THE LEMNACEAE, OR DUCKWEEDS

A REVIEW OF THE DESCRIPTIVE AND EXPERIMENTAL LITERATURE

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INTRODUCTION

As the simplest and smallest of flowering plants, the Lemnaceae are usually relegated to the category of botanical curiosities. It is easy to understand why inconspicuous plants of no economic importance should be so dismissed, but it would be difficult to overestimate their potential value as experimental organisms for morphogenetic, physiological and biochemical research. When fruitflies and breadmolds are contributing so much to the general field of genetics, the student of higher plants may well consider using organisms offering some of the same advantages.

The valuable characteristics of the Lemnaceae include their small size, rapid growth and relative structural simplicity. All can be grown in aseptic culture, simplifying work with organic compounds. Reproduction is usually vegetative, so that genetic variability can be eliminated by using a single clone for all experiments. Controlled conditions of temperature, light and nutrition are far easier to maintain than for most other angiosperms. Recently, control of flowering has been achieved in at least two species.

While some excellent experimental work has been done with the Lemnaceae, most of these characteristics have not been fully exploited. One reason may be that no general account of these plants has appeared since the last century. The purpose of this review is to provide one, particularly of work since that time, which may serve as a guide for future investigators. The major emphasis will be placed on experimental work, but not to the exclusion of the descriptive disciplines. The divisions employed are necessarily somewhat arbitrary, particularly in the experimental section where a given factor or process may have been studied from more than one point of view. While adequate coverage of all work with a particular group of plants must perforce touch most fields of botanical research, it is impossible to consider each of the problems in its general context; to do so would be to write an encyclopedia. Thus both the bibliography and the detailed text discussions have been limited strictly to work with the Lemnaceae, leaving the reader to supplement them from previous knowledge and from other specialized sources. The aim of this article is to approximate the compactness and efficiency of its subjects, though it is admittedly not the smallest and simplest of reviews.

DESCRIPTIVE

SYSTEMATICS

All the Lemnaceae are floating plants found at, or just below the surface of, relatively still fresh water. The individual green structures, never more than one centimeter long, are usually called "fronds" to avoid commitment as to their derivation. Although this term may be misleading in suggesting a pteridophyte relationship, it has been common in the literature for at least 80 years (cf. Dutailly, 1878) and will be adopted here. The term "Glied" (cf. Landolt, 1957), member or limb, may be preferable, but is found only in German.

TAXONOMY AND NOMENCLATURE. Four genera are usually recognized: *Spirodela, Lemna, Wolffiella, Wolffia.* The fronds of *Spirodela* and *Lemna* are flat, more or less oval in outline, and leaf-like. *Spirodela* bears two or more thread-like roots on each frond, *Lemna* one only. The two genera have been grouped in a tribe (Lemneae) (Hegelmaier, 1895) or subfamily (Lemnoideae) (Lawalrée, 1945); *Spirodela* has also been considered a subgenus of *Lemna* (Hutchinson, 1934). *Wolffiella* and *Wolffia* (Tribe Wolffieae, Hegelmaier; subfamily Wolffioïdeae, Lawalrée) are thalloid and have no roots. *Wolffiella* fronds are normally much longer than broad (e.g., 6-8 by 1-2 millimeters) and strap- or sickle-shaped, while *Wolffia* fronds are from 1 to 3 millimeters long and egg- or boat-shaped; Mason (1938) questioned their separate generic status.

The entire family is in need of modern taxonomic treatment. The last full discussion was that of Hegelmaier in 1895; Thompson revised the North American species in 1898. It is perhaps unavoidable that many of the diagnostic characters chosen, such as seed ribbing or fruit shape, are nearly useless for a family in which flowering is relatively rare, but certain specific and varietal characteristics also designated by Hegelmaier (1868, 1895) are clearly undependable due to their physiological plasticity (cf. Thompson, 1898; Hicks, 1937; Mason, 1938; Landolt, 1957). For this reason no attempt will be made to present the detailed taxonomy of the group. Hicks (1937), Fernald (1950), Fassett (1957) and Mason (1957) provide keys to most of the North American species; the drawings and descriptions in the last are particularly useful. Since many species of *Spirodela* and *Lemna,* at least, are cosmopolitan, these keys may be adopted for use elsewhere.

Some nomenclatorial matters which may cause confusion on reading the literature will be briefly mentioned. "Lemna major" is *S. polyrrhiza*. *Wolffiella* is classified under *Wolffia* in most of the older literature. "Bruniera" is *Wolffia*. "L. cyclostasa" is properly *L. valdiviana* according to Fernald (1950) and Mason (1957), although Landolt (1957) still used the former name. Much of the experimental work in Japan has been done with *L. paucicostata;* Thompson (1898) found that American collections of this species were indistinguishable from *L. perpusilla,* so that the status of the former species is in doubt. At least two distinct strains of *L. minor* have been collected in the United States; these differ in size and in ability to flower in culture (Landolt, 1957). *L. perpusilla* and non-gibbous forms of *L. gibba* may easily be mistaken for *L. minor* (cf. Mason, 1957), and such mistaken identifications are probably preserved in the physiological literature. Even *S. oligorrhiza* has been called *L. minor,* although the error was later corrected (Thimann and Skoog, 1940; Thimann and Edmondson, 1949).

In view of such problems, all strains used for experimental work should be preserved alive in culture, both for purposes of identification and for later attempts to repeat or continue the work in question. Any future taxonomic treatment of the family should also make use of varying culture conditions to ensure that characteristics chosen as diagnostic are at least relatively stable to environmental changes.

PHYLOGENY. The Lemnaceae are monocotyledons, and most investigators place them in the order Spathiflorae (Arales), relating them to the Araceae through the water-lettuce *Pistia*. This concept was already embodied in Hegelmaier's monograph (1868); the four genera can be read as a reduction series with the Lemneae primitive and the Wolffieae advanced, perhaps in the order *Spirodela, Lemna, Wolffiella, Wolffia* (Thompson, 1898, Brooks, 1940). A *Spirodela*-like form with ovule number and structure similar to *L. gibba* might be regarded as ancestral; no such form exists at present (Hegelmaier, 1895). The homologies of the family (especially *S. polyrrhiza*) to *Pistia* have been set forth in detail by Engler (1877), Arber (1919) and Brooks (1940), and will be discussed further when the nature of the frond is considered.

