened hundreds of extracts for inhibitory activity against *S. aureus*, *Salmonella typhimurium*, and *Mycobacterium tuberculosis*, and subsequent studies on the most promising plants by this group led to the isolation of many active principles (185–188). Other surveys for antimicrobial activity in plants native to the U.S. have been conducted using plants collected in Southern California (189, 190), Pennsylvania (119), Florida (191, 192), Indiana (193), Ohio and Michigan (194–197), Vermont (198), and Hawaii (199). The study on Hawaiian plants was based on a selection of those used in that area as home remedies. Tucakov (200), Kliewe and Hathmacher (201), Maruzzella and Henry (202), and Okazaki and Oshima (203, 204) studied the antimicrobial effects of a number of essential oils, a group of compounds in which antifungal activity is predominant. Burlage *et al.* investigated several plants reported to be toxic (205, 206). Studies involving fewer numbers of plants, plant parts, or extractives have also been reported (207–217).

Osborn (218) has surveyed more than 2300 different species of plants collected in England, while Australian plants have been extensively examined by Atkinson *et al.* (219–221). The Australian group also surveyed a number of higher fungi for antimicrobial activity (222). Similar surveys have been conducted on the flora of Nova Scotia (223–225), Brazil (226–228), Mexico (228–230), India (231–234), Japan (168, 235–243), China (244), the Philippines (245), as well as the U.S.S.R. (246–255) and other scattered areas (256–260). The antimicrobial substances from algae (261) and mosses have recently been reviewed (262), and their presence was demonstrated in a number of lichens (263, 264). Active substances in seaweed extracts have also been reported (265, 266).

Nickell (267), in a review on this area of investigation, has tabulated results of studies through 1959 in which vascular plants were demonstrated to elicit inhibitory action on microbes. Included in his tables are the plant name, family, class of organism(s) inhibited, type of extract utilized, and the plant part(s) tested. At about the same time, reviews on antibiotics from higher plants were published by Arnold (268), Virtanen (269), and Drobot'ko *et al.* (270) which were preceded by an earlier review by Freerksen (271). Antifungal substances from higher plants similarly were reviewed in 1961 by Sehgal (272).

Of particular interest is a study by Winter (273) in which he compared the antimicrobial properties of 2 groups of plants. One group included randomly collected native plants, whereas the second group comprised plants mentioned in a 300-year-old herbal which suggested that they were useful for the treatment of infections. Only 29.5% of plants from the randomly selected group exhibited antimicrobial activity, while 65% of plants selected because of their mention in the herbal were found to be active.

Karel and Roach (274) and Baron (275) have compiled lists of antibiotic substances isolated from higher plants as well as from microbes and other sources.

Because of the presence in crude plant extracts of substances which could exert antagonistic effects during the testing procedures, or which contain substances that stimulate the growth of the test organism and hence negate the effect of inhibitory substances present, new procedures should be developed. It would seem reasonable to utilize the new technique of thin-layer chromatography for these studies, since a resolution of components in the plant extracts could be expected, thus separating antagonistic and/or microbial stimulant constituents from those which might be active. Developed chromatograms then could be evaluated by means of bioautography, as suggested by either Kline and Golab (276) or Meyers and Smith (277).

Antiviral Activity.—Until the recent introduction of 5-iodo-2′-deoxyuridine (IDU), antiviral therapy in humans was nonexistent. Today, IDU is useful for the treatment of acute ophthalmic herpes simplex infections and is the only drug licensed by the U. S. Food and Drug Administration for the treatment of viral infection. Experimentally, IDU appears to be useful in the treatment of human vaccinia as well as smallpox infections (278). Aside from IDU, other synthetic compounds that appear interesting as antiviral agents are hydroxybenzylbenzimidazole, guanidine, and *N*-methyl-isatin-β-thiosemicarbazole. These compounds, as well as the problems associated with virus chemotherapy, have been reviewed recently by Kaufman (278).

Herrmann (279) has suggested that natural products should be preferable over synthetics as a source for new antiviral agents because naturally occurring mixtures, whether they be plant extracts or antibiotic filtrates, provide both a mixture of compounds, any one of which could be

active, and a diversity of chemical structures not easily obtained from chemical syntheses (279). However, little has been published on attempts to detect antiviral activity in plant extracts. Cochran and Lucas (280) investigated extracts from 46 members of the *Orchidaceae*, 24 species of other higher plants, and 6 species of mushrooms for their ability to protect mice against the polio virus. The orchids were selected because natives of New Guinea were said to use the flowers of certain of these species as drugs for the treatment of contagious diseases. Holobasidiomycetes were included in the study because of previous reports that extracts from selected members of this group, *i.e.*, *Boletus edulis* (281) and *Calvatia* species (280), were active tumor inhibitors. Most of the remaining higher plants tested were selected on the basis of suggested antimicrobial activities from the folklore. It was determined that 21 of 46 strains or species of orchids, 6 species of mushrooms, and *Hypericum perforatum*, *H. prolificum*, *Allium ampeloprasum*, *Kalmia latifolia*, *Maclura pomifera*, *Phellodendron amurense* *Medicago sativa*, and *Ribes hirtellum* exerted varying degrees of protection against poliomyelitis in the infected mice. Goulet *et al.* (282) studied the effects of extracts from 12 species of higher plants and basidiomycetes on 13 ECHO viruses, finding that many of the species were active, but only against specific viruses.

Taylor *et al.* (283), utilizing yolk-sac adapted vaccinia, influenza, eastern equine encephalomyelitis, and ornithosis viruses, evaluated extracts from 44 different species of plants. Extracts from 10 plants were effective in prolonging the survival time of vaccinia-infected chick embryos 20% or more. Four plants were effective against encephalitis, 6 against ornithosis, and 10 against influenza. Only 3 of the plants (*Allium halleri*, *Ambrosia apters*, unidentified native plant) produced 20% or greater survival against one or more viruses, thus substantiating the specificity pointed out by Goulet *et al.* (282). Twenty-two of the 44 species tested were active against one or more of the viruses.

More recently, a study in our laboratories involved the testing of extracts from 200 native plant species against vaccinia, polio type III, and pseudorabies viruses (119). It was found that 6 of the 200 species evaluated were found to have activity against one or more of the test viruses (*Apocynum* sp., *Asclepias incarnata*, *Artemisia* sp., *Aster divaricatus*, *Xanthium* sp., *Sium suave*). Two hundred types of crude drug plants were studied by Goro *et al.* (168) in a broad screening program in which extracts were evaluated for antitumor, antimicrobial, and antiviral activity. Influenza virus was used in this study.

With only a limited number of studies to evaluate the screening of plant extracts for antiviral activity, it appears that there is justification for more extensive studies in this area. As with the distribution of antineoplastic activity in the plant kingdom, antiviral inhibitory effects have been observed in diverse taxa. However, from preliminary observations, it can be noted that at least 13 genera of higher plants distributed in 10 families have been shown to elicit antiviral activity (*Artemisia*, *Ambrosia*, *Aster*, *Xanthium*, *Asclepias*, *Apocynum*, *Kalmia*, *Hypericum*, *Phellodendron*, *Medicago*, *Ribes*, *Maclura*, *Allium*). An inspection of the distribution of antineoplastic activity in the plant kingdom (Table II) discloses that only 5 of the above 13 genera have not given rise to at least 1 active antineoplastic species (*Ambrosia*, *Kalmia*, *Hypericum*, *Medicago*, *Ribes*). This observation, although admittedly based on insignificant numbers, especially those representing antiviral activities, suggests a relationship between antiviral and antineoplastic-cytotoxic activity. Only time and continued research will provide enough evidence to confirm this correlation.

Antimalarial Activity.—Whereas a number of isolated examples can be found in the folklore concerning plants alleged useful for the treatment of malarial infections, "fevers," or for use as "antiperiodics," little experimental evidence has been put forth to substantiate these claims. The most extensive survey of plants intended to detect substances having potential antimalarial activity was that reported in 1947 by Spencer *et al.* (284). They studied the effects of several solvent extracts, from more than 600 species distributed within 126 plant families, on chicks and ducklings infected with *Plasmodium gallinaceum*, *P. cathemerium*, and *P. lophurae*. Plants judged most active as a result of this evaluation were *Hymenocallis caribaea* and *Cooperia pedunculata* (*Amaryllidaceae*); *Castela tortuosa*, *Simaba cedron*, and *Simaruba amara* (*Simaroubaceae*); *Cornus florida* (*Cornaceae*); *Dichroa febrifuga* (*Saxifragaceae*); *Gentiana* sp. (*Gentianaceae*); *Croton* sp. (*Euphorbiaceae*); *Cissampelos pareira* (*Menispermaceae*); *Aristolochia* sp. (*Aristolochiaceae*); *Datisca glomerata* (*Datiscaceae*); and *Eryngium foetidum* (*Umbelliferae*). Eleven of these 13 species have been shown to elicit varying degrees of antineoplastic and/or cytotoxic activity (Table II). Only *Simaba cedron* and *Eryngium foetidum*, of the 13 plants indicated, have not been reported to elicit antineoplastic activity. *Castela tortuosa* also has not been reported, but the related *C.*

nicholsoni is active against sarcoma 37. Kupchan *et al.* have shown that cissampareine is the tumor inhibitor of *Cissampelos pareira* (143) and that aristolochic acid is the antitumor agent in *Aristolochia indica* (137). *Datisca hirta* is a hybrid of *Rhus glabra* and *Rhus typhina*, and while it has not been evaluated for antineoplastic activity *per se*, both of the hybridizing species have been shown to be active against carcinomas (Table II).

Malaria is still a disease of importance in many parts of the world today, and it is necessary to insure the availability of safe and effective drugs for the treatment of this condition. Recent reports indicate that the malarial parasite may well be developing a degree of resistance to the synthetic antimalarial drugs which may return the quinine alkaloids to their position of former importance as therapeutic agents. However, at least at the present time, a shortage of these alkaloids exists (285). In any event, future needs may require a continuation of surveys such as the one initiated by Spencer *et al.* (284), and to a lesser degree by Carlson *et al.* (195), as well as a study of the leads that have evolved from these screening reports.

Insecticide Activity.—Rotenone, nicotine, and the pyrethrins are important naturally derived insecticidal materials. Because of the economic value of insecticides in general, and particularly with regard to these 3 substances, a great deal of interest has been generated over the years to find new naturally occurring as well as synthetic substitutes. Included as part of this interest has been the initiation of several plant collection and screening programs, in addition to subsequent phytochemical investigations on the most promising plants discovered in this manner. McIndoo (286) has compiled all available literature reports on plants containing potential insecticidal substances up to 1941, and Jacobson (287) has extended these compilations through 1953. Since 1953, reviews have appeared by Sobotka in 1956 (288), by Ts'e in 1958 (289), by Hsiung (290) and Liu (291) in 1959, and most recently by Lipa in 1962 (292).

Hypoglycemic Activity.—Recent evidence indicates that orally effective hypoglycemic agents can be obtained from plant sources. Svoboda *et al.* (293) have demonstrated that 6 alkaloids from *Catharanthus roseus* (leurosine sulfate, lochnerine, vindoline, vindolinine dihydrochloride, catharanthine hydrochloride, and tetrahydroalstonine) are at least equal to tolbutamide in hypoglycemic action when administered orally to rats. It is interesting to note that the crude extracts from which the alkaloids were derived failed to elicit a hypoglycemic response (293). Also, the predominant folkloric use for *C. roseus* has been as an oral substitute for insulin; however, several studies have failed to confirm this action. These reports have been summarized by Farnsworth (294). Examples such as this, where biologically active substances can occur in a crude plant extract which apparently is devoid of activity, serve to point out the frustrations encountered in natural product biological evaluations.

Garcia has investigated a number of Philippine plants for which oral hypoglycemic activity is alleged through reports of their use in native medicine for this purpose (295–300). An extract from some of these plants, referred to as "plantisul," has been used with some degree of success as an insulin substitute according to reports in the literature (296, 298–300). *Tecoma* species of plants have been used orally by the natives of Mexico as antidiabetic remedies (301, 302), and a recent report by Hammouda *et al.* (303) claims that tecomine and tecostanine, 2 alkaloids from *Tecoma stans*, are potent hypoglycemic agents. Unfortunately, this report compares the activity of the 2 alkaloids after i.v. administration with tolbutamide administered orally. Similarly, vincamine, the major alkaloid of *Vinca minor*, has been reported to lower blood sugar when administered i.v. (304, 305). A number of common edible plants have been screened for hypoglycemic activity because of previous reports that each had been used for this effect (306, 307), and Mukerji (308) has reviewed the indigenous plants of India alleged to exert hypoglycemic effects. Other plants having potential oral hypoglycemic activity have been pointed out by Aliev and Rachimova (309).

The literature on plants claiming antidiabetic properties is voluminous. However, the validity of data interpretations in many such reports must be considered equivocal. Surely there is a need to evaluate plants which have been widely reported of value when used orally for the treatment of diabetes.

Although many compounds that show promise as hypoglycemic agents (leurosine, lochnerine, vindoline, vindolinine, catharanthine, tetrahydroalstonine, tecomine, tecostanine, vincamine, and others) have been isolated from plants, further studies could well prove them to be undesirable due to toxicity or side effects. In any event, these compounds represent new structures which could serve as models in the synthesis of active and safe hypoglycemic agents.

Cardiotonic Activity.—Hoch (310) has surveyed the literature up to 1961 concerning the

occurrence of cardiac glycosides and genins. His listings, which include only compounds that have been isolated in a pure state, indicate that these phytoconstituents are distributed in 39 genera of 14 plant familes. The importance of the cardiotonic glycosides in medicine has led to several extensive surveys of plants for their presence. Only a few surveys, however, have utilized biological testing exclusively to detect typical cardiotonic activity. Thorp and Watson (311), utilizing anesthetized guinea pigs, evaluated extracts from all of the available apocynaceous and asclepiadaceous plants of Australia. Evidence for the presence of cardiotonic activity was considered positive, following intravenous administration of a hydroalcoholic plant extract, when the heart slowed and contractions became more forceful. Typical cardiotonic activity was observed with extracts of *Carissa*, *Cerbera*, *Thevetia* (*Apocynaceae*), and *Gomphocarpus* (*Asclepiadaceae*) species. A total of 26 species was evaluated. More recently, Patel and Rowson (312) evaluated 33 species of Nigerian plants alleged to be useful as drugs. They utilized the isolated toad heart for detection of cardiotonic activity and found that species of *Allamanda*, *Callichilia*, *Hedranthera* (*Apocynaceae*); *Calotropis*, *Marsdenia*, *Pergularia* (*Asclepiadaceae*); *Vernonia* (*Compositae*); *Urginea* (*Liliaceae*); *Antiaris* (*Moraceae*); *Nauclea* (*Rubiaceae*); *Schwenkia* (*Solanaceae*), and *Mansonia* (*Sterculiaceae*) were active. However, subsequent chemical tests for the typical cardenolide nucleus were negative in all of the apocynaceous plants tested, as well as for the species of *Nauclea* (*Rubiaceae*) and *Schwenkia* (*Solanaceae*). Krider *et al.* (313) evaluated a number of plant extracts first chemically for the presence of typical cardenolides and then, for confirmation, tested all positive results by biological assay using the frog heart. A number of additional surveys of plants for cardiotonic activity have been reported; however, these involve only chemical tests which were designed to detect either the presence of 2-deoxy sugars or the unsaturated lactone, moieties considered characteristic for cardio-active substances. These surveys will be considered under *Phytochemical Screening Approaches*.

Androgenic, Estrogenic, and Related Activities.—The majority of literature reports involving a search for plant androgens, estrogens, and related materials have involved studies on specific folkloric remedies, those which have been alleged useful either as oral contraceptives, as ecbolics, or as emmenagogues. Undoubtedly, the majority of the emmenagogue and ecbolic plants derive their activity from an irritant mechanism such as that induced by the well-known plant purgatives and vesicant oils. Casey (314) has tabulated some 298 Indian plants used for these purposes in native medicine, and therefore the title "298 Alleged Anti-Fertility Plants of India" is misleading. Saha *et al.* (315) have also contributed a list of 277 Indian plants used for these purposes, many of which duplicate Casey's tabulations. The work by Saha and co-workers (315, 316) also included some experimental studies on a few of the most promising folkloric ecbolic plants found on their list. They evaluated extracts from these plants *in vitro* on excised guinea pig uteri and found several to have marked uterine stimulant activity (315, 316). A similar study, comprising 18 species of Indian ecbolic plants, showed 10 to exert varying degrees of stimulant activity on uterine tissue *in vitro* (317).