Strong disagreement with the Araceae-*Pistia* phylogeny has been recorded by Lawalrée (1943, 1945). Modifying the concept of Goebel (1921) that the frond of Lemnaceae represents a monocotyledonous embryo, Lawalrée considers the Wolffieae primitive. Homologizing cer-

tain lamellae present in the early vegetative development of *S. polyrrhiza* to the "squamulae intravaginales" of the Helobiae (Najadales), he revives the idea of "notre compatriot DuMortier" that the Lemnaceae properly belong in that order, suborder Potamogetoninae, near the Najadaceae (1945). Evidence from habitat and from embryo and flower development are also used to support this view. Particularly important is the subsequent report (Lawalrée, 1952a) that the early development of the endosperm in *L. minor* is helobial—that is, nuclear or acellular.

The bulk of embryological evidence, however, supports the Araceae relationship and specifically contradicts the report of an helobial endosperm. In an unpublished dissertation not even directed at this controversy, Brooks (1940) showed clearly that the endosperm of *L. minor* is cellular from the beginning. More recent work on both *Lemna* and *Wolffia,* as well as on the Aracean *Arisaema,* fully confirms the older view (Maheshwari 1954, 1956a, 1956b; Maheshwari and Khanna 1956). On the endosperm of *Wolffia,* Maheshwari states that the data "preclude any close relationship between the Lemnaceae and the Helobiales . . . on the contrary, they support the view that the Lemnaceae are closely allied to the Arales" (1956a). Still more recently, embryological evidence has been presented that *S. polyrrhiza* occupies a true intermediate position between the Araceae and the other Lemnaceae genera (Maheshwari, 1958).

CHROMOSOMES. The few data on chromosome numbers have not elucidated the relationship of the Lemnaceae to other groups or the taxonomic problems within the family itself. Counting somatic (root tip) chromosomes, Blackburn (1933) found $2n$ equal to 40 in *S. polyrrhiza* and *L. minor,* 44 in *L. trisulca,* 64 in *L. gibba,* 28 in *Pistia.* All the Lemnaceae chromosomes were extremely small, averaging 0.1 μ by 0.5 μ in *L. trisulca. Wolffia* (somatic, frond tissue) appeared to have about 50 chromosomes "inextricably tangled at the metaphase and considerably larger" than those of the Lemneae. Lawalrée (1943) counted 44 or 46 for *Wolffia arrhiza.* Brooks (1940) found n equal to 21 in microsporocytes of *L. minor,* and suggested that Blackburn may have missed a pair of very small chromosomes. A survey of chromosomes in all the genera should have considerable taxonomic value.

MORPHOLOGY AND ANATOMY

THE NATURE OF THE FROND. The simple structure of the Lemnacean frond has occasioned much controversy over its derivation. The Lemneae

produce new ("daughter") fronds from two pockets on each side of the narrower end of an older ("mother") frond, very near the point at which the root or roots arise (the "node"). This end of the frond is usually designated as "proximal," since, in an attached daughter frond, it is the portion closest to the mother; the wider end is denoted "distal." Each daughter frond becomes a mother in its turn, usually while still attached to its own mother; such groups of attached fronds may be called "colonies". In addition to being less highly differentiated, fronds of the Wolffieae have only one reproductive pocket; daughter fronds are produced asymmetrically to the main axis in *Wolffiella* and along it in *Wolffia*. Colonies in *Wolffiella* may consist of large numbers of individuals in stellate or wheel-shaped clusters (Hicks 1937, Mason 1957). *Wolffia* colonies rarely consist of more than three visible fronds.

In all four genera, each mother frond produces a considerable number of daughters during its lifetime, the exact number depending on various factors. In *Lemna and Spirodela,* daughter fronds are produced alternately from side to side, developing earlier in one pocket than in the other. Clones of the same species differ as to which pocket is "plus", producing the first daughter, but this normally remains constant within a clone. When flowering occurs, the inflorescence always appears in the "minus" pocket (cf. Hegelmaier 1868; Kandeler 1955; Landolt, 1957).

The asymmetry is already present in the embryo, and must be determined at a very early stage. If either "right"- or "left-handed" clones of *L. gibba* are allowed to self-pollinate and the seeds germinated, the resulting new clones show about a 1:1 ratio of right- to left-handedness, irrespective of parentage, suggesting that this is a random effect not under direct genetic control (Kandeler, personal communication; Hillman, unpub.).

Discussions of the relationship of these structures to a more typical monocotyledonous organization are necessarily connected with, and subject to, the same difficulties as the phylogenetic problems outlined earlier. These difficulties are basically those of describing with any good probability events which may have happened in the past and can neither be observed nor modified in the present. The frond has been viewed as entirely axial, with traces of leaf-like appendages remaining only in *Spirodela* (Hegelmaier 1868, 1895) or as fundamentally leaf-like, with certain embryonic characteristics (Goebel, 1921; cf. Lawalrée, 1943). Most writers have suggested a complex structure representing both leaf and stem: a modified shoot (Caldwell, 1899); a structure similar to

the *Erythronium* runner (Blodgett, 1915); or a structure homologous in all its parts to that of the sympodium of *Pistia* (Engler, 1877; Arber, 1919; Brooks, 1940). For detailed expositions, see the references cited.

The concept of reduction from a *Pistia*-like organization is attractively consistent with other evidence on the phylogeny of the family; this may or may not bear on its accuracy. A major advantage is that the reproductive pockets might easily be derived from similar structures in *Pistia* (Arber, 1919). Other hypotheses have to provide special explanations for these characteristic organs—for example, formation of laterals by the splitting of a terminal bud meristem early in development (Blodgett, 1915). In addition, the homologies to *Pistia* as outlined (with differences in detail) by both Arber and Brooks would necessitate the observed asymmetry in flowering habit, since the meristems within the two pockets of *Lemna* or *Spirodela* would have slightly different origins. Under this general hypothesis, the greater (distal) part of a *Lemna* or *Spirodela* frond represents a flattened petiolar leaf (phyllode), while the proximal end represents fused leaf sheaths, axial tissues and ligules as well as an inflorescence when present.

Goebel's (1921) interpretation of the frond is based on the idea that the main shoot never develops in the seedling—the distal portion represents a cotyledon; the proximal, a hypocotyl; the side members (daughter fronds) continue to repeat this pattern. Here the "tendency to asymmetry" is stressed as an important characteristic of the family, but no explanation of its origin is advanced. Lawalrée (1943) has modified this concept in a manner consistent with his hypothesis of helobial affinities, and considers the complexity of the Lemneae as derived anew from the simple, embryo-like Wolffieae. The observation that the young embryo of *Lemna minor* closely resembles an adult *Wolffia* frond (Lawalrée, 1952) is also used as evidence here, but most workers derive the Wolffieae by further reduction from the Lemnaceae pattern. It seems to the writer that any hypothesis reading the Lemnaceae as a series advancing with increased complexity has to explain away the increasing vascularization in the direction Wolffieae to Lemneae, a trend of no evident selective value given the environment and size of the plants.