A search for effective oral contraceptives has been accelerated due to the alarm generated by the rapid increase in world population. Most studies have involved the synthesis of anovulatory compounds, several of which have been made available and apparently are widely used. Administration of antispermatogenic compounds to the male does not appear to be a popular approach to the problem of population control; however, there is some evidence that this may be effective as a means to prevent conception (318).

Jackson (319), in a review on antifertility substances, has pointed out a few alleged antifertility plants, as have Henshaw (320) and Jochle (321) in similar general reviews. DeLaszlo and Henshaw (322), on the other hand, have compiled a list of 60 antifertility plants used by primitive people.

Lithospermum ruderale, first reported by Train *et al.* (61) to be used by squaws of the Nevada Indian tribes as an oral contraceptive, has received considerable attention in laboratory studies. Several phytochemical investigations (323–325) have failed to yield the active antifertility compound generally acknowledged to be present in this plant (319, 325, 326). Gassner *et al.* (325), however, point out that certain extraction conditions used when processing this plant can alter its biological activity. Similar antifertility effects have been noted for *L. officinale* (327, 328), but attempts to isolate the active component(s) have not, as yet, been successful (327, 329). Other plants alleged to have antifertility effects in humans, and for which some

validating evidence in animals has been reported, are *Mallotus philippinensis* (330, 331), *Polygonum hydropiper* (332), *Psoralea corylifera* (333), *Capsella bursa pastoris* (334), *Sanguisorba officinalis* (335), *Withania somnifera* (336), *Punicum granatum* (331), and *Jatropha curcas* (337), as well as others (338).

Although no antifertility screening programs involving large numbers of plants have been reported in the literature, scattered laboratory evidence exists suggesting this area as a fertile one for development. The discovery of an orally effective, nonsynthetic antifertility agent, occurring free in nature could possibily be an acceptable answer to the question of population control.

More specifically, several examples of estrogenic and androgenic activity in plant extracts have been reported. Androgenic activity has been associated with cinchona bark (339) and with *Rhynchosia pyramidalis* (340), but these reports lack confirmation. In the latter instance, we have found no evidence of activity in *R. pyramidalis* using the standard androgen assay with castrate rats, following either oral or i.p. administration of extracts (341). No example of a steroid estrogen isolated from plants thus far has been reported, the majority of the estrogenic activities being attributed to isoflavones or related structures. These compounds, (isoflavones), probably because of their structural relationship to stilbestrol, are generally acknowledged to be weakly estrogenic, *i.e.*, genistin, genistein, biochanin A, prunetin, coumestrol (342), phloretin (343), and the plant stilbenes (342). This area has been reviewed extensively by Biggers (342), Bradbury and White (344), and by Cheng *et al.* (345).

Although estriol (346) and estrone (347) have been reported isolated in crystalline form from female willow catkins and palm kernel residues, respectively, and represent the only apparently clear cut examples for the isolation of steroid estrogens from plants, these reports have recently been challenged by Jacobsohn *et al.* (348). Jacobsohn and co-workers were unsuccessful in their attempts to duplicate the isolation of estrone from palm kernels, even though the methods used must be considered highly sensitive and usually effective for this type of work. There is little doubt that Butenandt and Jacobi (347) did, in fact, isolate authentic estrone (18 mg. from 50 Kg.) of palm kernel extracts. However, the extract from which the isolated estrone was derived had been previously prepared and supplied by the Schering-Kahlbaum, AG, Berlin (348), a fact that does not preclude the possibility of estrone contamination of the palm kernel residues during processing (348). Skarzynski's (346) isolation of estriol from willow catkins, on the other hand, was not so clearly established. He isolated 7.5 mg. of crystals from 65 Kg. of starting material and found them identical with estriol on the basis of comparision of microscopic appearance, solubility in several solvents, ultraviolet spectrum, melting point of acetyl derivatives (126°), and, in addition, the mixed melting point of the isolate and authentic estriol was depressed only 1°. However, the major difference was that the estrogenic activity of the isolate was only one-fourth that of estriol (346–348). From these data, and on the basis of subsequent negative efforts by Jacobsohn *et al.* (347), it appears that the presence of estrone and estriol in higher plants, as well as steroid estrogens in general, remains to be demonstrated unequivocally.

Conversely, a potent estrogenic activity from *Butea superba*, assaying at about 900,000 mouse units/Kg. has been reported (349). Physical data presented for the compound responsible for this activity are insufficient for its characterization; however, from all available data, it appears to lack the qualifications of a steroid (349). Much of the controversy that has developed over the relative estrogenic potency of plant extracts resides, no doubt, in the selection of an appropriate biological assay. Biggers (342) has critically reviewed methods that have been applied to the detection of estrogenic activity in plant extracts, while Cheng *et al.* (345) have specifically surveyed the literature on the estrogenic activity of naturally occurring isoflavones. Also, a broad review on plant estrogens, encompassing all areas of the subject, has been prepared by Bradbury and White (344). In the latter publication, a list of 55 species of estrogenic plants, representing 49 genera and 28 families, has been tabulated.

Further exploitation of the plant kingdom for this interesting and potentially useful group of phytoconstituents, through a broad biological screening program, could very well be rewarding.

General Pharmacological Screening.—Malone and Robichaud have remarked on general screening as follows:

"The basic premise of pharmacologic screening is not to allow true biologic activity to go undetected even though the activity may be new, unexpected and unique. The initial screening procedure must unequivocally establish this activity as well as its probable nature in order to indicate the course of further, more specific pharmacologic evaluation." (98).

These workers have proposed a "Hippocratic Screen," utilizing normal unanesthetized rats, for the detection of biological activities in crude plant extracts. Using a modification of this procedure, which we refer to as the "Mouse Behavior Screen," extracts from 200 native plants have been evaluated for biological activity with the results being categorized as follows: (*a*) CNS depressant (weak, toxic, etc.), (*b*) CNS stimulant (weak, toxic, etc.), (*c*) autonomic (weak, toxic, etc.), (*d*) mixed CNS stimulant and CNS depressant, (*e*) other types of activity (119). It is our intent to continue this type of evaluation and attempt to correlate characteristic biological effects of plants with their respective taxonomic position and thus develop an area of so-called "Biotaxonomy."

Similar evaluations of plant extracts have been made on 163 West Indian medicinal plants (95–97). In addition, these workers have included *in vitro* or *in vivo* studies of 55 extracts (95) on guinea pig ileum, rat uterus, rabbit duodenum, isolated rabbit heart, and also on dog respiration and blood pressure, the rat stomach fundus (5-HT activity), and the rat diaphragm phrenic nerve preparation. The remaining 108 plants were evaluated in a similar manner, although not so completely (96, 97).

Train *et al.* (61), using plants alleged to be useful in Nevada Indian folk medicine, evaluated some 100 species of plants for their effect on rabbit ileum and for effects on dog blood pressure and respiration.

If an investigator in search of biologically active plant materials has no prior ethnobotanic or biosystematic knowledge of his investigational material, studies such as these are invaluable to supply a justification for continued and detailed phytochemical analysis.

Miscellaneous Phytopharmacological Surveys.—In addition to the more extensive screening programs described above, a number of additional efforts have been carried out, usually on fewer specimens of plants, involving the search for biologically active compounds. Borchers and Ackerson (350), in a search for trypsin inhibitors, examined the seeds of 38 species of plants. Similar substances which inhibited the growth of rats and chicks had been reported in legume seeds (350). The inhibitor was found in all *Leguminosae* seed samples analyzed, but it was absent from 8 seed samples representing other plant families.

In a search for new human plasma cholinesterase inhibitors, Orgell has studied extracts from a large number of plant species (351–353), in addition to several alkaloids (354) and other purified natural substances (354). Inhibitors have been found in extracts from plants in 11 families, and among the inhibitory compounds are riboflavin, bishydroxycoumarin, aloin, naringenin, and some 26 different alkaloids, the majority of which are indoles (354). Orgell has suggested that cholinesterase inhibition techniques may be useful for the detection of certain alkaloids and glycosides in plant extracts or crude drugs (354).

Srivastava *et al.* (355) screened 11 species of Indian plants for fibrinolytic and anticoagulant activity using rather simple *in vitro* methods. Recently, a simple test tube arrangement designed for the rapid evaluation of compounds for fibrinolytic activity has been described that could be adapted for use in the rapid screening of large numbers of plant samples (356).

Several extensive surveys of plant material have been reported in the literature with the intent to discover new hemagglutinins (phytohemagglutinins) that might serve as useful blood-typing reagents (357–370). The most recent of these surveys included the evaluation of seed extracts from 311 species of plants, representing 42 families, against 24 different hemagglutinating antigens (368). Specific positive reactions were given by 17 species in 12 families, exclusive of the *Leguminosae*, whereas 32 of 45 *Leguminosae* species gave similar specific results. A number of additional plants from both groups gave nonspecific hemagglutination reactions. Schertz and co-workers have drawn attention to the need for a continuation of similar screening programs and for the subsequent investigation of the more promising plants which give specific reactions (368).

Although remarks in this section of the review have been directed principally to problems and approaches involved in the detection of biologically active substances in higher plants, other fertile natural product areas remain essentially untapped. The biological activities of algae (371, 372), plankton (371), and marine biotoxins (373, 374) point to this fact, and the prospect of discovering new antibiotic producing organisms in the sea should stimulate the fins and gills in many a natural product investigator.

PHYTOCHEMICAL SCREENING APPROACHES

Ultimately, the goal in surveying plants for biologically active or medicinally useful compounds should be to isolate the one or more constituents responsible for a particular activity.

Hence, with the selection of a specific plant for phytochemical investigation, either on the basis of one or more approaches set forth under *Phytopharmacologic Approaches*, or through some other avenue, phytochemical screening techniques can be a valuable aid.

Certain investigators feel that an initial selection of investigational plants should be made, not on evidence that extracts elicit a particular and interesting biological activity, but rather on the basis that certain chemicals are present in the plant, relatives of which can usually be associated with biological activity. Thus, some investigators will select initially only alkaloid-containing plants for study on the premises that (*a*) alkaloids normally exert some type of pharmacologic activity, usually on the central nervous system, but not always so; (*b*) the greatest majority of natural products used in medicine today are alkaloidal in nature; (*c*) tests for the presence of these compounds in plants are simple, can be conducted rapidly, and are reasonably reliable, and (*d*) because of their chemical nature, alkaloids are more easily manipulated making extraction and isolation less of a problem. In addition, economics, as well as other factors associated with biological testing, often force the investigator to pursue a phytochemical approach. However, should a phytochemical group other than alkaloids be selected for investigation, say the flavonols, the diversity of expected biological activities can be enormous. Willaman has surveyed the literature and has found that at least 137 natural flavonoids are known, occurring in some 62 families, 153 genera, and 277 species of plants (415). Also, some 33 different pharmacologic or biological activities have been reported for one or more of 30 flavonoids (415). More recently, Horhammer and Wagner have reviewed the same area, and these numbers are therefore to be increased (416, 417). Also, Orzechowski has considered the role of flavonoids as therapeutic agents (418). Along similar lines, the coumarins have been reported to exert some 31 different biological effects, and according to Soine, their full range of pharmacologic activities is not appreciated by most investigators (419). Other examples pointing out the complexity of expected biological effects for any one category of phytoconstituents could, of course, be made. In any event, publications representing the phytochemical screening approach far outweigh those following phytopharmacologic avenues, not only in numbers of reports, but in representation of total plants examined.

Since the number of chemical categories of plant constituents is great, and each is capable of eliciting biological activity, no attempt will be made in this review to be all inclusive. This section of the review will be restricted to some general considerations of phytochemical screening methodology, followed by discussions of those categories of phytoconstituents which have been represented in major published surveys of screening programs. These will include: alkaloids, glycosides as a general class (heterosides), saponins (steroid and triterpenoid), sterols, cardiac glycosides, cyanogenetic glycosides, *iso*thiocyanate glycosides, anthraquinones, flavonoids and related compounds, tannins, and coumarins and related compounds. Surveys which have been conducted for each of these categories will be discussed along with the general methodology involved. The examples to be cited are intended to be representative of each class and are not meant to include all available published data.

General Considerations

A method for use in phytochemical screening should be (*a*) simple, (*b*) rapid, (*c*) designed for a minimum of equipment, (*d*) reasonably selective for the class of compounds under study, (*e*) quantitative in so far as having a knowledge of the lower limit of detection is concerned, and if possible, (*f*) should give additional information as to the presence or absence of specific members of the group being evaluated. Most published procedures adhere to criteria (*a*) through (*d*), but few are designed to provide the information included in (*e*) and (*f*). In fact, certain procedures cannot be duplicated because of insufficient details included in some reports. For example, Arthur and Cheung (420), in a phytochemical survey of Hong Kong plants, screened 332 species for alkaloids. They equated the precipitates observed following the addition of standard alkaloid precipitating reagents to results obtained by adding the same reagents to standard solutions of 1:100, 1:500, 1:2500, and 1:10,000 quinine sulfate. It is implied that water was the solvent. However, the solubility of quinine sulfate is stated to be 1 Gm. in 810 ml. of water (421). Along similar lines, Wall *et al.* (422) have used the cyanidin test for the detection of the γ-benzopyrone nucleus as indicative of the presence of flavonoids. They compare a test result color with a similar color produced by a 0.1% solution of rutin and equate it as a (+) reaction. Their extraction solvent is 95% ethanol (but fresh plant material was often extracted which would decrease this percentage considerably), and rutin is stated to be only slightly soluble in ethanol and soluble about 1

Gm. in 8 L. of water (421). We find that the maximum solubility of rutin at room temperature is about 0.02% for both 80 and 95% ethanol.

Webb, using a field method, estimated alkaloid precipitates with reagents on a + to ++++ basis but used no reference for comparison (423, 424). He also states, ". . . On the other hand, while the method may yield a percentage of 'false positives,' it has never failed to detect species with alkaloids" (424). If the initial field test did indeed fail to detect alkaloids, perhaps because of a low concentration in the plant, how could it be determined that the test was infallible when only field test positive species were collected for more specific laboratory examination?

One of the most important and fundamental considerations in designing a phytochemical screening procedure is the selection of a proper extraction solvent. It is often difficult to follow general or expected solubility rules for a given class of phytoconstituents since there are often substances of unknown character present in crude plant extracts that affect solubility. For example, Woo (425) has reported the effect of saponin in plant extracts on the solubility of certain normally insoluble compounds using selected solvents. Apparently saponin acts as a wetting agent to enhance the formation of micelles; thus, an increase in solubility of certain constituents is effected. This phenomenon has been noted through the use of synthetic detergents to enhance the solubility, and thus extractability, of alkaloids from Cinchona (426). Since saponins, or other similar surface-active agents, do not occur universally in plants, prediction of general solubilities for a class of phytoconstituents precipitates a major problem. In our laboratory *n*-hexane-soluble extractives from *Catharanthus lanceus* were found to be rich in alkaloids. Subsequent isolation of individual akaloids from the crude mixture proved them to be totally insoluble in *n*-hexane. Presumably the alkaloids occur in the plant, at least in this instance, dissolved in some lipid material, the latter being soluble in *n*-hexane.

No solution is offered for these problems involving solubility except to say that extract residues should always be examined with a variety of solvents to determine whether abnormal solubility phenomena have occurred.

Even though a great many problems are presented by the diverse methodology utilized by investigators in phytochemical screening, much useful information can be derived from published studies. Positive test results are usually clear cut and only the possibility of false-positive results need be further explored. Negative results, on the other hand, must be carefully weighed in terms of being due to a real absence of the test material in the sample being evaluated, or to the methodology employed.

Alkaloid Screening

Prior to a consideration of screening plant material for alkaloids, it would seem in order to define the term "alkaloid" as used in this review; however, the nature of the word itself precludes anything more than a vague definition. Anyone familiar with alkaloids surely has a knowledge of their character, but seldom can one give an acceptable definition. Most authorities agree that chemical, botanical, and pharmacologic implications must be reflected in an acceptable definition. Hegnauer's (427) suggestion that:

> "Alkaloids are more or less toxic substances which act primarily on the central nervous system, have a basic character, contain heterocyclic nitrogen, and are synthesized in plants from amino acids or their immediate derivatives. In most cases they are of limited distribution in the plant kingdom."

seems as acceptable as any. For purposes of this discussion we will utilize Hegnauer's concept except, of course, we cannot be concerned with the site or mechanism of synthesis. Thus, compounds such as aliphatic nitrogenous bases (ephedrine), amides (colchicine), and the amino acids (thiamine) themselves will not be considered as alkaloids.