FROND AND ROOT ANATOMY. For detailed drawings and descriptions see Hegelmaier (1868) and also Lawalrée (1943). With the exception of reduced vascular tissue in the Lemneae, and of the meristematic areas within the reproductive pockets, the bulk of the frond

is composed of chlorenchymatous cells, often separated by large intercellular spaces. These spaces are filled with air or other gases and provide buoyancy; they are particularly prominent in certain forms of *L. gibba* (see Hegelmaier, 1868, plate XIII). Certain cells in *Lemna* and *Spirodela* are filled with needle-like raphides, presumably calcium oxalate.

The upper epidermis in the Lemneae differs considerably from the lower (except in the submerged vegetative fronds of *L. trisulca*), being highly cutinized and unwettable. Stomata are located on the upper surfaces of all four genera, in *Wolffiella* perhaps not in strictly vegetative plants (Mason, 1938). Dotted about the upper epidermis of many Wolffieae are brown or reddish-colored "pigment-cells" which become particularly visible upon drying. *Spirodela* and some species of *Lemna* may form large amounts of anthocyanin pigments, and these have been the subject of several experimental studies.

Both *Spirodela* and *Lemna* fronds have greatly reduced vascular bundles. These typically have one xylem element above and one sieve element, with two companion cells, below (Schenck, 1886: *L. trisulca*). The xylem elements are elongated cells with spiral or ring thickenings; they usually occur in single file and are surrounded by other elongated but parenchymatous cells. A large bundle runs from the node toward the proximal end of the frond; under certain conditions, daughter fronds may remain attached to the mother by this bundle alone. In *S. polyrrhiza* the proximal end of the bundle is clearly free from the end of the frond for a short distance, so that bundle and frond together form an asymmetrically peltate structure. At the node, several bundles diverge into the distal portion of the frond, forming the veins or "nerves" of the taxonomists. There are five or more nerves in *S. polyrrhiza* and one to three of them in most *Lemna* species (cf. Sargent, 1957). There is no information on the function of the vascular system in these plants. No such system is present in the Wolffieae except occasionally as very small traces in the filament.

Roots in both *Spirodela* and *Lemna* are regarded as adventitious, and arise at the node just beneath the lower epidermis. Often the epidermis forms a short sheath about the upper few millimeters of root or roots, and a prominent root-cap is usually developed. The roots are usually less than 0.5 millimeters in diameter; the length varies widely from species to species and depending upon environmental conditions. The

writer has seen *L. minor* roots 14 centimeters long, but most are much shorter.

In cross-section the roots show a reduced vascular strand similar to that in a frond nerve and surrounded by a ring of elongated cells. These are in turn surrounded by larger, more isodiametric cortical cells which include symmetrically arranged air spaces, and by an epidermis.

Many root cells contain chloroplasts, and these are apparently active photosynthetically (Pirson and Göllner, 1953). There are no root hairs. Since the entire lower surface of Lemneae fronds can absorb nutrients from the medium, and the plants can grow well under conditions which entirely prevent root elongation, the functional importance of the roots is difficult to evaluate. It has been suggested (cf. Arber, 1920) that they serve chiefly as anchors to keep the fronds right side up, and to form the tangled masses which are of some significance in dispersal, and protection from water motion.

This brief outline indicates that although the gross morphology and vegetative reproduction of the Lemnaceae are somewhat unusual, their anatomy, particularly the prominent air spaces and reduced vascular structures, resembles that of many aquatic angiosperms.

FLOWERS. For detailed descriptions of flowers and flower development, see Hegelmaier (1868, 1895), Thompson (1898), Caldwell (1899), Brooks (1940) and Lawalrée (1952); for photographs, see Saeger (1929). There is some question as to whether one should refer here to an inflorescence composed of pistillate and staminate flowers or to a single flower, a difficulty which the reader will recognize by now as typical of the Lemnaceae literature. Since most authors do not consider the anatomical evidence conclusive, the second view will be adopted for the sake of simplicity. The flower arises in or near the same meristematic area which produces daughter fronds. As far as is known, a frond produces only one flower in its lifetime; the primordia are usually well-differentiated while the frond itself is still young, even entirely enclosed within the mother frond.

In the Lemneae the flower arises only in the minus reproductive pocket (see above). Each flower consists of one pistil and usually two, occasionally one, very rarely three (in *Spirodela*), stamens. These organs are surrounded during development by a membranous sack-like "spathe" typically only one cell layer thick and either opening or torn towards maturity. The pistil usually matures earlier than the stamens,

and one stamen earlier than the other. The flask-shaped pistil has a stylar canal which opens at the stigma; a large pollination drop is exuded at maturity. Both pistil and stamens turn up above the water surface as they grow out of the reproductive pocket. The ovules are anatropous to orthotropous, usually one or two, but as many as six may occur, particularly in *L. gibba.* The anthers are biloculate. A vascular bundle runs from the frond node to the base of the flower and there diverges into three traces, one to the pistil and one to each stamen.

Flowers in the Wolffieae differ in having a single stamen, a single always orthotropous ovule, and in the absence of a "spathe". Occasionally stamens of certain *Wolffia* species may show very reduced vascular traces. The flower usually projects directly upwards out of a floating frond. Flowering in *Wolffiella* has been observed and described by Giardelli (1935) and Mason (1938). Maheshwari (1954) has published a detailed account of flowering and fruiting in *Wolffia microscopica.*

Lemnaceae pollen grains are small (10-25 μ in diameter) and spinulose. Erdtman (1952) has figures of *L. gibba* and *L. trisulca* pollen; see also the other references above.

EMBRYOLOGY; SEEDS. The embryology of the Lemnaceae has been examined frequently, but Brooks (1940) and Maheshwari (1954, 1956a, b, 1958) provide the most reliable recent information, including critical considerations of the earlier literature. Embryo sac development in *Lemna* and *Wolffia* is bisporic, of the *Allium* or *Scilla* type; the mature embryo sac is 8-nucleate. *S. polyrrhiza* embryo sac development is monosporic, and the basal apparatus shows suggestions of a more aggressive development than in the other genera. All have cellular endosperm. In *L. minor,* root development begins in the embryo, from the first frond primordium (Hegelmaier, 1868; Caldwell, 1899, Brooks, 1940; Lawalrée, 1952a), but not in *L. perpusilla* (Blodgett, 1923; Brooks, 1940) or *L. paucicostata* (Maheshwari, 1956). There is no evidence of root development in the Wolffieae.