Estimates for the distribution of alkaloids in vascular plants have been placed as high as 15–20% (427), although this figure appears somewhat high with respect to data derived from several extensive phytochemical screening programs. Wall *et al.* (423, 428–435) have screened more than 4000 species of plants and report a distribution of about 10% alkaloids. Webb (424) in his experience with some 1700 species indicates alkaloid occurrence to be about 14%, whereas the Smith Kline & French survey found that about 10% of 25,000 species screened were positive for alkaloids (436). Since a few of these undoubtedly will be determined through future studies to be false-positive alkaloid containing species, 9–10% seems to be the more logical estimate representing alkaloid-yielding plant species.

Alkaloids are widely distributed in the plant kingdom, although certain groups have been shown to be characteristically devoid of them. Excellent essays on this subject have been published by Willaman and Schubert (93, 94) and by Webb (424). The handbook of alkaloid-bearing plants by Willaman and Schubert is also

useful to establish this relationship among plant taxa (437).

Since alkaloids usually occur in plants as their water-soluble salts, some workers believe that extraction with acidulated water can result in a crude extract which can be tested directly with one or more standard alkaloid precipitating reagents. Other workers feel that the presence in such an extract of materials that are capable of giving false-positive alkaloid tests necessitate a purification procedure before valid results can be obtained. This is usually accomplished by the addition of base and subsequent extraction with a water-immiscible organic solvent. The organic extract can then be tested by application to filter paper, drying, and dipping or spraying with an alkaloid detecting reagent that gives a chromogenic response with alkaloids. If the latter method is not preferred, the organic solution can be re-extracted with dilute acid and the usual alkaloid precipitating reagents added to separate portions of this acid extract.

Another method of removing impurities that are capable of giving false-positive tests (*i.e.*, proteins) from an initial aqueous acidic extract is to "salt out" these materials by the addition of powdered sodium chloride. An additional procedure for alkaloid detection could be based on the addition of alkali directly to the powdered plant sample, followed by extraction with an appropriate organic solvent. This extract could then be purified by partition as described above, or be tested directly.

With respect to these general methods, certain anomalies have been reported in the literature which should be pointed out. There is no implication that these examples are frequently encountered in alkaloid screening; however, one should be aware that they do exist. Certain plants (*i.e.*, *Saussurea lappa*) are known to contain labile nonbasic constituents and may yield nitrogenous materials (pseudoalkaloids) on extraction with ammoniacal solvents (438), while others contain alkaloids that are susceptible to modification by acidic reagents (438). That proteins, which may be present in aqueous or acidic aqueous plant extracts, can precipitate on the addition of heavy metal alkaloid precipitating reagents and thus yield false-positive tests, is well established (438–444). Such proteins can be removed by treatment of the extract with sodium chloride prior to the use of the heavy metal reagent, a procedure which usually salts out the protein (438). However, alkaloids such as alstonine may be quantitatively precipitated as hydrochloride under these conditions (438). In the treatment of a crude plant extract to remove impurities by the acid–base–organic solvent –acid procedure, it is quite possible that plants containing water-soluble alkaloid bases will go undetected. Quaternary bases, amine oxides, betaines, and choline would fall into this category (438).

Variability of results in alkaloid testing of plant material can be induced by a number of factors such as age, climate, habitat, plant part tested, season, time of harvest, chemical races of plants, sensitivity of alkaloid type to reagents, etc. A few examples regarding these factors should serve to point out their importance. *Geijera salicifolia* was found by Webb to give consistently better alkaloid tests as the broad leaf form, than the narrow leaf form, even when the 2 were growing side by side in the field (424). In certain groups of plants (*i.e.*, *Compositae*), alkaloids often are found only in or near the flower tops (438), and in the *Apocynaceae*, alkaloids generally tend to concentrate in the root or bark, often to the exclusion of other parts of the plant (438); thus, the proper selection of plant parts for testing is quite important. To obtain equivalent results, quantitation of precipitates obtained with alkaloid reagents is not always possible, especially when comparing different genera or families. This is exemplified through knowledge that *Galbulimima baccata* (*Himantandraceae*) was found to be rated a ++++ in field tests and subsequent analysis resulted in a yield of 0.01–0.05% of 4 alkaloids. A ++++ rating for *Daphnandra aromatica* (*Monimiaceae*) was determined in the field and subsequent analysis in the laboratory yielded 6+% of crude alkaloids (424). *Antireha putaminosa* (*Rubiaceae*) loses 50% of its alkaloid after 2 months' storage, and high alkaloid decomposition rates have also been noted for *A. tennuifolia*, *Randia racemosa*, and *Gardenia vilhelmii*, 3 additional rubiaceous plants (424). Silica gel drying of *Antirhea tennuifolia* for 1 month resulted in material that gave a ++++ alkaloid test, whereas this same plant dried in the shade for 1 month gave a negative alkaloid test (424). *Acronychia baueri*, on the other hand, gave strong alkaloid positive tests when 124-year-old herbarium specimens were evaluated (424, 445). Along similar lines, Raffauf and Morris have reported that a plant sample identified as *Nicotiana attenuata* (*Solanaceae*), and estimated to be some 1300 years old, gave positive alkaloid tests (446). *Duboisia myoporoides* yielded 3% of hyoscyamine when harvested in October, but when harvested in April of the same year, 3% hyoscine was isolated (424). Examples of alkaloid decomposition as a result of milling

TABLE III.—SOME USEFUL ALKALOID PRECIPITATING REAGENTS

Name	Comp.	Ref.
Bouchardat	Iodine–potassium iodide	(493, 504)
Dragendorff	Bismuth potassium iodide	(447, 496, 504, 632)
Ecolle	Silicotungstic acid	(447, 732)
Gold chloride	Chlorauric acid	(493)
Hager	Picric acid	(448)
Kraut	Iodine–zinc chloriodide	(447)
Marme	Cadmium potassium iodide	(447)
Mayer	Potassium mercuric iodide	(447, 448, 452, 453)
Platinum chloride	Chloroplatinic acid	(448, 493, 781)
Scheibler	Phosphotungstic acid	(448)
Sonnenschein	Ammonium phosphomolybdate	(496)
Valser	Potassium mercuric iodide	(452)
Wagner	Iodine–potassium iodide	(496)
	Bismuth antimony iodide	(782)
	Bromauric acid	(783)
	Bromoplatinic acid	(497)
	Bromothallic acid	(784)
	Picrolonic acid	(785)
	Sodium tetraphenylboron	(786)
	Trinitroresorcinol	(787)

dried plant material have also been cited (424). These examples should suffice to point out just a few of the problems encountered by the natural product investigator who is interested in the detection and isolation of biologically active alkaloids.

Alkaloid Detecting Reagents

For detecting alkaloids in phytochemical screening, two types of reagents are available, *i.e.*, alkaloidal precipitants and spray or dip reagents. Table III lists 20 precipitating reagents commonly used for the detection of alkaloids, whereas Table IV presents 15 reagents that were used in 45 recent phytochemical surveys for alkaloids. At least 2 reagents were used in 38 of the surveys, while 7 surveys depended solely on 1 reagent to establish the presence of alkaloids. Because of the variable sensitivities of these reagents and because of their nonspecificity for alkaloids, many investigators utilize 4 or 5 reagents in their screening of plant extracts, and only samples yielding precipitates with all reagents are considered to contain alkaloids. Fulton (447) has tabulated some 200 of these reagents and presents a great deal of information concerning their specificity and sensitivity. A series of papers by Munch *et al.* (448–451) is concerned with the effect of 17 different alkaloid detecting reagents on several classes of nitrogenous bases. Travell (452) has studied the sensitivity of Mayer's and Valser's reagents, both solutions of potassium mercuric iodide, with the former prepared from mercuric chloride and potassium iodide and the latter from mercuric iodide and potassium iodide.

TABLE IV.—ALKALOID DETECTING REAGENTS EMPLOYED IN SCREENING PROGRAMS

Reagent	Surveys Used in,[a] No.
Mayer's reagent	39
Silicotungstic acid reagent	23
Dragendorff's drop reagent	19
Wagner's reagent	11
Dragendorff's spray reagent	10
Sonnenschein's reagent	9
Hager's reagent	7
Bouchardat's reagent	3
Phosphotungstic acid	2
Valser's reagent	1
Chloroplatinic acid reagent	1
Chlorauric acid reagent	1
Sodium tetraphenylboron reagent	1
Ammonium reineckate reagent	1
Tannic acid reagent	1

[a] Two or more reagents were used in 38 randomly selected surveys; 1 reagent only was employed in 7 surveys.

The reagent used by most investigators for phytochemical screening is essentially the same formula that Mayer originally introduced in 1862. Several investigators have demonstrated, however, that the original formula is perhaps the least sensitive for alkaloid detection, in comparison with many proposed modifications (452–454), and Travell (452) has conclusively demonstrated the superiority of Valser's over Mayer's reagent. In our laboratories we have compared the sensitivity of several common alkaloid precipitating reagents using 40 different alkaloids and representing several different chemical types (454). The reagents tested were Mayer's (3 formulas), Valser's, Wagner's (2 formulas), Bouchardat's, Hager's, Scheibler's, silicotungstic acid, Dragendorff's, Marme's, gold chloride, and Sonnenschein's. It was demonstrated in this study that the various reagents exhibit wide differences in sensitivity for structurally dissimilar

alkaloids. None of the reagents would detect ephedrine at a concentration of 0.1%, but Wagner's, Bouchardat's, Dragendorff's, and Scheibler's each detected all of the other alkaloids at concentrations ranging from 0.001 to 0.1%. Hager's, Marme's, and gold chloride reagents were by far the least effective detecting reagents, failing to react with 13, 12, and 10, respectively, of the 40 test alkaloids. All 3 of the Mayer's formulations were inferior to Valser's reagent with respect to sensitivity and specificity of alkaloid detection (454). It should be pointed out that the majority of these precipitating reagents must be used to detect alkaloids only in acid solution, and furthermore, that a large number of naturally occurring non-nitrogenous plant principles will react to give false-positive tests. These will be discussed subsequently.

Investigators who prefer to use spot tests, or those who prefer to chromatograph concentrated plant extracts for the detection of alkaloids, have a variety of available reagents. The most widely utilized, however, are modifications of the original Dragendorff drop test reagent, which produce orange to red colors with most alkaloids. Although a number of modified formulas have been proposed, each reported to have advantages over the others, the 2 most frequently utilized in phytochemical studies are the 1951 Munier and Macheboeuf (455) and the Thies and Reuther (456) modifications. A literature search has revealed the availability of at least 15 modifications of the Dragendorff spray reagents (457–471). We were prompted to study the stability and sensitivity of one of these modified reagents since a number of published reports had commented on the need for their storage under refrigeration with concomitant protection from light. It was determined that prepared concentrates of the 1951 Munier-Macheboeuf Dragendorff's reagent required a storage period of about 1 week prior to its use in the preparation of the diluted reagent. Also, the diluted spray reagent should be stored for a minimum of 1 week prior to its use for alkaloid detection in order to obtain maximum sensitivity. The reagent maintained its stability and sensitivity for at least 6 months and no special storage conditions were found necessary (472).

Some alkaloid detecting reagents are available which on reaction with certain groups of alkaloids, or with specific functional groups, produce characteristic chromogenic responses. These can be of considerable value in screening work, but only after alkaloids have been determined in the sample being evaluated. A selected list of general as well as specific chromogenic reagents has been prepared and is presented in Table V.

False-Positive Alkaloid Reactions.—Mechanisms for the reaction between alkaloids and detecting reagents are dependent chiefly on the chemical character of the reagent. Fulton (447) classifies alkaloidal precipitants as (*a*) those which react with basic compounds (alkaloids) to form insoluble salts; examples are silicotungstic, phosphomolybdic, and phosphotungstic acids. (*b*) Those which react with alkaloids as loose complexes to form precipitates; examples are Wagner's and Bouchardat's reagents (iodine-potassium iodide). (*c*) Those which react to form insoluble addition products through the alkaloid nitrogen; examples are the complex heavy metal salt reagents, Mayer's, Valser's, Marme's, and Dragendorff's. And (*d*) those which react through the attraction of organic acids with basic alkaloids to form insoluble salts. An example of such a reagent would be Hager's (picric acid). Obviously, these are rather nonspecific reactions and a number of nonalkaloidal plant constituents should be expected to precipitate also from solution on the addition of these reagents to crude plant extracts. These false-positive reactions are most liable to occur when testing an extract that has not been treated by at least one acid-base–organic solvent purification.

The most frequent false-positive reactions have been attributed to the presence of proteins which precipitate on the addition of heavy metal containing reagents (423, 438–444). Included in this category are "albuminous substances" (452), peptones (466), and ptomaines (441, 447). At least one textbook has indicated that amino acids will also precipitate with the general alkaloid reagents (444). However, a study by Winek and Fitzgerald (473) appears to disprove this allegation. Among other substances reported in the literature as the cause of false-positive alkaloid reactions are certain glycosides (444, 474), and carbohydrates (474), betaine (423, 439, 474), choline (474), purines (439), methylated amines (439), tannins (423), and ammonium salts (439, 466). Recently, we were able to show that previous positive alkaloid tests reported for extracts of *Piper methysticum* were due to the α-pyrones: kawain, dihydrokawain, methysticin, dihydromethysticin, and yangonin (475). This prompted an investigation of the mechanism by which nonalkaloidal compounds are able to elicit a positive reaction with an alkaloid detecting reagent, in this case the modified Dragendorff reagent. It was de-

TABLE V.—GENERAL AND SPECIFIC ALKALOID REAGENTS FOR CHROMATOGRAPHY

Alkaloid Type	Reagent	Ref.
Simple amines	1-Fluoro-2,4-dinitrobenzene	(461, 788)
	Ninhydrin	(789, 790)
Free and phenolic-bound OH groups	Potassium ferricyanide–$FeCl_3$	(461)
Aryl amines	Chromosulfuric acid	(461)
	Glucose–H_3PO_4	(461)
Secondary aliphatic and alicyclic amines	Sodium nitroprusside	(461)
Methylene oxide group	Chromotropic acid	(791)
Piperidine–pyrrolidine	Isatin–acetic acid	(792, 793)
Pyridine	Konig's reagent	(461)
Purines	Bromine vapor	(794)
Morphine	Ehrlich's reagent	(795)
Steroid glycoalkaloids	Vanillin–H_3PO_4	(796)
Choline and related compounds	Chargraff's reagent	(461)
	Hydroxamic acid–$FeCl_3$	(461)
	Dragendorff's, modified	(797, 798)
	Dipicrylamine	(798)
Indoles	Aminopyramidine	(799)
	Ceric ammonium sulfate–H_3PO_4	(800–803)
	Ceric sulfate–H_2SO_4	(804–806)
	Cinnamic aldehyde–HCl	(461)
	Perchloric acid–$FeCl_3$	(461)
	2,6-Dichloroquinonechlorimide	(461)
	Van Urk's reagent	(807)
	Prochaska's reagent	(807)
	Keller's reagent	(808, 809)
Indoles, β-substituted	Van Urk's reagent	(808–810)
Indoles, 5,6-dihydroxy	Ehrlich's reagent	(811)
	Hopkin's-Cole reagent	(811)
	Salkowski reagent	(811)
	Gibb's reagent	(811)
	Folin and Ciocalteu's reagent	(811)
	p-Dimethylaminocinnamaldehyde	(811)
	Acidic oxidizing reagent	(811)
	Terephthalaldehyde	(811)
	Nitrose reagent	(811)
	Formaldehyde–HCl reagent	(811)
	Xanthydrol reagent	(811)
	Diazotized *p*-nitroaniline	(811)
	Sodium molybdate–HCl reagent	(811)
	$FeCl_3$–potassium ferricyanide	(811)
	Ferric chloride solution	(811)
	Ammoniacal silver nitrate	(811)
Indoles, ergot	Ehrlich's modified reagent	(461, 808, 812)
	Ehrlich's reagent	(808–810)
	Van Urk's reagent	(808–810)
	Alport-Cocking's reagent	(808)
	Glyoxalic acid	(808)
Indoles, yohimbine type	Modified Keller reaction	(813)
Miscellaneous (special applications)	Sodium tellurite–H_2SO_4	(814)
	Ceric sulfate–trichloracetic acid–sulfuric acid	(461)
	Nessler's reagent	(461)
	Sulfuric acid–methanol (fluorescence)	(461)
	Nitric acid–methanol (fluorescence)	(461)
	Levine-Chargraff reagent	(815)
	Sulfuric acid	(816)
	Dichromate–H_2SO_4	(817)
	Ninhydrin reagent	(818)
	Xanthydrol reagent	(784, 818)
	Hydroxylamine	(819)
	Hydroxylamine–$FeCl_3$	(820)
	Sodium nitroprusside	(821, 822)
General alkaloid reagents	Mayer's	(823)
	Potassium iodoplatinate	(461, 824–832)
	Iodine	(461)
	Dragendorff's reagent, modified	(457–471)

termined that any non-nitrogenous organic compound having conjugated carbonyl (ketone or aldehyde) or lactone functions would react in a manner typical of alkaloids (476). These minimum qualifications are quite prevalent in natural products and undoubtedly many false-positive alkaloid reactions are promulgated through this mechanism. Fortunately, the majority of com-