The fruit is a thin-walled bladdery utricle containing one to several variously ribbed seeds. These are characterized by a prominent operculum at the micropylar end, noted by Hegelmaier (1868) in *Lemna* and since observed in all the genera. Several detailed descriptions of seed germination have been given (Hegelmaier, 1868; Caldwell, 1899; Goebel, 1921; Blodgett, 1923; Mason, 1938; Maheshwari, 1956) and differ somewhat in the morphological values assigned to various por-

tions of embryo and seedling. Several vegetative generations of fronds may be present in fully developed *Lemna* embryos; flowering can occur in young *L. perpusilla* seedlings still attached to the seed coat (Blodgett, 1923).

Natural History

Geographical and Geological Occurrence. The family is world-wide. About half of the 20 to 30 species are primarily tropical or subtropical, but the others occur in temperate regions. *S. polyrrhiza, L. gibba, L. minor, L. perpusilla* (if identical with *L. paucicostata,* see above) and *L. trisculca* appear to be cosmopolitan, with the last, however, restricted to cooler areas. Most other *Spirodela* species are from Australasia, and the Wolffias from American, African and Asiatic tropics; *Wolffiella* is primarily a new-world genus (Hegelmaier, 1868, 1895). However, a new species of *Spirodela* has been described from South America (Koch, 1932). Saeger (1934) has reported *S. oligorrhiza,* an Australasian species, from Missouri, but suggests its probable escape from cultivation in fish ponds. Further investigation might show more of the species as world-wide or nearly so, even without recourse to such devices.

Lemnaceae fossils are first found in the Upper Cretaceous deposits of the Rocky Mountain area (Moret, 1943; Bell, 1949), and relatively freely thereafter.

Habitat; Physiological Strains. Cursory observations on habitat abound in the literature, but there are few sources of precise, quantitative information. Chief among these is the work of Landolt (1957) who studied many species throughout California. The discussion below is drawn primarily from this source. See also Hicks (1937) and Luther (1951) for other details on habitat. Arber (1920) provides a good general treatment of Lemnaceae ecology in Britain.

The Lemnaceae occur in still or slightly moving water; more rapid motion sweeps them away unless they become entangled in anchored plants or debris. Growth may also continue for sometime out of the water on wet mud (Guppy, 1895). Flourishing growth is frequently found in stagnant small ponds or ditches rich in organic matter, or near sewer outlets and the like (Hicks, 1937; Rao, 1943; Luther, 1951). Some species also occur in brackish water; according to Landolt, a salinity of 0.05 molar is the upper limit tolerated, but Luther reports ca.

2.5 per cent (0.43 molar as NaCl) as the upper limit for *L. minor,* and even higher values for *L. trisulca.* Species differences with respect to optimum mineral concentrations may result in seasonal successions in which, for example, *L. minor* and *L. gibba* will give way to *Wolffia punctata* as the mineral concentration falls. All species appear to grow well within the pH range of 4.5–7.5, with outer limits at 3.5 and 8.5. There may be small differences in the pH tolerance of various species (Hicks, 1937).

Lemnaceae are found at all light intensities from full sunlight to below 50 foot-candles (noon intensity) in dense shade, with considerable differences in the apparent preferences of various species. *L. gibba* and *L. perpusilla* are notably sun-plants. Different strains of the same species may show different light-requirements under experimental conditions (cf. White, 1936c). Growth often continues until the water surface is covered with several layers of plants, with the lower plants still growing. *Wolffiella lingulata* can grow under a one-centimeter-thick layer of other Lemnaceae. It is probable that at least some of the growth under these conditions is supported heterotrophically at the expense of dissolved organic matter.

Temperature requirements of various strains and species differ, particularly in the minimum which can be survived. In general, temperatures between 20° and 30° C. are most favorable to growth, and occur during most of the growth period; temperatures near or above 30°C. are easily reached in the upper few millimeters of water in full sunlight. *S. polyrrhiza* and *L. perpusilla* have high temperature optima, and *L. trisulca* is low among the group; their local and geographical distribution varies accordingly.

Landolt (1957) studied 60 strains (clones) comprising 12 species, both in the field and under controlled (aseptic) environmental conditions; he concluded that few ecological races are present within the Lemnaceae. In most cases, larger physiological differences were observed between species than between strains within a species, but with the notable exception of *L. valdiviana,* in which the various strains showed few physiological characteristics in common. The strain differences within a species did not appear to be of any ecological significance, but probably arose by chance and were perpetuated vegetatively. The existence of these strains is, however, of considerable importance for experimental work, particularly on processes such as flowering, since work done with one strain may not be precisely repeat-

able with another. The situation is in part analogous to that obtaining in micro-organisms, and for this reason stock cultures of any strain used for experimental work should be maintained and kept available for future investigators.

FLOWERING AND FRUITING. Reports of flowering and fruiting have been reviewed and summarized by Saeger (1929) and Hicks (1932a). Since then flowering has been reported for the first time in *Wolffiella* (Giardelli, 1935; Mason, 1938) and in *Wolffia microscopica* (Maheshwari, 1954), so that almost all species have now been observed in flower. There is little information concerning the conditions inducing flowering in nature, and probably no general statement for the entire family would be valid. Certain species, particularly *L. gibba* and *L. perpusilla,* are frequently found in both flower and fruit (Landolt, 1957); it is significant that these two are the only species in which experimental control of flowering has been unequivocally achieved (Kandeler, 1955; Hillman, 1958). Flowering in other species, notably *S. polyrrhiza,* is extremely rare. It would be unwise to conclude for any species or strain, however, that "the ability to produce flowers apparently has been so completely lost that probably they are never produced by plants in nature" (Hicks, 1932a); this statement was made about *Wolffiella,* which may flower and fruit abundantly (Mason, 1938).

Photoperiodism and high temperatures have been implicated in flowering in nature (Kandeler, 1955; Landolt, 1957). However, several attempts to affect flowering by daylength and temperature have proved unsuccessful with most or all of the species used. While at least six species flower regularly in nature in California, only three of these, and only some strains of them, flowered under a large variety of controlled conditions in aseptic culture (Landolt, 1957). Crowding, light intensity or changes accompanying drought are other factors which have been suggested. Much of the speculation in the literature has dealt with the possibility that unknown changes in the composition (inorganic or organic) of pond water may induce flowering. It has often been observed that a pond with one species in flower will frequently have several others in flower as well, while the same species together in nearby ponds may be entirely vegetative (Saeger, 1929; Hicks, 1932). Whatever the factors responsible, when flowering occurs it is normally in summer or late spring. Most writers report that flowering fronds and colonies are often distinctly different in size and shape from the vegetative; although Landolt (1957) demurs, the writer's observations

on *L. gibba* and *L. perpusilla* in culture agree with the older view, particularly in noting an increased frond asymmetry accompanying flowering.

The mechanism of pollination is not clear. Most of the workers cited by Knuth (1909) agree that in *Lemna* the pistil matures before the anthers dehisce; the stigma may remain receptive even after dehiscence, however, so that self-pollination is possible. Pollen might be carried from frond to frond by various agencies: wind, water and the small fauna associated with masses of Lemnaceae have been suggested. The spinulose character of the pollen has been cited as evidence for entomophily (cf. Blodgett, 1923), and the large droplet usually secreted by the stigma may function to attract insects.

Cross-pollination is clearly unnecessary for certain strains of *L. gibba* and *L. perpusilla* which will fruit freely in single-clone cultures, but it is not known whether each individual flower self-pollinates or whether floating pollen from neighboring fronds is required. These two species fruit most freely in nature. Species which rarely fruit may be self-sterile and thus fruitless due to the fact that all the fronds in a small pond often belong to a single clone (Landolt, 1957). In addition, the embryo sac of *L. minor* frequently disorganizes before fertilization can take place (Caldwell, 1899).

Fruits may float or sink, and the seeds may germinate either on the surface (occasionally while still attached to the parent frond) or below it; if the latter, development of air spaces soon floats the young seedlings (Hegelmaier, 1868).

DORMANCY. When either drought or low temperatures bring about conditions unfavorable for growth, many species of Lemnaceae are able to persist until growth is again possible. In some cases this may be in the form of seeds *(L. gibba, L. perpusilla)* or as ordinary fronds *(L. trisulca)*, but many species also produce more or less specialized turions, or resting stages, which are more resistant than normal fronds. Turions of *S. polyrrhiza* are the most specialized and best known. These are modified fronds smaller and thicker than the normal, and dark green or purple; air spaces are reduced or absent, and the cells are heavily loaded with starch grains. There may be a few unelongated roots (Hegelmaier, 1868; Biscoe, 1873; Guppy, 1895; Jacobs, 1947). Some species of *Lemna* produce similar turions, but usually less modified from the normal fronds than those of *S. polyrrhiza* (Thompson, 1898; Hicks, 1937; Landolt, 1957). *L. gibba* turions are thin fronds

entirely without air-spaces (Guppy, 1895), and *Wolffia* turions are usually similar to normal fronds but heavy with starch (Hegelmaier, 1868; Landolt, 1957).

All of these structures sink to the bottom or remain imbedded in mud until favorable conditions resume. Although they have been called "winter buds", especially in *S. polyrrhiza,* this term is misleading, since they are formed from early summer onward as well as in completely frost-free climates (Thompson, 1898; McCann, 1942; Jacobs, 1947). Guppy (1895) thought that turion formation was favored by high temperatures and light intensities; several recent experimental studies have dealt with turion formation and will be discussed later.

LEMNACEAE AND OTHER ORGANISMS. A heavy growth of Lemnaceae modifies the aquatic environment beneath it, reducing gas exchange with the air and photosynthesis by submerged plants. The low oxygen tension is associated with a high organic content, particularly organic nitrogen, and such conditions tend to favor the growth of blue-green algae (Myxophyceae or Cyanophyceae) (Stephanova, 1928; Rao, 1953).

Various Lemnaceae are found in all possible combinations with each other and with other floating plants such as *Azolla, Riccia, Eichhornia* and *Nymphaea;* emergent or slightly submerged plants may serve as anchorage in areas where the plants would otherwise be swept away (Hicks, 1937; Jacobs, 1947; Landolt, 1957). While tall plants usually shade out Lemnaceae entirely, *L. paucicostata* and others may occur in rice fields as weeds sufficiently troublesome to warrant the development of selective herbicides (Yui and Koike, 1955; Yui, 1956).

Mats of Lemnaceae harbor a varied fauna of small invertebrates; this has been studied chiefly in the northern U. S. A large number of insects are obligate or facultative associates of *Lemna.* Among the former are the ephydrid fly *(Lemnaphila scotlandae)* and the rhyncophorous beetle *(Tansyphyrus lemnae)*; eggs are laid on the fronds which then serve as food for both larvae and adults. Facultative associates include aphids, collembolans, thysanurans and several carnivorous forms as well as spiders. All may play a part in pollination while moving from frond to frond. In addition, hydras, flatworms *(Euplanaria)* and snails (e.g., *Physa*) are also common just beneath the surface; the latter feed freely and may oviposit on the fronds. Jacobs (1947) noted that *Physa* ate *S. polyrrhiza* fronds but not the turions. Combinations of Lemnaceae (particularly Wolffieae) and *Azolla* together can be ef-

fective in preventing mosquito breeding, but apparently not Lemnaceae alone (Ancona, 1930).

Duckweeds are used as food by a number of vertebrates including, of course, ducks and other water birds, fish, particularly carp *(Cyprinus),* and muskrats *(Ondatra)* (Hicks, 1937; Jacobs, 1947). Both birds and muskrats play a role in dispersal, for plants clinging to feet, fur or feathers can be carried overland, particularly in humid weather when the fronds do not dry out (Jacobs, 1947).

The wisdom of lemnophagy appears to be confirmed by recent reports that, at least for farm animals, the nutritional value of these plants compares favorably with that of alfalfa (Lautner and Müller, 1954; Müller and Lautner, 1954).

EXPERIMENTAL

Introductory

Extensive use of Lemnaceae as experimental materials began in the 1920's, involving either *L. minor* or *S. polyrrhiza.* Much of the early work dealt with mineral nutrition and the "auximone" question, to be outlined below, and many of the papers are of historical interest only. For the first two decades, most of the work came from the laboratories of E. Ashby in Great Britain, N. A. Clark in Iowa, and A. C. Saeger in Missouri.

Aseptic Culture Techniques. Hansteen (1899) and Mameli and Pollacci (1914) reported successful sterilization of living *Lemna* fronds, but true aseptic culture techniques were introduced by Clark (1930) and Saeger (1930); they have been used in most investigations since that time with the inexplicable exception of work in Britain. Once a single aseptic frond is obtained alive, its offspring can be subcultured indefinitely under aseptic conditions. The various techniques used in the early work to maintain asepsis need not be considered here, since standard bacteriological and tissue-culture methods can be used, essentially unmodified. Methods of obtaining aseptic fronds have included repeated washings with sterile water, treatment with ultraviolet radiation, and immersion in solutions of hypochlorite (sodium or calcium) or potassium mercuric iodide. Probably the best procedure is as follows:

Wash fronds of the desired strain vigorously in tap water to remove excess algae and debris, and place them in a mineral medium

supplemented with 0.2 percent sucrose and 600 mg/L tryptone, casein hydrolysate or other source of organic nitrogen. After several days in such a medium, bacterial growth will be heavy, and few if any resistant spores will remain ungerminated. Now immerse fronds for varying lengths of time (30 seconds to 5 minutes) in a 0.5 percent hypochlorite solution (1/10 dilution of most household bleaching products such as Clorox, Purex, etc.) and transfer directly to a sterile medium with organic supplements; rinsing is usually unnecessary. Some of the fronds will survive the hypochlorite treatment and be aseptic.