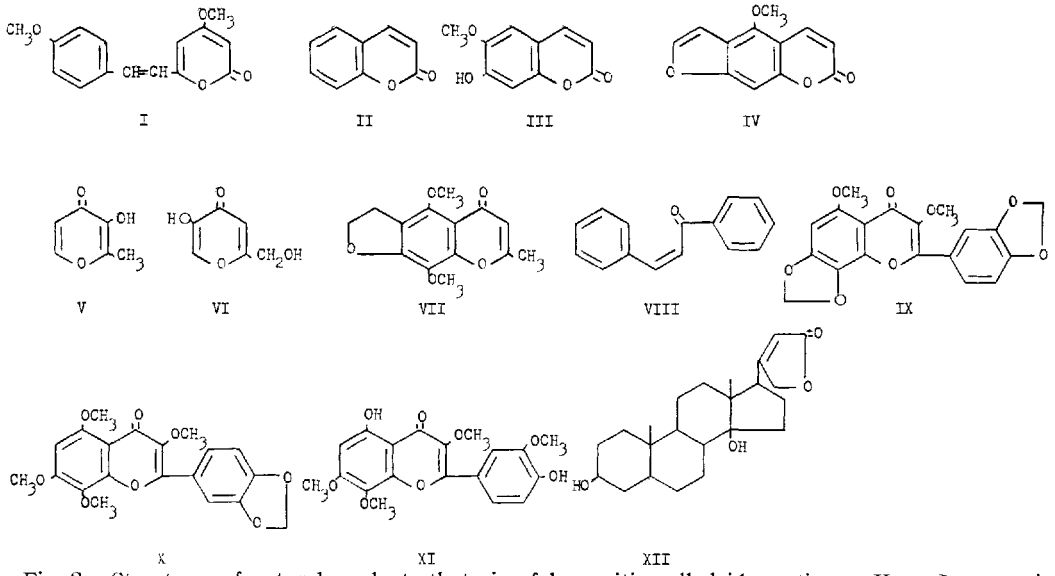
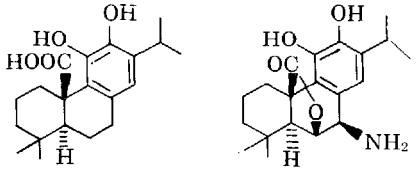


Fig. 2.—Structures of natural products that give false-positive alkaloid reactions. Key: I, yangonin (476); II, coumarin (476); III, scopoletin (476); IV, bergapten (476); V, maltol (476); VI, kojic acid (476); VII, khellin (476); VIII, chalcone (476); IX, meliternatin (477); X, meliternin (477); XI, ternatin (477); XII, digitoxigenin (476).

pounds with these functionalities can be separated from the alkaloids by treatment of the extract with base, followed by extraction with organic solvent which, in turn, is extracted with dilute aqueous acid. Another interesting report is that by Briggs and Locker (477), who in 1949 isolated 3 compounds from *Melicope ternata* which gave precipitates with the usual alkaloid reagents and crystalline salts with acids. However, these compounds contained no nitrogen and proved to be the completely alkylated hydroxyflavones, meliternatin, meliternin, and ternatin (Fig. 2). It would appear that, in the light of our work, the false-positive test for alkaloids was due to the presence of the conjugated carbonyl in each molecule rather than, as stated by the authors, the fact that each molecule was completely alkylated. Presumably other flavones would react similarly, as would most of the cardenolides and bufadienolides.

Householder and Camp (850) have recently pointed out that treatment of plant extracts with ammonium hydroxide and acetone can give rise to artifacts which give positive reactions with the standard qualitative alkaloid test reagents. These investigators were unable to identify the condensation products formed in this reaction but presented evidence to show that the rate of formation was affected by exposure to light and the atmosphere.

A recent report by Russian workers (851, 852) presented evidence for the isolation of an alkaloid named rosmaricine from *Rosmarinus officinalis* (*Labiatae*). This anomaly of an alkaloid from a member of the mint family prompted Wenkert and co-workers to investigate the validity of the Russian work (853). They found that rosmaricine was indeed not present in the plant prior to the addition of ammonia (used by the Russian workers in their isolation experiments), and that this "alkaloid" was undoubtedly formed as a result of the action of the base on the precursor carnosic acid (853).



Carnosic acid Rosmaricine

Other anomalous alkaloid reactions have been mentioned in the literature but as yet they cannot be explained. For example, *Samolus repens* (*Primulaceae*) extracts give a black color and precipitate with Dragendorff's reagent (478). We have observed this phenomenon frequently in the field testing of fresh plant material and have assumed the reaction to be one of free iodine in the reagent combining with starch to give a typical blue-black color. Extracts from *Plagianthus divaricatus* (*Malvaceae*) have been reported to give a pink color, but no precipitate, with Dragendorff's reagent (478).

Webb has pointed out that about 9% of species tested in the field for alkaloids were found to be false-positive reactions following subsequent detailed laboratory analysis (424). On the basis of experience resulting from tests on some 25,000 plant species, Douglas has estimated that not more than 5% of initial positive alkaloid tests have been found to be due to nonalkaloid entities (436).

False-Negative Alkaloid Reactions.—If one considers the nonheterocyclic nitrogen bases (protoalkaloids) as alkaloids, it will be noted that the greatest majority of these fail to react with the usual alkaloid precipitating reagents. Certain examples of this can be documented (454, 466). Also, unless certain precautions are observed in the test procedure, quaternary alkaloids and amine oxides (nupharidine, dilupine, trilupine) will not be detected (466). That is, if an acidic plant extract is treated with base and extracted with an immiscible organic solvent, both the aqueous basic layer and the organic layer must be tested for alkaloids. In most alkaloid screening procedures that have been reported, the basic layer has been neglected. Arguments for this omission have been based on the assumption that quaternary alkaloids or amine oxides would not be expected to occur in plants to the exclusion of tertiary bases which would be detected by this procedure. Raffauf points out that this may be an erroneous assumption (466).

Alkaloid Testing of Herbarium Specimens.—The validity of alkaloid tests on plant material derived from herbarium sheets is open to question. Often, for many and varied reasons, it is difficult to find certain plants in their native habitat at the time of collection of indigenous flora, and this alternative to collection has been used by several investigators (423, 424, 478–482) to survey a broad distribution of plant taxa. An obvious disadvantage in the use of such material is that usually only leaves and branches are available and instances are known wherein plants contain alkaloids in other organs, but their leaves and stems are relatively alkaloid-free (424). Also, herbarium material is often quite old, and a number of examples can be cited correlating alkaloid decomposition as a function of time (424). On the other hand, plants 1300 years old have been reported still to give alkaloid positive tests (445). It is common practice in some herbaria to treat specimens with formalin or mercuric chloride as preservatives. Formalin could very well decompose many alkaloids, and mercuric chloride reacts with certain alkaloidal precipitants to form abnormal precipitates. With Mayer's reagent this is evidenced by a bright orange precipitate with yellow streaks which eventually become red, and with Bouchardat's reagent a pale purplish brown precipitate is observed (424). In some herbaria, it is common to mark specimens in a manner that treatment of this type can be easily ascertained, while in others this practice is not carried out. Even with these problems, extensive herbarium specimen testing for alkaloids by Webb has allowed him to acknowledge this procedure as a valuable adjunct to the testing of fresh material (424). Cain *et al.* (479) have indicated that equivalent results were obtained in their studies with fresh plant material and dried herbarium specimens.

Field Tests for Alkaloids in Plants.—Investigators searching for new alkaloid bearing species in remote or distant areas of the world often find it difficult to return to make additional bulk plant collections for laboratory study. Therefore, simple field tests for alkaloids have been developed which are sufficiently reliable to distinguish alkaloid-containing plants, *i.e.*, those containing at least 0.01% of alkaloids, thus enabling bulk collections of these species initially and eliminating the need for a return expedition. These field tests can be classified in the following manner.

Organoleptic Evaluation.—It has been suggested by Webb that at least some of his collections of species for laboratory examination were made on the basis of taste, in conjunction with some knowledge of the botanical characteristics of the samples being evaluated (483). That is, he avoided tasting plants in such families as the *Anacardiaceae*, *Euphorbiaceae*, etc., but bitterness in a group such as the *Lauraceae*, particularly if a *Cryptocarya*, would suggest alkaloids. In Webb's opinion, differentiation of bitter alkaloids and saponins can be made on the basis of taste, but only after considerable experience (483). Also, bitterness in the inner bark of such groups as *Evodia*, *Acronychia*, and *Melicope* (*Rutaceae*), in conjunction with an observation of yellow pigmentation, is suggestive of the presence of alkaloids (acridones). While judgments such as these may be justified by an investigator who has had considerable experience, and whose botanical and chemical background are complementary, the average alkaloid hunter could hardly justify such an approach.

Spot Tests Using Alkaloid Test Paper.—Kraft (484) has developed a simple device for alkaloid detection in fresh plant material, a process which consists of impregnating filter paper with Dragendorff's reagent, followed by

TABLE VI.—PHYTOCHEMICAL SURVEYS FOR ALKALOIDS

Area	Author(s)	Yr.	Species Tested, No.	Ref.
Argentina	Barnes and Gilbert	1960	71	(492)
	Codoni	1947	17	(493)
Australia	Webb	1949	753	(423)
	Webb	1952	1040	(424)
China	Nikonov *et al.*	1961	35	(494)
Costa Rica	Saenz	1964	59	(495)
Hawaii	Swanholm *et al.*	1959	96	(496)
	Swanholm *et al.*	1960	29	(497)
	Scheuer *et al.*	1962	71	(498)
Hong Kong	Arthur	1953	116	(499)
	Arthur and Cheung	1960	332	(420)
	Arthur and Chan	1962	400	(500)
Japan	Kariyone *et al.*	1956	85	(501)
	Goto *et al.*	1959	220	(168)
Malaya	Amarasingham *et al.*	1964	542	(490)
	Douglas and Kiang	1957	[a]	(502)
	Kiang and Douglas	1957	214	(503)
	Kiang *et al.*	1961	708	(504)
	Nakanishi *et al.*	1965	89	(176)
Malgache	Meyer and Pernet	1957	[a]	(505)
	Pernet	1956	5	(506)
Mexico	Dominguez *et al.*	1960	8	(507)
New Zealand	Cain *et al.*	1961	21	(479)
	Cambie *et al.*	1961	697	(478)
	Cambie *et al.*	1961	74	(480)
	Cambie *et al.*	1961	251	(481)
	Cain *et al.*	1962	320	(482)
Nigeria	Patel and Rowson	1964	33	(312)
	Persinos *et al.*	1964	10	(99)
	Quimby and Persinos	1964	12	(100)
North Borneo	Arthur	1954	205	(491)
Papua—New Guinea	Webb	1955	295	(483)
Sweden	Hulton and Torssell	1965	191	(854)
Taiwan	Hsu	1957	51	(508)
	Huang *et al.*	1959	61	(509)
	Koo *et al.*	1965	1000	(510)
	Yeh *et al.*	1959	72	(511)
Thailand	Nilanidhi	1964	21	(512)
Tibet	Blinova and Stukkei	1960	113	(513)
U.S.S.R.	Aliev	1962	80	(514)
	Efros	1946	30	(515)
	Ismailov	1958	140	(516)
	Lazur'evskii and Saidykov	1939	259	(517)

(*Continued on next page.*)

drying. A plant part is incised with a razor blade and a small amount of juice is applied to the test paper which, if alkaloids are present, will give the characteristic orange color indicative of a positive test. This method has been applied to the field testing of about 1200 species of plants by Nikonov and Ban'kovskii (485), who found it to be acceptable with certain reservations. They found it unsuited for plants containing pigments in the sap which masked positive reactions, and it was further determined that protoalkaloids such as ephedrine were not detected. An important point that must be emphasized is that these workers stress that the typical color of an alkaloid-positive reaction must be observed within 30 sec. from the time that the sample was applied to the paper for a test to be considered valid (485). We have used paper similar to this in our laboratory for the detection of alkaloids in organic solvents during chromatographic separations and found that it was necessary to apply water to the paper, after the sample solvent had evaporated, in order for the reaction to take place.

Spot Tests on Paper Using Liquid Reagents.—The most extensive phytochemical survey for alkaloids in the plant kingdom is being conducted by scientists from the Natural Products Section, Smith Kline & French Laboratories, Philadelphia, Pa. This program was initiated in 1954, but extensive alkaloid testing did not begin until 1958. Briefly, their approach consists of a semirandom collection of plants from all parts of the world, with tests for alkaloids made in the field on all accessible parts of each species. Those found to be promising as a source of alkaloids are collected at the test site in sufficient quantity to enable laboratory extraction of the alkaloids for subsequent pharmacologic study. Extracts from

TABLE VI.—(*Continued*)

Area	Author(s)	Yr.	Species Tested, No.	Ref.
	Kurinnaya	1956	[a]	(518)
	Kuvayev and Blinora	1960	[a]	(519)
	Massagetov	1946	113	(520)
	Nikonov and Ban'kovskii	1959	1200	(485)
	Oparin and Chepurin	1953	[a]	(521)
	Orechoff	1934	368	(522)
	Sokolov	1956	[a]	(523)
	Soskov *et al.*	1963	[a]	(524)
	Stepanyan	1963	35	(525)
	Yakunina *et al.*	1961	55	(526)
	Zolotnitskaya	1954	231	(527)
World-wide	Wall *et al.*	1954	292+	(422, 428)
	Wall *et al.*	1954	598+	(429, 430)
	Wall *et al.*	1955	606+	(431, 432)
	Wall *et al.*	1957	432+	(433)
	Wall *et al.*	1959	921+	(434)
	Wall *et al.*	1961	1030+	(435)
Apocynaceae	Abisch and Reichstein	1960	31	(486)
	Abisch and Reichstein	1962	4	(487)
Asclepiadaceae	Abisch and Reichstein	1962	64	(488)
Campanulaceae	Gertig	1962	11	(528)
Caryophyllaceae	Naumenko	1957	[a]	(529)
Leguminosae	White	1943	145	(530)
	White	1944	3	(531)
	White	1951	53	(532)
	White	1951	55	(533)
	White	1957	54	(534)
Orchidaceae	Luning	1964	525	(535)
Periplocaceae	Abisch and Reichstein	1962	6	(488)
Pinaceae	Tallent *et al.*	1955	27	(536)
Ranunculaceae	Winek *et al.*	1964	10	(537)
Solanaceae	Scott *et al.*	1957	61	(538)
Seeds	Earle and Jones	1962	900	(5)
Seeds[b]	Kazimierz	1962	27	(539)
Misc. plants[c]	Paris and Moyse-Mignon	1956	73	(540)
Misc. plants	Stein and Kamienski	1957	220	(541)
Fungi	Worthen *et al.*	1965	37	(542)
	Worthen *et al.*	1962	3	(543)
	Tyler	1961	8	(544)
	Tyler and Stuntz	1962	160	(545)
	Tyler and Stuntz	1963	94	(546)

[a] Only an abstract of the paper was available; data not included. [b] Analyzed for choline. [c] Analyzed for choline and betaine.

all plants shown to contain alkaloids by this field test are screened for several types of pharmacologic activity (436).

The field test for alkaloids used by this group has been described (466) and is essentially the same as that utilized by Nikonov and Ban'kovskii (*vide supra*) with the exception of the special test paper. Instead, plant sap obtained by making an incision of the appropriate plant part, is applied to filter paper, dried, and a micro drop of specially prepared Dragendorff's reagent is added (466). Positive tests are evaluated as previously described. All positive field tests are confirmed by means of a laboratory alkaloid detection procedure (*vide infra*).

Of 25,000 species evaluted in this manner to date, about 10% have been recorded as alkaloid-positive (436). About 5% of the plants shown to contain alkaloids by the field test were not confirmed by the laboratory procedure (436).

Abisch and Reichstein (486–488) have also utilized this spot test technique for alkaloid detection in their study of plants of the *Apocynaceae*, *Asclepiadaceae*, and *Periplocaceae*. However, their extracts were prepared from dry plant material.

Test Tube Spot Tests.—Culvenor and Fitzgerald (489) have described a simple kit that can be taken into the field for use in testing samples of plant material for alkaloids. About 2–4 Gm. of fresh plant part is ground in a small mortar with sand and sufficient chloroform to make a slurry. Ammoniacal chloroform is added and the mixture stirred for 1 min. prior to filtration into a small test tube. Extraction of the alkaloids from the chloroform is accomplished by shaking the solution with 0.5 ml. of 2 *N* sulfuric acid and separation of the acid layer by means of a medicine dropper. A few drops of this acid extract are then tested with either Mayer's reagent or

silicotungstic acid to ascertain the presence of alkaloids. When samples were analyzed by both the field method and a laboratory procedure, it was found that a number of weakly positive tests recorded through use of the laboratory test were found to be negative in the field test (489). The method, of course, fails to detect quaternary alkaloids and this appears to be its major drawback.

Presumably, many of the plants collected for laboratory alkaloid testing by Webb (423, 424, 483), Amarasingham *et al.* (490), and Arthur (491) were field analyzed in a similar manner to that described above. However, their respective reports failed to point out any consistency with regard to this matter.

Alkaloid Surveys.—Although surveys for alkaloids, representing tests on more than 15,000 species of plants, have been published (5, 99, 100, 168, 176, 312, 420, 423, 424, 428–435, 478–483, 485–488, 491–546, 854) (Table VI), the data that they present are often inconsistent because of variations in testing methodology. That is, some of the procedures will detect both quaternary and tertiary alkaloids, but the former group is omitted from most survey reports. Certain procedures involve treatment of the alkaloid fraction to remove substances that often give rise to false-positive alkaloid reactions, whereas others do not include this extra step. Some methods are semiquantitative, while others lack this desirable feature. A survey of the most extensive and more frequently reported methods allows them to be classified into 6 major categories (Table VII). Perhaps the simplest method is that represented by group *A* in which either an acidic or aqueous plant extract is prepared, with or without the use of heat, followed by filtration and the addition of one or more alkaloidal reagents to separate portions of the filtrate. Most investigators assess a rating of 0, or +1 to +4 on the lack of, or degree of precipitation following use of the reagents. However, there is seldom any indication of the alkaloid equivalent of these ratings. This undoubtedly could present a problem to either a novice or one who is attempting to duplicate results in a different laboratory. On the other hand, a person experienced in alkaloid screening can usually assess this +1 to +4 rating system by a rule of thumb. The major drawback of this method is that it results in the greatest number of false-positive reactions. An inspection of the compounds presented in Fig. 2, which are representative of a great number of nonalkaloidal plant constituents capable of giving false-positive alkaloid reactions, shows that for the most part they would be soluble in either aqueous or acidic media. Also, although this method would not differentiate between quaternary and tertiary alkaloids, neither would it fail to detect one or the other.

Group *B* testing differs from group *A* only in that the filtrate is made basic and extracted with an organic solvent (usually chloroform or ether), followed by extraction of the alkaloids from the organic solution with dilute aqueous acid. The usual alkaloidal precipitants are then added to separate portions of the acid extract. This method has the advantage over the group *A* procedure of eliminating a great number of compounds from the final test extract that are capable of eliciting false-positive alkaloid reactions; however, any quaternary alkaloids present would also be eliminated. Simple modifications in this method would allow one to test for the latter group of alkaloids.

TABLE VII.—ALKALOID TEST METHODS USED IN PHYTOCHEMICAL SCREENING

Group	Method	Ref.[a]
A	Acidic or aqueous extracts.	(5, 420, 423, 424, 478–483, 490, 496, 497)
B	Acidic or aqueous extract, followed by alkali treatment, immiscible solvent extraction, and partition with dilute acid.	(507, 536)
C	Alcohol extraction followed by concentrating and addition of acid.	
	1, No additional treatment.	(100[b], 422[b], 428–435[b], 478–482, 542[b], 545[b], 546[b])
	2, Partition purification, test made only on tertiary alkaloid fraction.	(99, 100, 422, 428–435, 507, 538, 545, 546)
	3, Partition purification, tests made both for tertiary and quaternary alkaloids.	(312, 486–488, 498, 509, 511, 535, 542)
D	Extraction of alkalinized sample with organic solvent.	(502–504)
E	Prollius fluid extraction, concentration, addition of acid.	(420, 423, 424, 483, 491, 496)
F	Procedures involving chromatography.	(99, 100, 176, 537)

[a] Only laboratory methods are presented; see earlier discussions for field testing methods. [b] Preliminary test only; additional testing employed.

Since water will extract a number of nonalkaloidal constituents from plants, and because there is a possibility of free alkaloid bases existing in the plant as such and these would be water insoluble, most investigators utilize alcohol (methanol or ethanol) or alcohol–water mixtures as a primary extraction medium. Group *C* test methods involve preparation of an alcohol extract followed by removal of solvent and the addition of dilute acid to dissolve any alkaloids. Some investigators test the resultant acid extract directly (312, 478–482) and stop at this point (group *C-1*). The advantages and disadvantages for this type of testing are similar to those discussed for the group *A* methods (*vide supra*). Others will confirm initial positive reactions following a base–organic solvent–acid extraction. These are the group *C-2* methods which are designed primarily to eliminate substances capable of eliciting false-positive alkaloid reactions. The group *C-1* and *C-2* methods were designed and used most extensively by Wall and co-workers (422), but there are 2 important features that should be discussed concerning these procedures. First, the test involves precipitation of free alkaloid bases from the initial acid extract with NaOH rather than with NH_4OH. Thus, if a majority of the alkaloids in the sample were phenolic in character (highly improbable), the phenolates formed on the addition of fixed alkali would be insoluble in the immiscible organic solvent used for the extraction of the basic alkaloid-containing solution. Subsequent extraction with dilute acid would then result in a solution free from phenolic alkaloids and would therefore not be representative of the true alkaloid content of the sample. A second problem associated with this method was recognized by the workers themselves after screening the first 4000 accessions. Because they were experiencing a lesser number of positive results than would be expected from statistical averages, they increased the concentration of test solution so that 1 ml. would be equivalent to 4.0 Gm. of dry plant material (434, 435). Previous test results in the series (422, 428–433) were reported on solutions which represented only 0.2 Gm. of dry sample. This, of course, made any negative alkaloid test results reported in the first 4000 accessions (422, 428–433) open to question. Recognizing this problem, plants yielding negative results from the latter group, if available, were retested and the results included in reports on the final 2000 accessions (434, 435). The method does not include specific provisions for the detection of quaternary bases, but as indicated previously, modifications could be made so that this procedure would detect these compounds.

Various modifications of group *C-2* methodologies have been proposed in order to detect quaternary alkaloids and report them as a separate group. In group *C-3* methods the aqueous alkaline solution, after extraction with an organic solvent, is treated with a mineral acid until it is distinctly acid to litmus, followed by the subsequent addition of any of the usual alkaloidal precipitants to this acidic solution. It should be emphasized that a weak positive test at this point need not necessarily imply the presence of quaternary alkaloids. To the contrary, a weak test must be expected because of incomplete extraction of tertiary alkaloids with the organic solvent, and would be evidenced by a slight cloudiness of the solution following addition of the reagent. A positive test, on the other hand, would be noted as a definite heavy flocculation or precipitation on addition of the reagent.

Group *D* methods involve the addition of alkali to the drug, followed by extraction with an organic solvent and partition of the concentrated extract with dilute aqueous acid prior to the addition of precipitating reagents. These procedures fail to detect quaternary alkaloids, but the final test solution is relatively free from many substances associated with false-positive reactions.

Extraction of dry plant material with Prollius fluid (ether–chloroform–ethanol–NH_4OH) (25:8:2.5:1) (547), followed by evaporation of the solvent and addition of dilute acid, is the representative procedure for group *E* methods. Webb (423) has indicated that certain plants give negative tests with Prollius fluid, but +4 reactions when acidic aqueous extracts of the same plant were tested (group *A*). He suggests that this is due to a poor solubility of quaternary bases in Prollius fluid. However, several instances wherein the reversal of these positive and negative tests using Prollius fluid and dilute acid extracts were also observed (423). In our laboratories, we found this method to be unsatisfactory when compared with others in an evaluation of testing procedures using plant samples of known alkaloid content (548).

Group *F* methods involve the use of chromatography to detect alkaloids in dry plant material. These allow not only for the detection of alkaloids, but also for an estimate of the number present. In addition, the use of selective chromogenic spray reagents could serve to tentatively classify alkaloids in the samples into general groups. We have described a method

utilizing thin-layer chromatography which requires only small samples, eliminates most compounds suspected of yielding false-positive reactions, and differentiates tertiary and quaternary alkaloids (549). Several alkaloid detecting reagents used in chromatography are presented in Tables IV and V.

The comparative efficiencies of the alkaloid screening procedures of Wall *et al.* (422), Webb (423), Kiang and Douglas (503), Swanholm *et al.* (496, 497), Abisch and Reichstein (486), and Arthur (491) have been studied using 28 plant samples known to contain alkaloids and 8 samples known to be devoid of alkaloids. The latter group included several plants known to yield false-positive alkaloid reactions. Alkaloid-containing plants were selected so that they represented several different chemical classes of alkaloids (549). The surveys under comparison employed groups *A*, *C-1*, *C-2*, *D*, and *E* methodologies. It was determined that the Wall *et al.* (422) and Kiang and Douglas (503) methods were most satisfactory, and that the direct acid extraction method of Webb (422) was the most rapid and also gave acceptable results. The Prollius fluid extraction (422) and the Abisch and Reichstein (486) techniques gave the poorest results, and the latter procedure was quite time consuming (549). All plant samples known to contain alkaloids were detected using the Kiang and Douglas (503), and Webb (423) acid extraction methods. However, these 2 procedures also gave the greatest number of false-positive reactions with the plant samples known to be devoid of alkaloids (549). False-positive alkaloid reactions were completely eliminated by use of either the Wall *et al.* confirmatory test (422) or the Abisch and Reichstein method A (486). Other studies have shown that significant differences exist in these methods with regard to their ability to remove alkaloids for testing from plant material (454).

Screening for Heterosides (Glycosides).—Heterosides are organic compounds in which a hemiacetal linkage usually connects the anomeric carbon of a sugar (glycone) with an alcohol or phenolic hydroxyl of a second nonsugar molecule (aglycone). This type of linkage gives rise to the so-called *O*-heterosides (*e.g.*, salicin), the most common type of heteroside found in plants. If the anomeric carbon of the glycone is attached to an aglycone through sulfur, the *S*-heterosides are formed (*e.g.*, sinigrin). A third group are the *N*-heterosides which involve attachment of the glycone to an amino group of an aglycone (*e.g.*, vicine, crotonoside). Finally, the C-heterosides involve a carbon to carbon linkage of glycone and aglycone (*e.g.*, aloin).

As a general rule, plant heterosides are easily hydrolyzed with dilute acids or appropriate enzymes. The C-heterosides are a notable exception, as they are resistant to the usual type of acid hydrolysis, and require ferric chloride for this purpose.

A number of different sugars are known to occur in plants in combination with an equally large number of diverse aglycones. Paris (550) has recently reviewed plant heterosides with particular reference to the types and distribution in plants.

In most instances, the biological activity of heterosides can be attributed to the aglycone moiety. The glycone is mainly associated with the degree or modification of activity, primarily induced by the aglycone. However, the cardiac heterosides can be pointed out as a group that have no useful biological activity unless the heteroside is intact (310). Thus, we have the economically important saponin heterosides and the medicinally useful anthraquinone, flavonoid, cyanogenetic, *iso*thiocyanate, and cardiac groups.

From a chemical point of view, there are 3 parts of the heteroside molecule that can be used as a means of detecting this group of compounds in plant material. First, the hemiacetal linkage between aglycone and glycone is usually not associated with biological activity, nor can it be associated with any specific aglycone.[2] This part of the molecule does not appear attractive as a means of detecting plant heterosides. Because of the usual correlation of biological activity with the aglycone moiety of heterosides, and because this part of the molecule often has chemical properties amenable to ready detection, most investigators have used it as a means of screening plant material indirectly for heterosides.

If, however, heterosides must be intact to exert their potential biological activity, it would appear most fruitful to detect the hemiacetal linkage in plant extracts as an identifying feature of the presence of heterosides. Several investigators have proposed methods to accomplish this, but a lack of published applications of these to the screening of plants for heterosides, attests to their complexity or inefficiency. Bourquelot (551) proposed a method for detecting and identifying heterosides based on the determination of an "Enzymolytic Index of Reduction" obtained by measuring the optical

[2] The notable exception is concerned with deoxysugars commonly found only in combination with cardiac heterosides.

rotation of a heteroside-containing plant extract before and after hydrolysis with specific enzymes. Although the method has some value, it is time consuming and requires large amounts of plant material; therefore, it would be difficult to adapt to a large-scale screening program. Bliss and Ramstad (552), devised a simple procedure that could be adapted for routine screening. It consists of (*a*) separation of the heterosides in an extract by paper chromatography, (*b*) hydrolysis of the heterosides on the chromatogram with proper enzymes (*i.e.*, α-glucosidase–invertin; β-glucosidase–emulsin), and (*c*) location of the reducing sugars formed on the chromatogram by means of an appropriate reagent spray. This method appears to be least objectionable of many proposed. However, it will detect only those heterosides for which the selected enzymes have a hydrolytic specificity. Also, optimal reaction conditions such as time, temperature, and pH would have to be determined for a large number of substrate heterosides to propose operating conditions that would allow detection of the greatest number of compounds. Janot *et al.* (553) and Paris (554) have suggested chromatographic methods for detecting heterosides similar to the method of Bliss and Ramstad, but acid hydrolysis of the sample is included to supplement the action of enzymes. Other methods have been proposed, but either they have not been applied successfully to plant samples, or certain limiting factors make them of doubtful value for general screening (555, 556).

Knapp and Beal (557) have proposed a method involving (*a*) the selective extraction of heterosides from plant material using 80% ethanol, (*b*) oxidation of the free sugars in the extract to their corresponding carboxylic acids so that they will not be detected after hydrolysis of the heterosides, (*c*) hydrolysis of the heterosides in the extract using 0.15 *N* sulfuric acid and heat (100°) and (*d*) detection of hydrolyzed glycones by means of paper chromatography. The major objection to this procedure is that holosides, especially sucrose which is widespread in plants, are detected; thus, the method is of decreased value.

Abisch and Reichstein (486) have utilized a rather simple procedure which involves the preparation of an extract devoid of free sugars, hydrolysis of the extract with the Kiliani acid mixture, and testing of the hydrolysis products with Fehling's solution for evidence of reduction. These investigators have pointed out the nonspecificity of the test; however, in a broad screening program, false-positive reactions must be accepted, especially in the absence of a completely acceptable and specific method of detection.

It does not appear that adequate methodology has been developed to allow for an extensive screening of plants for heterosides based on the approaches described above. As indicated previously, the majority of studies involving a search for heterosides in plant material have been concerned with tests designed to detect specific aglycones. The more important of these will now be considered.

Screening for Saponins and Related Compounds

Several types of compounds must be considered whenever saponin testing is to be conducted. Of major import are the steroidal and triterpenoid saponins and their respective sapogenins, as well as saturated sterols, saturated terpenes, diterpenes, and other steroidal plant constituents (*e.g.*, cardenolides). The economic importance of steroidal sapogenins, mainly because of their facile conversion to the medicinally useful steroid hormones, has been reviewed by Correll *et al.* (11). Discussions concerning this group of compounds will be directed primarily at the detection and/or differentiation of steroidal saponins from other types of saponins and polycyclic phytoconstituents.

A number of surveys have been conducted which have been designed to detect saponins in plant material (99, 100, 168, 422, 428–435, 478–483, 490–492, 494, 499, 506, 507, 513, 529, 558–580). Saponins have several characteristic properties that can be used as a basis for simple detection tests. (*a*) They are all capable of hemolyzing red blood cells, (*b*) in aqueous media they will produce a characteristic honeycomb froth which persists for at least 30 min. after vigorous shaking of the solution; (*c*) they are toxic for fish, causing paralysis of the gills; and (*d*) they produce characteristic color reactions in the Liebermann-Burchard test. All of these properties have been utilized in one or more screening tests for the detection of saponins in plant material, and each merits discussion to show the limitations and value of these test procedures (Table VIII).