This method is substantially like that used for many strains by Landolt (1957) except that the pretreatment in supplemented medium makes sterilization of even delicate submerged forms, such as *L. trisulca,* relatively easy by bringing most of the microorganisms out of resistant stages into active growth.

Permanent stock cultures should be maintained on media enriched with sugar and organic nitrogen sources to ensure detection of contaminations, and kept at low temperatures and light intensities to obviate the need of frequent transfers. Medium solidified with agar will support good growth, and is useful if the cultures are to be transported.

GROWTH MEASUREMENTS. Growth of Lemnaceae cultures can be followed by taking fresh or dry weights, and various simple techniques for this appear throughout the literature. Dry weight measurements can be misleading, since they frequently reflect primarily changes in starch content rather than growth (Gorham, 1950). Probably the most reliable measures of growth are those representing increases in frond number: the simplest such value is merely the percentage increase in frond number over a given time. Under conditions giving approximately exponential increases, a growth constant, K, can be derived by calculating the slope of the line representing the logarithm of the number of plants on successive days (Clark, 1925). K and another value frequently used, "multiplication rate" (MR), are defined and related as follows:

$$K = \frac{MR}{1000} = \frac{\text{Log}_{10}\,(Fd) - \text{Log}_{10}\,(Fo)}{d} = \frac{\text{Log}_{10}\,\frac{[Fd]}{[Fo]}}{d}$$

Fo is the original frond number, Fd the frond number on day d, and d the number of days involved. Thus an MR of 301 (K = 0.301)

represents a doubling of frond number each day, since 0.301 is Log_{10} 2; and an MR of 150 represents a doubling every two days, and so forth. It is important to recognize that these values are really appropriate only for exponential growth; under other conditions a statement of percentage increase conveys as much information. Most workers, however, have used MR indiscriminately because of the ease with which values from one period may be compared to those from another.

For all values depending on frond number it is best to count all fronds, no matter how small, visibly projecting beyond the mother fronds, in order to avoid subjective decisions as to frond maturity. Note that MR and K are expressions of change in frond number, not number of colonies. The average number of fronds per colony is another value which may be of importance under certain conditions.

Total and average frond areas have frequently been followed in *Lemna* and *Spirodela* cultures. Such values may be obtained by projecting images of the floating fronds onto a ground glass screen on which tracings can be made for subsequent measurement, or by focussing their shadows directly onto a photocell and relating area to the decrease in light transmitted (Ashby, Bolas and Henderson, 1928; Ashby and Oxley, 1935). A further refinement is to photograph the fronds for subsequent planimetric or photoelectric estimations of area (Gorham, 1941). In any such procedure, the fronds should not overlap.

Measurements of root length are also encountered in the literature. Individual plants may be followed from time to time with a horizontal microscope (Pirson and Göllner, 1953a, b); however, when an estimate of average root length in a treatment is desired, some principle of selection must be followed, such as measuring the length of the longest root on each of ten colonies taken at random from each culture (Hillman, 1954).

All values, including weight, multiplication rate and frond area, are usually derived from more than one culture per treatment, and may be subjected to various statistical procedures as appropriate. Landolt (1957) found that three replicate cultures would usually give reliable estimates of multiplication rate.

Due to the small size of Lemnaceae, measurements of respiration and photosynthetic activity can be conducted by standard manometric techniques without significant modifications.

KINETICS OF GROWTH IN LEMNACEAE CULTURES. The fact that frond multiplication is exponential under many conditions may lead

to the notion that growth in *Lemna* cultures precisely resembles that in a bacterial, yeast or algal culture. This is not so. Under optimal conditions the individual microorganisms will continue to divide and produce essentially equivalent daughter cells with at least potentially unlimited capacities to continue to do so. A cell O becomes two cells (A, B) which in turn become four cells (A1, A2, B1, B2); after three generations all eight cells (A1A, A1B, A2A, . . . etc.) are of the same age, ideally at least, and none of the original cells is present. In a *Lemna* culture, however, each frond can produce only a certain number of daughter fronds before it dies, and the early daughter fronds produced are not equivalent to those produced later by the same frond. A discussion of this phenomenon will appear later. In addition the "generation" concept in a *Lemna* culture is not like that described above. A frond O gives rise to two daughters (A, B). Each of these may then produce two daughters—A1, A2 and B1, B2—but O meanwhile is producing frond C. Thus at the 8-frond stage the culture has, morphologically speaking, four "second-generation" fronds (A1, B1, A2, B2), three "first-generation" fronds (A, B, C) and the inoculum frond O. O can continue to produce fronds D, E and F; while these have developed after the first four "second-generation" fronds, and may often show the effects of new medium, growth regulators, or flower-inducing conditions more strongly than they, morphologically they are simply late daughter fronds of the first generation. Though confusing, such considerations must be borne in mind throughout; the similarity between Lemnaceae cultures and those of microorganisms reproducing by division is more apparent than real. The concept of "generation time" (cf. Clark, 1925) is thus completely misleading, and should be avoided.

It has been observed by many workers that the stock conditions from which experimental inocula are taken may strongly affect the results, particularly in short-term growth studies. This is of course a reflection of the fact that the development of a given frond starts long before it becomes visible in the culture. It is thus good practice to take all the inoculum fronds for a given experiment or series from the same stock conditions, and to keep these conditions as close as possible to the experimental conditions under study. In most aseptic work, cultures are usually started with one to several colonies. In spite of physiological differences between early and late daughter fronds, the characteristics of a clone cannot be altered by selection and propagation of particular fronds (Mendiola, 1919).

GROWTH CYCLES. A number of reports have appeared concerning cyclic growth of Lemnaceae cultures under presumably constant conditions. Dickson (1938a) observed both short (4-6 day) and long (25-40 day) cycles in the multiplication rate and dry weight increase of *L. minor* in non-aseptic culture, but later (1938b) concluded that these were probably due to the sampling techniques employed. Apparent growth cycles can also be induced by transfer to a potassium-deficient medium, but the effect is of relatively short duration (White, 1940).