All known triterpenoid and steroidal saponins are hemolytic (584, 585), as undoubtedly are certain other plant constituents. The former group occurs both as the heteroside and as free triterpenes, whereas steroidal saponins are never found as free sapogenins in plant material (584, 585). This is an unusual phenomenon since specific steroidal saponases are known to be present in steroidal saponin-containing plants

TABLE VIII.—SURVEYS FOR SAPONINS IN PLANTS

Detection Method	Source of Plants	Species Tested, No.	Ref.
Hemolysis	India	38	(562–564, 581)
	Mexico	8	(507)
	Nigeria	22	(99, 100)
	Poland	4	(574)
Hemolysis + froth	Brazil	71	(492)
Hemolysis + steroidal sapogenin identification	Brazil	21	(559)
	Liliaceae	15	(579)
	Philippines	222	(571–573)
Froth test	India	38	(562–564, 581)
	Malaya	542	(490)
	North Borneo	205	(491)
	Papua—New Guinea	116	(558)
Froth test + Liebermann-Burchard test	Australia	1136	(558)
	New Zealand	1533	(478–483)
Fish toxicity	India	38	(562–564, 581)
Isolation of steroidal sapogenins	India	38	(562–564, 581)
	Mexico	150	(567–570)
Isolation or detection of triterpenoid sapogenins	India	6	(565)
	Poland	8	(580)
	U.S.S.R.	5 families	(577)
Undetermined methods	Bulgaria	72	(560)
	Chile	2894	(561)
	China	3	(582)
	India	4	(566)
	Japan	220	(168)
	Malgache	5	(506)
	Spain	27	(575, 583)
	Tibet	113	(513)
	U.S.S.R.	[a]	(576, 578)

[a] Original paper unavailable; data not included in abstract.

(586). Since all saponins are soluble to some extent in 80% alcohol, they are usually extracted with this solvent (584, 585), and if such a plant extract is mixed with a standardized red blood cell suspension (585) and hemolysis of the cells takes place, it can be assumed that saponins are present. On the other hand, terpenoid and steroidal saponins act similarly in this respect and cannot be differentiated on this basis alone. Wall *et al.* (585), Walens *et al.* (587), and Rothman *et al.* (588) have developed standard procedures which have been successfully used by many investigators for the detection and estimation of steroidal sapogenins, and which differentiate triterpenoid from steroidal types. The procedure involves (*a*) hemolytic detection of saponins in the plant extract (585), (*b*) isolation of crude sapogenins following hydrolysis (585), and (*c*) subjecting the crude sapogenin acetates to infrared (585) or ultraviolet (587) spectral analysis. Initially, the extraction procedure eliminates to a great degree the starch, sugars, and protein which, if present, would produce troublesome tars as a result of the acid hydrolysis (588). The presence of these materials in the extract would necessitate larger amounts of acid, with subsequent destruction of some sapogenin. It has been determined by using the recommended extraction procedure (585), that 2 *N* HCl in alcohol at reflux temperature or 0.5 *N* HCl under pressure will completely hydrolyze saponins (584, 588). Characteristic infrared and ultraviolet absorption spectra of the hydrolysates then serve to indicate whether the saponins causing hemolysis in the sample are steroidal or triterpenoid.

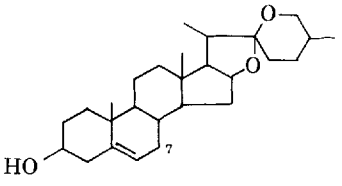
The appearance of a characteristic honeycomb froth, which persists for at last 30 min. after shaking an aqueous boiled (3–5 min.) mixture containing the plant material, is presumptive evidence for the presence of saponins. This method of detection is rapid, simple, and requires little equipment, but it will not differentiate triterpenoid from steroidal saponins. It has been used, however, by several investigators (Table VIII). If only a small froth is produced by this treatment, which is stable for only a few minutes, proteins, certain plant acids, or a low concentration of saponin may be the cause (558). Evidence exists that the froth test is not infallible since the saponin of *Castanospermum australe* is an active hemolytic agent at a dilution of 1:20,000; however, at this dilution no frothing occurs (558). The addition of aqueous sodium carbonate to a boiled aqueous plant extract which froths poorly, or not at all, may result in the production of a stable and dense froth. If this occurs, the presence of free acids (*e.g.*, stearic acid, diterpene acids, triterpene dicarboxylic acids) is indicated (558).

TABLE IX.—DIFFERENTIATION OF POLYCYCLIC SUBSTANCES IN PLANTS

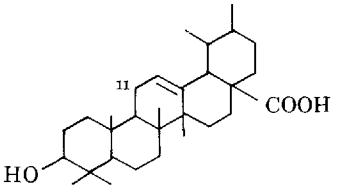
Hemolysis Test	Froth Test	Liebermann-Burchard Test	Substances Present
+	+	+ Blue or green	Saponins, probably steroidal.
+	+	+ Red, pink, purple or violet	Saponins, probably triterpenoid.
+	+	− Pale yellow	Saponins, possibly heterosides of saturated sterols or saturated triterpenes.
±	−	+ Red, pink, purple or violet	Saponins absent, free triterpenes, diterpenes, sterols or related polycyclic substances present.
−	−	− Pale yellow	Saponins absent, also unsaturated triterpenes, sterols, etc., absent, but may contain saturated sterols or saturated triterpenes.
−	+[a]	± Pale yellow, red, pink, purple or violet	Saponins absent, probably free diterpene acids.

[a] Froth only evident after addition of sodium carbonate and shaking.

Some investigators have included a test for unsaturated sterols in their phytochemical surveys. In most instances, the Liebermann-Burchard (L-B) test[3] has been used to detect this class of compounds (422, 428–435, 478–483, 491, 558, 616), and it has been used to advantage in the differentiation of triterpenoid and steroidal saponins (558). According to Simes *et al.* (558), blue or blue-green colors are formed in the L-B test with steroidal saponins, and red, pink, or purple colors result if triterpenoids are present. However, when these workers applied the L-B test either directly to powdered plant material, or to a solution of extracted material, they noted that there was variation in the colors produced, depending on the manner in which the test was conducted. For example, ursolic acid (triterpenoid) gives a blue-green color in solution, but if the test is applied directly to solid material, the colors noted are only purple or violet (558). Simes *et al.* (558) do not comment on the time required to observe these characteristic reactions, whereas Wall *et al.* (422), using chloroform extracts of plant material, point out that interfering substances such as carotene and xanthophylls produce immediate color changes in the L-B test, as also do saturated sterols. However, when interfering substances are absent, unsaturated sterols give a minimal color density at the start of the test, and slowly reach a maximum after about 15 min. This delayed color reaction has also been observed by others (589, 591). Brieskorn and Herrig (592), in an investigation of the mechanism of the L-B reaction, found the following features essential for color formation. In the steroids, two conjugated double bonds in ring B, or one double bond and an unhindered methylene group at C_7 which can undergo oxidation and dehydration, are essential. In pentacyclic triterpenes, however, it is the methylene group at C_{11} in ring C that is involved. Steroid esters were shown to give a more intense color reaction in the L-B test than the corresponding alcohols, while the opposite was true for pentacyclic triterpenes and their esters.



Steroid Sapogenin



Pentacyclic Triterpenoid Sapogenin

The use of the froth test, in conjunction with the L-B reaction, has been found useful for the detection and differentiation of triterpenoid and steroidal saponins, triterpenoid sapogenins, free sterols, and other polycyclic substances (558). An outline of methods used for these identifications using the froth test, L-B reaction, and hemolysis, is presented in Table IX.

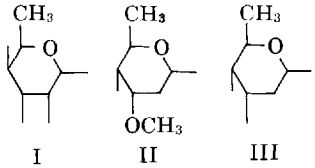
Willaman and Wadley (593) have found an interesting correlation existing between *Agave* and *Dioscorea* species with respect to the presence or absence of unsaturated sterols and steroidal saponins. They point out that strong positive sterol (L-B) tests on extracts from these 2 genera usually indicate an absence or very low steroidal saponin content. However, with *Yucca*, and undoubtedly with other groups of plants, no such association was indicated.

[3] The L-B test is usually conducted by adding a small amount of acetic anhydride–sulfuric acid mixture (19:1) to a solution of the sterol in suitable anhydrous solvent. A history of the test has been given by Dam (590).

It would appear that chromatographic differentiation of polycyclic substances in plant extracts could be used to advantage, especially since the advent of thin-layer chromatography which allows the use of corrosive reagents, since a great variety of steroid detecting reagents are available, *e.g.*, vanillin–phosphoric acid (594, 595), antimony trichloride (596–600), antimony pentachloride (596, 601), sulfuric acid (602), 50% sulfuric acid (603, 604), sulfuric acid–acetic anhydride (599, 605), chlorosulfonic acid (599), silicotungstic acid (596), phosphotungstic acid (606), zinc chloride (607), anthrone (608), furfural–sulfuric acid (609), sodium nitroprusside (610), Nessler's reagent (611), Millon's reagent (596), tetrazolium blue (612), phosphomolybdic acid (596, 613, 614), and Dragendorff's reagent (341). Axelrod and Pulliam (615) have developed several micro-methods for the detection of characteristic functional groups on the steroid nucleus which can be adapted to paper or thin-layer chromatography and which should be of great value in phytochemical screening.

Cardiac Glycosides

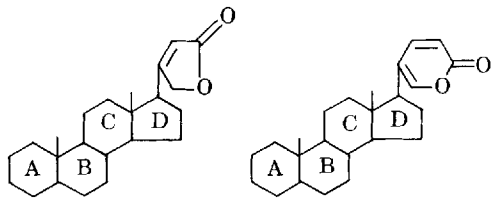
The importance of cardiac glycosides as therapeutic agents requires no elaboration. Their status in medicine, however, is not reflected in the number of published efforts to seek new and better agents of this type in the plant kingdom. As previously mentioned, a few surveys have been conducted in which plant extracts were evaluated for cardiotonic activity either through *in vitro* or *in vivo* biological tests (311–313). In the case of cardiac glycosides, the minimum structural features required for biological activity are so well defined that the substitution of a strictly chemical means of detection is an accepted procedure. All cardioactive glycosides are classified as steroids (sterols), having the cyclopentanoperhydrophenanthrene nucleus, an α-β-unsaturated lactone ring (5- or 6-membered) at C_{17}, a β-oriented hydroxyl at C_{14}, a *cis* fusion of the C and D rings at C_{13}-C_{14}, and the attachment at C_3 of one or more sugars, usually deoxyhexomethyloses (310). A 5-membered unsaturated lactone at C_{17} categorizes the glycoside



I II III

I, L-Rhamnose
II, D-Cymarose
III, D-Digitoxose

as a cardenolide, whereas a 6-membered unsaturated lactone at the same position exemplifies the bufadienolides. Of these 2 groups the cardenolides are the most frequently encountered and the most useful as therapeutic agents.



Cardenolides Bufadienolides

Detection of cardiac glycosides in plants has been effected by means of chemical tests applied either directly to a crude or semipurified extract, or following chromatographic separation of the glycosides. The latter method is to be preferred since a judicious selection of solvent systems and chromatographic conditions will free the glycosides from extraneous plant substances that either interfere with or mask the evidence of positive chemical tests.

Several methods for preparing an initial extract of the plant material have been reported, and methanol, 85% ethanol, or 80% ethanol appear to be the most useful solvents. The latter is advantageous since starch will not be extracted (617). Although a certain amount of enzymatic degradation of the native glycosides will occur, either as a result of the drying procedure or because of some processing step, this will not usually affect qualitative methods of detection since most of these are applicable to both native and secondary glycosides, as well as their aglycones. Bieleski (618) has considered the problem of halting enzyme action when extracting plant tissues, with respect to plant phosphatases, and although the situation may not be analogous, the general precautions and approach to the problem are worthwhile. The cardenolide detection method of Krider *et al.* (313) employs a paper chromatographic separation of a semipurified extract prior to the application of chemical detection methods. This purification is effected by means of lead hydroxide precipitation of nonglycosidic interfering substances. Certain extracts encountered in routine screening work, however, would require little purification, a step that is often time consuming and tedious.

Chemical tests applied to the detection of cardiotonic glycosides and/or aglycones are carried out by applying one or more reagents to a chromatogram of the extract (619–635) or to a piece of filter paper on which the extract was applied (486–488), or directly to the liquid extract (312). The available reagents are designed to detect

TABLE X.—CHROMOGENIC REAGENTS USED FOR THE DETECTION OF CARDIAC GLYCOSIDES

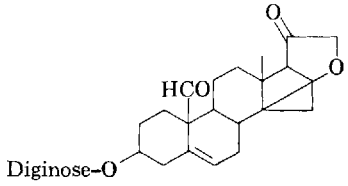
Reaction Site[a]	Common Name of Test	Major Constituent(s) of Reagent	Ref.
A	Baljet	2,4,6-Trinitrophenol–alkali	(630, 637, 833, 834)
A	Kedde	3,5-Dinitrobenzoic acid–alkali	(313, 627, 635, 638, 835)
A	Raymond	*m*-Dinitrobenzene–alkali	(630, 639, 640, 835)
A	Legal	Sodium nitroprusside–alkali	(630, 639, 641, 836)
A		1,3,5-Trinitrobenzene–alkali	(837, 838)
A		2,4-Dinitrodiphenylsulfone–alkali	(846)
A		1,2-Naphthoquinonesulfonate–alkali	(839)
A		Tetradinitrophenyl–alkali	(631)
A		*o*-Dinitrobenzaldehyde	(639)
A		1,3-Dinitrobenzaldehyde	(840)
A		Tetranitromethane	(841)
B	Kiliani	Ferric sulfate–sulfuric acid	(644)
B	Keller	Ferric chloride–acetic acid	(645, 842)
B	Keller-Kiliani	Ferric chloride–sulfuric acid–acetic acid	(632)
B	Pesez	Xanthydrol	(647, 649)
B	Tollens	Silver nitrate–ammonia	(646, 836)
B	Langejan	Orcinol–hydrochloric acid	(843)
B		Naphthoresorcinol–hydrochloric acid	(639)
B		*p*-Dimethylaminobenzaldehyde–phosphoric acid	(844)
C	Liebermann	Acetic anhydride–sulfuric acid	(589, 64)
C	Carr-Price	Antimony trichloride–acetic anhydride	(630, 649, 845)
C		Trichloracetic acid	(629, 630)
C		Trichloracetic acid–chloramine	(629, 630)
C		Sulfuric acid–ferric chloride–H_3PO_4	(847)
C		Sulfuric acid, 84%	(848)

[a] A, unsaturated lactone; B, desoxy sugar; C, steroid nucleus.

either the unsaturated lactone moiety at C_{17}, the deoxy sugar at C_3, or the steroid nucleus, the latter being the least specific for cardiotonic glycosides. These reagents are listed in Table X, together with common names frequently associated with tests involving their usage.

Most frequently utilized for the qualitative, as well as the quantitative evaluation of cardiac glycosides, have been the Baljet (2,4,6-trinitrophenol) (636, 637), Kedde (3,5-dinitrobenzoic acid) (638), Raymond (*m*-dinitrobenzene) (639, 640), or the Legal (sodium nitroprusside) reagents (641) which react with active methylene groups as found in the C_{17}-unsaturated lactone moiety (642, 643). These reagents give orange, purple, blue, and violet colors, respectively, with cardiac glycosides. Also utilized, but to a lesser extent, are reagents that react with the deoxysugar moiety of cardiac glycosides, negative tests of course being expected for aglycones. These include the Kiliani (ferric sulfate–sulfuric acid) (644), Keller (ferric chloride–acetic acid) (645), Tollens (silver nitrate–ammonia) (646), and Pesez (xanthydrol) (647) reagents. For detection of the steroid nucleus, the Liebermann (acetic anhydride–sulfuric acid) (648), and Carr-Price (antimony trichloride–acetic anhydride) (649) reagents are commonly used. Recent and thorough discussions of cardiac glycoside-detecting reagents have been published by Jensen (630), Rosenthaler (650), Cerri (651), and Frerejacque and DeGraeve (652). The latter paper is of particular value since it tabulates most of the useful reagents, gives formulas for their preparation, techniques for use, and presents remarks concerning the specificity and limitations of each.

In a phytochemical screening program for cardiac glycosides, initial positive tests on plant extracts using any one reagent should be confirmed with reagents specific for the 2 additional reactive sites (*vide supra*). For example, an initial positive Keller reaction is indicative only of the presence of a deoxy sugar. This should be followed by a second test which might be the Liebermann reaction for the steroid nucleus. Assuming that a positive reaction was also noted, a third and confirming test might be the Legal or Kedde test which denotes an unsaturated lactone at C_{17}. Positive tests with all 3 reagents offer reasonable assurance of the presence of cardioactive glycosides. If a negative test was noted with the Legal or Kedde reagent, in conjunction with positive reactions from the Liebermann and Keller tests, the presence of digitanol



Diginin—A Digitanol Glycoside

glycosides (diginin, digifolein, etc.) would be indicated since they lack the α-β-unsaturated lactone at C_{17} of the normal cardioactive glycosides and as a group are pharmacologically inactive substances (653).

Of particular importance in the interpretation of these tests is the observation of chromogenic reactions which may be transient, those which may change rapidly, or be dependent to a great degree on concentration of cardiac glycoside present. Certain of the older literature presents color reactions which are erroneous and confusing since the purity of either the glycosides being investigated, or the chemicals used in the reagents, must be questioned. Cook (589) has reviewed the color reactions of steroids in the Liebermann-Burchard reactions and presents a variety of possible colors for these compounds. In addition, he points out that in many instances, the chromogenic reactions are often delayed.

Using the tests enumerated herein may result in a few false-positive reactions for cardiac glycosides since deoxy sugars, α-β-unsaturated lactones, and steroids exist in other molecules found in plants (310). However, the use of tests for all 3 of these groups should greatly reduce the incidence of false-positive interpretations.

Table XI presents a summary of several phytochemical surveys that have been published concerning the detection of cardiac glycosides in a number of different plants. These and other data (310) point out that cardiac glycosides are present in at least 39 genera of 14 plant families.

Flavonoids and Related Compounds

The flavonoids are plant pigments based on the C_6-C_3-C_6 carbon skeleton as found in flavones, flavonols, isoflavones, flavonones, catechins, leucoanthocyanins, anthocyanins, aurones, and chalcones (Fig. 3). Although more than 33 different biological activities had been reported for some 30 of 137 known natural flavonoids up to the year 1955 (415), rutin is still the major useful member of this class of compounds as a medicinal agent. Several reviews on the biological activities and place in drug therapy of the flavonoids have been published (342, 344, 345, 415–418, 655), as have essays on their economic nonmedicinal applications (656–658). More recently, reports concerning the antiviral (659), anti-inflammatory (660), and cytotoxic (144, 661) activities of flavonoids have served to make them an exciting group of compounds for the researcher interested in biologically active phytoconstituents.

Flavonoids are widely distributed throughout the plant kingdom in the form of aglycones as well as heterosides. However, the latter are most frequently found in flowers, fruits, and leaves, whereas the former are usually most abundant in woody tissues. Some compounds are never found as heterosides, such as the nonhydroxylated and fully alkylated flavones, nobiletin, tangeretin, and meliternatin, and the polymethoxychalcone, pedicellin. These flavonoids have no hydroxyl groups with which a sugar residue can combine. It is difficult to state a general solubility rule for flavonoids because they range from water-soluble, ether-soluble (highly methylated nonheterosides), to ether- and alcohol-soluble (hydroxyflavone, flavanone, and isoflavone aglycones), to water-soluble, ether-insoluble (heterosides with up to 3 sugars) forms. However, when fresh plant material is extracted, methanol or ethanol will usually remove most flavonoids. Both flavonoid heterosides and aglycones are, as a general rule, insoluble in petroleum ether, and advantage can be taken of this fact to defat the sample prior to alcohol extraction. When dry plant material is to be extracted, several solvents should be sequentially used to insure complete removal of all flavonoids; however, in a screening program this is not usually feasible. Wall *et al.* (422, 428–435) have used 80% ethanol routinely in screening some 6000 plant accessions for flavonoids,

TABLE XI.—PHYTOCHEMICAL SURVEYS FOR CARDENOLIDES

Plant Source	Species Tested, No.	Method of Detection	Ref.
Nigeria	33	Keller-Kiliani and Kedde tests on plant extracts	(312)
Tibet	113	[a]	(513)
U.S.S.R.	[a]	[a]	(654)
Apocynaceae	22	Kedde test: paper chromatography	(620, 624)
Apocynaceae	5	Kedde, xanthydrol, $SbCl_3$ tests: extracts spotted on paper	(486, 487)
Asclepiadaceae	64	Kedde, xanthydrol, $SbCl_3$ tests: extracts spotted on paper	(488)
Moraceae	35	Kedde test: paper chromatography	(622, 625)
Periplocaceae	6	Kedde, xanthydrol, $SbCl_3$ tests: extracts spotted on paper	(488)
Misc. plants	17	Kedde test: paper chromatography	(313)
Misc. plants	10	Kedde, trichloracetic acid–chloramine and $SbCl_3$ tests: paper chromatography	(626)

[a] Original article unavailable; data not given in abstract.

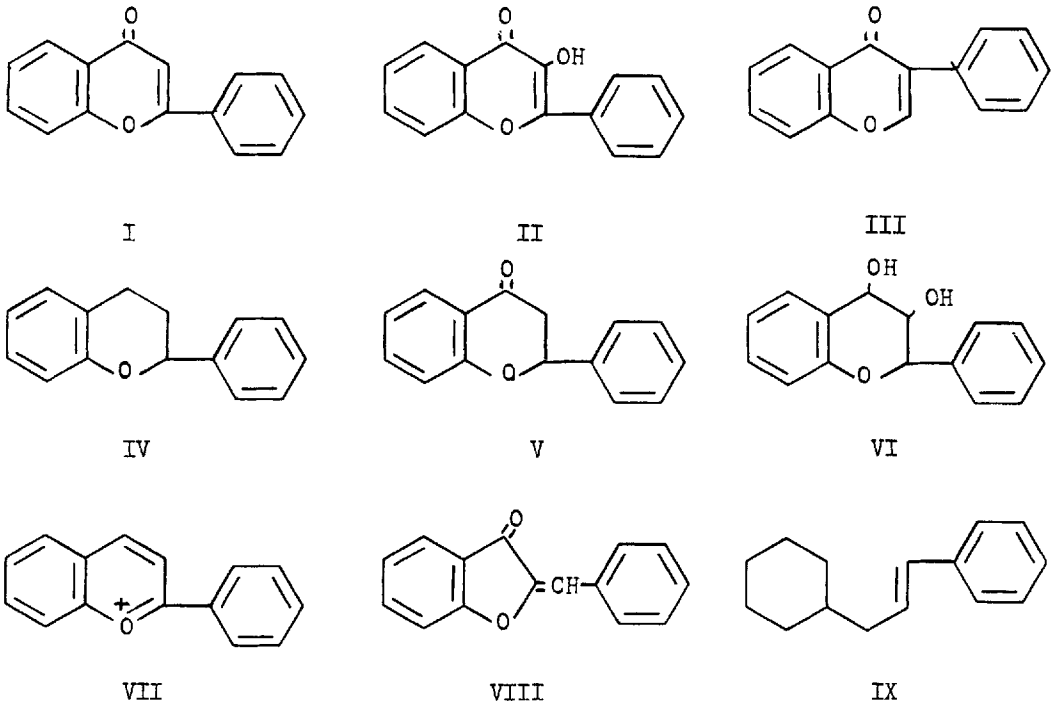


Fig. 3.—Types of flavonoids. Key: I, flavones; II, flavonols; III, isoflavones; IV, catechins; V, flavanones; VI, leuconanthocyanins; VII, anthocyanins; VIII, aurones; IX, chalcones.

the majority of the samples consisting of dry plant material.

A number of specific color reactions for various types of flavonoids have been reported that could be adapted to screening large numbers of plant samples, but specificity of a sort is usually not desirable for the initial testing. One of the most useful general tests is the so-called cyanidin reaction of Willstatter (662) which will detect compounds having the γ-benzopyrone nucleus. To an alcoholic solution of plant material is added a small piece of magnesium ribbon, followed by the dropwise addition of concentrated HCl.

Colors ranging from orange to red (flavones), red to crimson (flavanols), crimson to magenta (flavanones), and occasionally to green or blue are taken as evidence for a positive reaction for either the aglycone or heteroside. Colors usually develop within 1–2 min. following addition of the acid and are, of course, subject to variation in intensity depending on the concentration of flavonoid present in the sample (663). Problems often arise in the interpretation of positive tests when the plant extract being tested is highly pigmented. Bryant has modified the method so that heterosides and their aglycones can be differentiated (664). Xanthones have also been observed to give a positive cyanidin reaction (663).

Chalcones and aurones will not give the cyanidin reaction under the reducing conditions of this test. Direct addition of concentrated HCl to an ethanol extract of plant material containing either of these types of flavonoids, however, will result in an immediate red color (663).

Anthocyanins can be identified in plant material by extraction with cold 1% HCl followed by boiling and observation of the color at the boiling point. These plant pigments vary in color from orange-red to blue-red. Robinson and Robinson (665–668) first introduced this test which, through the use of immiscible solvent extractions, can differentiate many of the anthocyanins. A number of surveys for anthocyanins have been conducted, representing hundreds of plant species (665–680), and representative reviews on the subject of anthocyanins have been published (681–684).

Leucoanthocyanins can easily be detected by the method of Bate-Smith and Metcalfe (685) which has been utilized by Cain *et al.* (478–482) in their extensive screening of the New Zealand flora. The test involves digesting a small sample of plant material with 2 *N* HCl in 1-propanol for 15–30 min. A slow development of a strong red or violet color is indicative of a positive reaction.

Catechins give a blue or green color with ferric

chloride, but this test is of little value when crude plant extracts are being evaluated because of many other compounds that can give similar tests. Extraction of plant material with benzene, followed by extraction with ether, will yield a catechin-containing ethereal extract. Chromatography of this extract, followed by treatment of the chromatogram with 3% ethanolic *p*-toluenesulfonic acid and warming, will yield yellow spots if catechins are present (686).

A summary of flavonoid color reactions, following treatment with various reagents on paper chromatograms, is presented in Table XII.

In addition to the specific flavonoid surveys cited above, others of a more general nature have been published (99, 506, 507, 687–692). General review articles are available which should be of value to those who are interested in more specific information on testing for individual flavonoids such as the catechins (686), isoflavonoids (693–695), flavones (695–697), flavonols (697–698), dihydroflavonols (699), flavanones (700), chalcones (700), aurones (700), leucoanthocyanins (701), and the flavonoids generally (702–709).

Tannins

Two groups of phenolic constituents, hydrolyzable and condensed, comprise the tannins, substances which are important economically as agents for the tanning of leather, and for certain medicinal purposes. More recently, evidence has been presented in support of their potential value as cytotoxic and/or antineoplastic agents (146).

Hydrolyzable tannins are yellow-brown amorphous substances which dissolve in hot water to form colloidal dispersions. They are astringent and have the ability to tan hide. Chemically speaking, they are esters which can be hydrolyzed by boiling with dilute acid to yield a phenolic compound, usually a derivative of gallic acid, and a sugar. These are often referred to as pyrogallol tannins.

Condensed tannins (catechin tannins, phlobatannins) are polymers of phenolic compounds related to the flavonoids and are similar in general properties to the hydrolyzable tannins, but are not very soluble in water, and following treatment with boiling dilute acid, red-brown insoluble polymers known as phlobaphenes or tannin-reds are formed.

Tannins are detected most simply in plant extracts by the use of the so-called gelatin–salt block test (710) which has been utilized extensively in the phytochemical surveys of Wall *et al.* (422, 428–435). This test employs aqueous extracts prepared from 80% ethanol extracted plant material. A sodium chloride solution is added to one portion of the test extract, of 1% gelatin solution to a second portion, and the gelatin–salt reagent (710) to a third portion. Precipitation with the latter reagent, or with both the gelatin and gelatin–salt reagents is indicative of the presence of tannins. If precipitation is observed only with the salt solution (control), a false-positive test is indicated. Positive tests are confirmed by the addition of ferric chloride solution to the extract and should result in a blue, blue-black, green, or blue-green color and precipitate. Hoch (711) has applied some 33 different classical tannin detecting reagents to several tannin extracts; however, the nonspecificity of many of these would render them im-

TABLE XII.—COLOR REACTIONS OF FLAVONOIDS ON PAPER[a,b]

Type Flavonoid	Visible	U.V.	Ammonia Visible	Ammonia U.V.	Aluminum Chloride Visible	Aluminum Chloride U.V.	Na_2CO_3 Visible	$NaBH_4$ Visible	$ArSO_3H$[c] Visible
Flavones	Pale yellow	Dull-brown Red-brown Yellow-brown	Yellow	Bright yellow Yellow-green Dull-purple	Pale yellow	Fluorescent green Yellows Browns	Bright yellow	Colorless	Yellow
Flavonols	Pale yellow	Bright-yellow Yellow-green Browns	Yellow	Bright-yellow Yellow-green Green	Yellow	Fluorescent yellow or green	Yellow Yellow-brown Bluish	Colorless	Yellow
Isoflavones	Colorless	Faint purple Pale yellow[d]	Colorless	Faint purple Pale yellow	Colorless	Fluorescent yellow	Pale green	Colorless	
Catechins	Colorless	Colorless[d]	Colorless	Fluorescent pale blue Black	Colorless	Colorless Pale blue Yellow-white			Brown
Flavanones	Colorless	Colorless	Colorless	Colorless Pale yellow Yellow-green	Colorless	Fluorescent green-yellow Blue-white	Pale yellow-green	Magenta	Colorless
Leucoanthocyanins	Colorless	Colorless							Red Pink Purple
Anthocyanins	Pink Orange Red-purple	Dull red or purple Pink Brown[e]	Blue-gray Blue	Bluish					Unchanged
Aurones	Bright-yellow	Bright-yellow Green-yellow	Orange Orange-pink	Yellow-orange Orange Red-orange Brown	Pale-yellow	Fluorescent orange Brown Pink	Orange Brown Red	Colorless	Orange Pink
Chalcones	Yellow	Brown Black Yellow-brown	Yellow Orange Red-orange Pink	Orange Red Purple Black	Yellow Orange Yellow-orange	Fluorescent orange Brown Pink	Orange Brown Red	Colorless	Orange Pink

[a] Adapted from Seikel (849). [b] With ferric chloride and $K_3Fe(CN)_6$, all produce blue colors. [c] *p*-Toluenesulfonic acid, after heating. Dihydroflavonols change from colorless to yellow, becoming flavonols. [d] Short wavelength U.V.: isoflavones, yellow; catechins, black. [e] 3,5-Diglycosides are either fluorescent yellow or rose or bright red or purple. [f] Only derivatives of cyanidin, delphinidin, and petunidin change color.

practical for use in general phytochemical screening work.

Several phytochemical surveys for tannins in plants of Argentina (712), Bulgaria (713), China (495), Japan (168), Mexico (507), Nigeria (99, 100), Pakistan (714), Tibet (513), the U.S.S.R. (715–718), as well as from hundreds of additional miscellaneous plants (2, 5, 422, 428–435, 529), have been conducted. Happich *et al.* (2) have emphasized, from a practical viewpoint, the requirements necessary in a tannin for it to be of commercial value.

Coumarins

Coumarins are benz-α-pyrone derivatives found widely distributed in plants; however, they occur most frequently in members of the *Gramineae, Orchidaceae, Leguminosae, Umbelliferae, Rutaceae,* and *Labiatae.* They rarely, if ever, occur in the *Liliaceae, Onagraceae, Pinaceae,* or *Cactaceae.* The medicinal value of certain coumarin derivatives (bishydroxycoumarin, 8-methoxypsoralen) is well known; however, according to Soine (419), the full range of biological activities for these compounds is not appreciated by most investigators. Reviews by Bose (719) in 1958, and more recently by Soine (419) in 1964, have served to point out the biological activities of coumarins, a group which appear most interesting because of their anticoagulant, estrogenic, dermal photosensitizing, antibacterial, vasodilator, molluscacidal, anthelmintic, sedative and hypnotic, analgesic, and hypothermal effects (419, 719).

Several types of coumarin derivatives have been found in plants, the substituted coumarins, furanocoumarins, pyranocoumarins, benzocoumarins, phenylcoumarins, and others.