An annual growth cycle can occur in *L. minor* in aseptic culture under constant conditions of light and temperature. During the summer months, multiplication rate, root growth and root cell plasmolysis time reach their maxima, while minima are found for root osmotic value and permeability to urea. The inverse condition obtains in winter (Pirson and Göllner, 1953a). An annual cycle has also been reported (Henssen 1954) for the readiness of *S. polyrrhiza* to form turions. Although these results may indicate an annual rhythm of a strictly endogenous nature, no attempt was made to control factors other than light, temperature, inoculum and nutritional conditions; thus, factors such as air pollution may be at least partially responsible, particularly for lowered growth during winter. Note that small amounts of fuel gas can cause visible injury to *S. oligorrhiza* (Saeger, 1933b), and similar phenomena are well known in other plants. In contrast to the reports of annual cycles are the data of Landolt (1957) which show essentially constant multiplication rates obtained for a strain of *S. polyrrhiza* under constant conditions at various seasons of the year.

NORMAL VEGETATIVE GROWTH

This section will deal with most of the major external factors affecting vegetative growth in the light, as well as with certain basic processes such as photosynthesis and respiration. With the exception perhaps of the paragraphs on carbon sources, all the factors considered here are those which are likely to vary under "normal" conditions of growth. Most of them have also been varied in investigations directed to the more specific experimental questions which will be considered in the succeeding sections.

MINERAL NUTRITION. The mineral nutrition of *L. minor* in particular has been studied in a large number of early investigations; for individual citations see the bibliography. The sum of the evidence indicates

simply that *Lemna* responds as a typical higher plant and grows satisfactorily in light on a completely inorganic medium. Requirements for all the major elements plus iron, manganese, molybdenum, boron, zinc, copper and gallium have been demonstrated, though the evidence for the necessity of the last three is not entirely clear-cut (Steinberg, 1946). Chloride is required at the level of 50-100 μg/L., but can be partially replaced by 150 μg/L of bromide (Martin and Lavollay, 1958a,b). The pH optimum depends on many factors, including the use of ammonium or nitrate nitrogen sources and the degree to which iron and other micronutrients are kept available by chelators such as citrate or ethylenediaminetetraacetate (EDTA) (cf. Bitcover and Sieling, 1951; Landolt, 1957). Several nutrient solutions are in current use; probably the best for maintaining vigorous growth over long periods of time is Hutner's medium (Hutner, 1953; Landolt, 1957) which contains EDTA. The single known disadvantage is that EDTA and other chelating substances may modify flowering in some circumstances, and therefore may have other developmental effects as well. A modified Hoagland's medium has been used successfully by Gorham (1950); see also the medium used by Pirson and Seidel (1950).

The effects of varying or removing single minerals have been studied by numerous workers, and are of course subject to complex interactions with other factors. The chief body of work on such interactions of nitrogen and potassium levels with light intensity is that of H. L. White (1936-1940) which should be consulted for details; this work was not done in aseptic culture.

Low nitrogen (nitrate) levels reduce *L. minor* multiplication rate, frond area, chlorophyll content, protein content and respiration, and increase starch content and root length. The optimal nitrate level increases with increased light intensities. Root length is an indicator of apparent carbohydrate-to-nitrogen ratio, the longest roots occurring under conditions of relatively high carbon assimilation and low nitrogen (White, 1936b, 1937a,b; White and Templeman, 1937). Pirson and Göllner (1953) found that low levels of nitrogen or phosphate increased root growth and root cell length, lowered root photosynthesis and respiration, and slightly increased the osmotic value of the root cells. Actual deficiencies of these elements had similar effects except that total root length was reduced. Allison et al. (1948) detected no loss of gaseous nitrogen from *L. minor* grown with either nitrate or asparagine.

The effect of phosphate level on photosynthesis by *L. minor* was

studied by Lindeman (1951, 1952). In the region of light saturation, phosphate deficiency inhibited photosynthesis. Increasing the phosphate level removed this inhibition within a few hours. Most of the phosphate taken up was in the trichloro-acetic-acid-soluble fraction of the plants, and there appeared to be a linear relation between photosynthetic rate and the quantity of TCA-soluble organic phosphate.

Low potassium levels decrease root length, multiplication rate, net assimilation and frond area in *L. minor,* and the optimal potassium level is higher at higher light intensities (White, 1936a, 1938, 1939). The decrease in root length reflects a decreased cell length. Potassium deficiency also lowers root photosynthesis and reversibly increases respiration; gradients with respect to plasmolysis time and urea permeability are reduced. Similar effects are reported for calcium deficiency (Pirson and Seidel, 1950; Pirson and Göllner, 1953b). Bierhuizen (1954) confirmed these effects of low potassium, and also observed that rubidium, but not cesium or sodium, could partially replace potassium over a period of 18 days.

The symptoms of various micronutrient deficiences in *L. minor* have been described by Steinberg (1946). Martin (1955) showed that the toxic effects of excess zinc were reduced by increased levels of magnesium.

Most Lemnaceae grow as well or better in $\frac{1}{3}$ strength Hutner's medium as in the undiluted solution; dilution to 1/100 reduces growth in many species. In general, the semi-submerged species *(Wolffia, L. trisulca)* or those with thin fronds grow better than the others on dilute media (Landolt, 1957). Changes in medium composition and concentration may also affect the action of growth-regulating compounds. Hillman (1954) observed that *L. minor* grown in highly diluted Gorham's medium showed characteristic morphological changes—primarily great root elongation and frond expansion—which might be ascribed to the reduced osmotic value of the medium. This "dilution effect" could be reversed by appropriate concentrations of sucrose, but not by osmotically equivalent amounts of mannitol or lactose, which are not utilized as carbohydrate sources. This suggests that the dilution effect is not a simple osmotic phenomenon but may be due to complex nutrient interactions. Addition of sucrose is known to affect the pH drift in mineral media (Henssen, 1954) and also increases the efficiency of salt utilization under most conditions (Steinberg, 1946).

AUXIMONES. The question whether green plants require small

amounts of accessory organic growth factors, or auximones, occupies much of the early literature on *Lemna* and *Spirodela* nutrition. It had been reported that growth in purely inorganic solutions was extremely poor unless extracts of peat, leaf mold, soil or manure were added (Bottomley 1920a, b; Mockeridge, 1925; see these papers for earlier citations). These results were interpreted to mean that plants require auximones, possibly of a nucleic acid nature, as animals require vitamins. It was largely in attempts to supply conclusive evidence on this point that aseptic culture techniques were developed, and it was soon demonstrated, both with and without such methods, that organic materials were not required for prolonged growth (e.g., Clark and Roller, 1924, 1931; Saeger, 1925, 1930; Wolfe, 1926; Ashby, 1929b; Clark, 1932). Clark et al. (1938) showed that *Lemna* grown in aseptic, completely inorganic culture formed larger amounts of vitamins A, B and C than when grown in soil-water media.