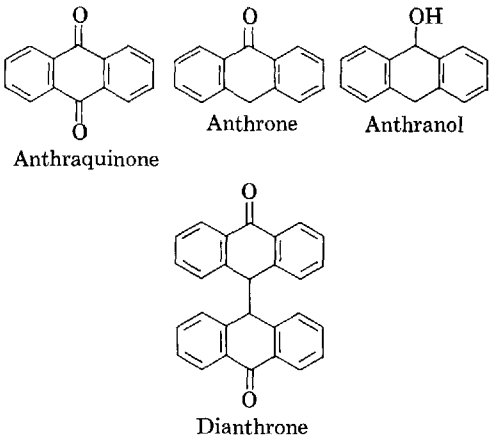
Coumarin itself can be easily detected in plant material simply by placing a small amount of moistened sample in a test tube and covering the tube with filter paper moistened with dilute sodium hydroxide solution. The covered test tube is then placed in a boiling water bath for several minutes, the paper removed and exposed to ultraviolet light. If coumarin is present, a yellow-green fluorescence appears within a few minutes (720). This procedure, however, is applicable only to coumarin and related volatile compounds. Most methods that appear useful for detecting coumarins in plant material are based on extraction of the plant sample, followed by chromatography of the extract and revelation of the coumarins with spray reagents such as diphenylboric acid, β-aminoethyl ester (721), KOH and diazotized sulfanilic acid (722), or uranyl acetate (723).

Plant material is best extracted by first quick-washing it with ice cold petroleum ether to remove lipids. Most coumarins are insoluble in this solvent, especially if it is kept cold. The defatted sample can then be extracted with warm, dilute alkali hydroxides which form the water-soluble coumarinate. Neutral organic impurities then are extracted with ether and on acidification of the aqueous coumarinate solution, the parent coumarin reforms and is removed by ether extraction. Acidic impurities are then removed by extraction with sodium bicarbonate solution. The ether solution can then be concentrated and used for chromatographic studies with specific reagents (*vide supra*), or as a spot test in which a drop of concentrate is treated, in a porcelain dish, with a drop of saturated solution of hydroxylamine hydrochloride in alcohol and 1 drop of saturated alcoholic solution of KOH. This mixture is heated over an open flame until it begins to bubble. After cooling, 0.5 *N* HCl is added to acidify the mixture, followed by a drop of 1% ferric chloride solution. A positive test consists of a violet color and is indicative of the presence of lactones (724). Other tests (725–728) have been utilized for the detection of coumarins in plant materials; however, only a slight interest has been noted in testing for coumarins, as evidenced by limited inclusion of tests for this group in phytochemical surveys (494, 513, 616).

Anthraquinones

The largest group of naturally occurring quinone substances are the anthraquinones. Although they have a widespread use as dyes, their chief medicinal value is dependent upon their cathartic action. They are of restricted distribution in the plant kingdom and are found most frequently in members of the *Rhamnaceae, Polygonaceae, Rubiaceae, Leguminosae,* and *Liliaceae.* As found in plants, they are usually carboxylated, methylated, or hydroxylated derivatives of the anthracenes, anthrone, anthranol, anthraquinone, or dianthrone. Hydroxylated anthracenes often occur as heterosides linked with various sugars through one of the hydroxyl groups (*O*-heterosides). Other types of anthracene heterosides are represented as C-heterosides in which the sugar and aglycone are linked by a carbon to carbon bond.

For the qualitative detection of anthraquinones in plant material, the Borntrager reaction, as modified by Kraus (729), appears to be simplest to perform in the application to phytochemical screening. The powdered sample (0.3 Gm.) is boiled for a few minutes with 0.5 *N* KOH (10 ml.) to which is added 1 ml. of dilute hydrogen per-



oxide solution. After cooling, the mixture is filtered and 5 ml. acidified with 10 drops of acetic acid. This acidulated mixture is then extracted by shaking with 10 ml. of benzene in a separator and the benzene layer takes on a yellow color. A 5-ml. sample of this benzene extract is shaken with 2.5 ml. of ammonium hydroxide, and a positive reaction for the presence of anthraquinones is evidenced by the formation of a red color in the alkaline layer. Normally, if C-glycosides are present in a sample being evaluated for anthraquinones, they will not be detected by the usual Borntrager reaction, as C-glycosides require special methods for cleaving the sugar from the aglycone. This can be done with ferric chloride, sodium dithionate or, as described above, with peroxide in an alkaline medium. It has been shown that this method results in a mixture of products (730); however, this is not a disadvantage for a general screening test. Other simple and rapid spot tests, which involve the direct addition of a reagent to the solid sample (powdered drug), have been described. They should be useful in phytochemical screening (731, 732), but to date have not been shown to be applicable for this type of work.

Phytochemical surveys for anthraquinones have been found only infrequently in the literature (494, 513).

Cyanogenetic Glycosides

Compounds in plants that liberate hydrocyanic acid (HCN) following hydrolysis are of major importance because of their potential danger as poisons to livestock as well as humans (733–735). Also, the use of at least one cyanogenetic glycoside, (−)-mandelonitrile-β-glucuronoside (laetrile), for the treatment of human neoplastic disease has been suggested (736), but this remains as a controversial subject. Should cyanogenetic glycosides prove useful in this respect, the plant kingdom should be expected to be a source for new and similar compounds.

It is generally recognized that a small amount of HCN occurs free in plants, but that the greatest amount is combined in glycosidic linkage (737). Although high concentrations of cyanogenetic glycosides have been reported present in the seeds of only a few species of plants, *i.e.*, those of *Rosaceae*, lesser amounts have been reported in almost every plant tissue from certain other plants. No general rule can be established with regard to the distribution of these compounds in various plant tissues. Cyanogenetic compounds are widely distributed, having been detected in more than 50 natural orders of plants, including ferns, basidiomycetes, and phycomycetes (737). Wherever cyanogenetic compounds are found in plants, specific hydrolytic enzymes are also known to exist, although a few exceptions are known (737). Also, there is some evidence that emulsin, ordinarily thought to be specific as a catalyst for the hydrolysis of all cyanogenetic glycosides, will hydrolyze certain of these compounds only with difficulty, and some are known to be unreactive in the presence of this enzyme (737).

Methods for the qualitative as well as quantitative determination of cyanogenetic glycosides in plant and animal tissues have been extensively reviewed by van der Walt, who considers such methods as the Guignard, Vortman, phenolphthalein, guaiac–copper, benzidine acetate–copper acetate, iodine–starch, Prussian blue, and the ferrous–uranic nitrate tests (735). The most widely used of these tests for the qualitative detection of cyanogenetic glycosides in plants has been the Guignard test. According to van der Walt (735), it is nonspecific since any volatile reducing agent such as hydrogen sulfide, sulfur dioxide, or aldehydes will give a positive test. Volatile reducing agents in plant extracts, however, are not frequently encountered and the Guignard test appears to be the simplest, most rapid, and accurate means by which the presence of cyanogenetic compounds can be established in plant specimens. This test, as described by Burnside (733), is conducted by placing about 2 Gm. of moist shredded plant material or crushed seeds in a small test tube, followed by the addition of 4 drops of chloroform (to enhance enzyme activity, see *reference 738*). Sodium picrate solution (5 Gm. Na_2CO_3, 0.5 Gm. picric acid, water *q.s.* 100 ml.; stable for 4 months if kept cool and well stoppered) is prepared and strips of filter paper are saturated with the solution. The strips then are blotted dry and inserted between

split cork stoppers which are then introduced into the neck of the test tube containing the reaction mixture. Care should be exercised to insure that the paper strips do not touch the inner sides of the test tube. The test tube and contents are then warmed at 30–35° for up to 3 hr. Large concentrations of HCN are detected within 15 min. as evidenced by a change in color of the yellow picrate test paper to various shades of red. Absence of a red color after 3 hr. is taken as a negative test.

Plant materials not analyzed for HCN at the time of collection can lose a large amount of their cyanogenetic glycoside content through spontaneous hydrolysis. Briese and Couch have shown that if chopped fresh plant material is stored in an aqueous mercuric chloride solution (1 Gm. $HgCl_2$ for each 100 Gm. of fresh plant material), the cyanogenetic glycosides present in the preserved specimens will remain stable for up to 6 months, presumably through an inactivation of the hydrolytic enzymes (739). van der Walt (735), however, claims that the relationship of mercuric chloride to the water volume in the preservative is more important than the mercuric chloride–plant material ratio for glycoside stabilization.

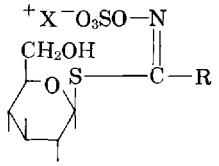
To insure that hydrolysis of cyanogenetic glycosides will indeed take place, some investigators have added emulsin (or other specific enzymes) to all plants being evaluated for the presence of these compounds. Other investigators have carried out the hydrolysis in the presence of buffer (740). Although it has been determined that cyanogenetic glycosides are hydrolyzed by dilute acids to yield HCN, this method has not been applied to the screening of plants for cyanogenetic glycosides (737). Concentrated mineral acids are to be avoided since HCN is not released under these conditions (737).

Although it appears that the Guignard test is adequate for screening plants for cyanogenetic glycosides, the precautions enumerated above should be taken into consideration if the results are to be meaningful.

Several screening programs have been conducted for the presence of cyanogenetic compounds in plants from Africa (735), Australia (423), Hong Kong (499), North Borneo (491), and the U.S.S.R. (741), in addition to others (734, 742–745).

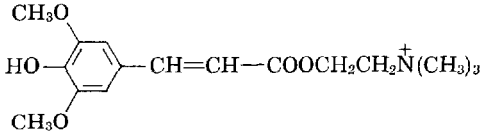
Isothiocyanate Glycosides

This group of interesting compounds will be discussed since plants containing isothiocyanates have been implicated in human as well as livestock poisoning (746), because of the antibacterial and fungistatic potential of the glycosides (747) and because of their unique, yet restricted distribution, which makes them valuable in chemotaxonomic studies (748–753). Isothiocyanate glycosides are found most abundantly in the *Cruciferae*, as well as in the related families *Capparidaceae*, *Moringaceae*, and *Resedaceae* of the Rhoeadales (746). Also, their unexpected occurrence in the taxonomically remote *Caricaceae*, *Euphorbiaceae*, *Limnanthaceae*, *Phytolaccaceae*, *Plantaginaceae*, *Salvadoraceae*, and *Tropaeolaceae* has been reported (746). Whenever mustard oil glycosides are found in a particular species, they appear to accumulate chiefly in seeds, as well as being found throughout the plant (746). The general type formula for these colorless, water-soluble compounds is illustrated.



Isothiocyanates

In all cases observed to date, glucose has been found to be the glycone moiety of isothiocyanate glycosides, thus substantiating the more common designation of this class of compounds as isothiocyanate glucosides. Because of the preponderence of potassium in plants, this element is usually found as the cation of the sulfuric acid residue. Sinalbin, the classical glucoside of white mustard, is unique in that it contains sinapine, a quaternary base, as its cationic moiety.



Sinapine

Myrosinase (myrosin), the specific enzyme catalyzing the hydrolysis of all isothiocyanate glycosides has been found in all plants containing this substrate. However, it accumulates in particular cells (idioblasts) which do not contain the glycosides (746). This enzyme has been stated to be most active at pH 6.5–7.5 and at a temperature of 30–40° (746).

About half of the known 30 or more natural mustard oils are nonvolatile isothiocyanates (cheirolin, erysolin, *p*-hydroxybenzylisothiocyanate) and as such are not steam distillable. Therefore, the practice of referring to this class of

compounds as "essential oils" should be discontinued.

Early methods for the detection of isothiocyanates consisted of hydrolysis followed by organoleptic evaluation of the liberated aglycone. As a result, only the volatile isothiocyanates were recorded. Today, however, with the availability of chromatographic techniques and rather specific detecting reagents, the analysis of plant material for isothiocyanates has become much more exacting. Although a number of microchemical tests for isothiocyanates and their glycosides have been reported (754–756), the preferred method for their detection in plant material appears to be through extraction of small samples (2–5 Gm.), followed by conversion of the natural *iso*thiocyanates into thiourea derivatives, and paper chromatographic separation of the mixture using water-saturated chloroform (757), ethyl acetate–water (758), 2-butanone–water (758), pyridine–amyl alcohol–water (759, 760), heptane–90% formic acid–*n*-butanol (759, 760), or *n*-butanol–ethanol–water (761). Visualization of the separated compounds is then accomplished by means of Grote's reagent (modified nitroprusside) (757), by silver nitrate (762, 763), or by the iodineazide reagent of Feigl (764) as modified by Kjaer (757).

A useful list of mustard oil-containing plants known up to 1938 has been published by Schmalfuss and Muller (765). Schneider (766) presents a reliable key to the older literature on isothiocyanates, and recent reviews on this topic have been published by Delaveau (748–750), Hegnauer (751), Zinner (752), and Kjaer (753, 767, 768). In addition, several surveys for isothiocyanates in plants have been conducted (767–773).

Essential Oils

Essential oils (volatile oils), in addition to their value as flavoring agents and perfumes, have been reported to have excellent antibacterial (201, 202, 204) and antifungal (202–204) properties.

A few reports have been published which include an evaluation of plant samples for the presence of essential oils (168, 491, 499, 500, 513, 774–778). For maximum efficiency, tests should be conducted on fresh material since most of the volatile constituents of plants are lost during drying. In most instances the methods that have been employed to detect essential oils have been crude since they have of necessity been conducted in the field as organoleptic examinations. More elaborate laboratory examinations have involved steam distillation followed by measurement of the water-immiscible oil and, in some cases, followed by the application of chemical tests for terpenes.

Arthur (491, 499, 500) studied more than 700 species in North Borneo and Hong Kong, and simply chopped a small amount of fresh plant with a razor, introduced this into a test tube, added hot water, and boiled the mixture. Any characteristic odor of essential oils was then recorded. Kohlmunzer (777) evaluated some 59 species of plants from genera known to have previously yielded economically important essential oils (*Salvia*, *Lavandula*, *Mentha*, *Rosmarinus*, *Thymus*, etc.). Following steam distillation and subsequent measurement of the separated oil, chemical tests for cineol were applied. On the other hand, Betts (779) has devised a method employing thin-layer chromatography for the evaluation of petroleum ether extracts, from umbelliferous fruits, in which essential oils are universally soluble. Fluorescein-treated plates of his extracts were first viewed under ultraviolet light to note the presence of dark quenching spots against a bright yellow background. Unsaturated compounds were then detected as spots by exposure of the plates to bromine vapor which converted the fluorescein to eosin, and subsequent ultraviolet examination then indicated unsaturated compounds against a dull background. Plates were then sprayed with 2,4-dinitrophenylhydrazine which revealed ketones and aldehydes as orange spots.

A simple microcohobation still for the estimation of quantities of essential oil ranging from 2–50 μl. in small (0.4 Gm.) samples of plant material has been shown to give accurate results and could be of value in screening large numbers of plant samples for essential oils on a quantitative basis (780). At least this would be some improvement over current organoleptic methodology.

SUMMARY

This review, to the best of this author's knowledge, is the first dealing with phytochemical and phytopharmacologic screening to be published. Therefore, out of necessity, a number of perhaps important topics were not discussed, and certainly some of those included were treated to a lesser degree than one might consider adequate.

It was the desire of this author to make several facts evident through a review of the areas covered in this manuscript: (*a*) that natural products have been, and still remain as, an interesting and important source of biologically active substances, the major sources of which remain untapped; (*b*) that although many methods are

available for the biological as well as chemical screening of plants, these are largely inadequate, and there is a great need for an increased interest in the development of improved methodology; (*c*) that a great deal of needless duplication of effort is evident in certain of the results presented in this review; and (*d*) correlations made and reported here point out the need for a restimulus of interest in this area of research.

The number of published reports on phytochemical and phytopharmacologic surveys is exceedingly small in view of the amount of effort that is undoubtedly being expended in these areas. Several explanations for this are suggested: (*a*) some investigators feel that this type of research should not be published because much of it represents so-called "negative" data; (*b*) others undoubtedly do not care to be associated with "screening" publications; and (*c*) a great deal of this work is being conducted in laboratories having a vested and commercial interest so that publication of their results would reveal methodology and approaches to this area of investigation that might be considered "unique" and/or classified. However, those reluctant to publish these types of data are usually exceedingly interested in hearing of, or utilizing similar results by others, in planning their own approach to phytochemical-phytopharmacologic problems, or in making certain correlations with their own data.

Natural product studies involving the search for new biologically active substances present unique challenges and problems not fully appreciated by many persons having only a limited appreciation and breadth of knowledge in biological areas. Many of the problems, either enumerated or suggested by studies reported here, can be solved if proper attention is given to the remedies. In the past, at least in this country, we have not had the organizational tools through which natural product studies could be considered in their own light. This is not true today, for with proper effort expended in its own behalf, the area of natural products can assume a more important place in the scientific community to serve humanity better.

Those of us interested in new biologically active phytoconstituents are constantly reminded, either verbally, through innuendo, or by means of restricted budgets and limited availability of research funds, of the "scientifically" unsound approaches that are used in our quest for new drugs. But, can methods other than those delineated in this review be suggested? And the work must continue.

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