The auximone question as such is probably conclusively settled, but it is still necessary to consider the body of evidence that in many nutrient solutions the growth of Lemnaceae is improved by added organic materials in amounts too small to supply a significant source of carbohydrates. The effects of auxins and other simple compounds will be considered later. The most probable line of explanation for the effects of manure, peat and so forth is suggested by the work of Olsen (1930) who found that such materials maintained the availability of iron as organic complexes even in solutions of high pH. Citrate ion proved equally effective (cf. Bitcover and Sieling, 1951). Many effects of organic substances are probably due to such action. Hutner et al. (1950) found that complex organic extracts failed to stimulate growth of *S. oligorrhiza* in suitably prepared medium. While it would be unwise to state dogmatically that all the growth promotions in the auximone literature are due to relatively non-specific complexing effects, it is clearly incumbent on an investigator to show that the inorganic nutrition is optimal before assigning specific roles to substances causing growth promotion.

Light Duration and Intensity. While levels of mineral nutrition, light, temperature and carbon sources all interact on vegetative growth, the effects of light duration and intensity appear to be relatively uncomplicated. At light intensities below about 700 foot-candles (ca. 7000) lux), the multiplication rate of *L. minor, S. polyrrhiza* and most other species studied increases with increasing daily duration of

exposure, reaching a maximum under continuous light (Clark, 1925; Ashby, 1929d; Landolt, 1957). Unlike many other plants, the Lemnaceae are probably not injured by continuous illumination or unnatural photoperiodic cycles; growth of *L. minor* under alternating two-hour periods of light and darkness does not differ significantly from that on a 12-hour alternating cycle (Ashby, 1929a).

Experimental studies have been performed with light intensities from 50 to 1600 foot-candles, mostly in the lower part of this range. Ashby (1929a) and Ashby and Oxley (1935) used a clone of *L. minor* in which multiplication rate (at 25° C.) reached its maximum at about 150 foot-candles continuous illumination with incandescent light. 1400-1600 foot-candles was deleterious. Assimilation (dry weight increase) increased with increasing light intensity up to about 1600 foot-candles, but starch and monosaccharides were present in the fronds at intensities as low as 150 foot-candles, leading to the conclusion that light affected multiplication rate through processes other than carbohydrate production. White (1936c) studied several strains at 25° C. constant temperature; one showed light saturation at ca. 1600 foot-candles, another at ca. 900 foot-candles (cf. Hicks, 1934). The same strain responded somewhat differently in different experiments, particularly at different times of the year.

Light intensity interacts with nitrogen level, a higher level of one factor requiring a higher level of the other. Where the optimum potassium level at 60 foot-candles is 2 mg./L., at 300 foot-candles it may be 200 mg./L. Protein content, respiration rate per unit area, and root length all increase with increasing light intensity; respiration on a dry weight basis falls (White and Templeman, 1937; White, 1937a, b; 1938; 1939).

Landolt (1957) used fluorescent light supplemented with incandescent to supply various photoperiods and intensities to a large number of Lemnaceae. Multiplication rate increased with intensity until a maximal value was reached. Depending upon the species, this maximal value (at 24° C.) was attained between 200 and over 900 foot-candles. Light saturation or even injury occurred at lower intensities at lower temperatures, or in the presence of sucrose. Sucrose promoted growth under non-saturating light conditions.

The effects of light and darkness on the uptake of nitrate, nitrite and ammonium ions was studied by Yoshimura (1952) in *S. polyrrhiza* and *L. valdiviana*. The consumption of ammonium was clearly acceler-

ated by light, that of nitrite accelerated under certain conditions and that of nitrate either unaffected or slightly inhibited. The evidence suggested that the effects of light were probably indirect and due to the increased partial pressure of oxygen resulting from photosynthesis.

TEMPERATURE. Ashby and Oxley (1935) found an optimum temperature of ca. 29° C. for multiplication rate in *L. minor,* but no change in dry weight increase as the temperature was raised from 18° to 29°. Growth fell off sharply at ca. 35°. Jacobs (1947) reported a temperature optimum of 25° for *S. polyrrhiza.*

Landolt (1957) investigated the temperature requirements of 60 strains, comprising 12 species. Temperature optima in Hutner's medium lay between 20° and 30°, usually ca. 26°, depending on the strain; addition of sucrose to the medium often increased the optimal temperature several degrees. *S. polyrrhiza, L. perpusilla* and some strains of *L. gibba* showed high optimal temperatures of 30° or over, while the lowest optima (ca. 23°) were those of *S. oligorrhiza* and some strains of *L. valdivana.* Temperature maxima lay mostly between 30° and 32°, with some higher. Temperature minima for growth ranged from below 4° to 18°. The effect of the inorganic composition of the medium on these values was not investigated.

Attempts to detect a thermoperiodic effect of alternating day and night temperatures gave negative results; multiplication rates under these conditions (26° for 16 hours, light — 14° for 8 hours, dark) were about the same as at a constant temperature (22°) representing the average for the treatment. Landolt points out that these results are consistent with the hypothesis that thermoperiodicity in plants is related to the transport of food materials, since the size and habit of Lemnaceae would reduce or eliminate this process as a limiting factor.

The same paper also contains data on the capacity of many strains to overwinter in the vicinity of Zürich, Switzerland.

Temperature studies under aseptic conditions may be complicated by the fact that even moderate light intensities will quickly raise the temperature in tubes or flasks considerably above that of the surrounding air. Where accurate temperature control is required, the best practice is to immerse the vessels in a constant temperature water bath at least up to the level of the nutrient solution inside (e.g., Kandeler, 1955, 1956). Casperson (1956) has shown that the actual frond temperature of *S. polyrrhiza* under 500 foot-candles of light may be from

one to three degrees higher than that of the medium. This effect is probably smaller for Lemnaceae with thinner or submerged fronds.

CHLOROPLASTS; PHOTOSYNTHESIS. *L. trisulca* has supplied material for an outstanding series of papers on chloroplast movements and their relation to photosynthesis. Plastid orientations in this plant are more sensitive to light than in most other organisms used in such studies: 1200 foot-candles is enough to cause complete parastrophe, or arrangement of the plastids along the cell walls parallel to the direction of the light (Zurzycki, 1953). Light intensities above 5,000 foot-candles cause inactivation of the photosynthetic mechanism (Zurzycki, 1957a). After the careful control of temperature necessary for such experiments was established (Zurzycka and Zurzycki, 1950), it was possible to study the effect of wavelength on the movements of the plastids. The change from apostrophe (the normal condition in darkness with plastids parallel to all cell walls) to epistrophe (condition in dim light with plastids along those walls perpendicular to the direction of the light) shows a maximum sensitivity in the blue, and is possibly due to absorption by carotenoids. The change from epistrophe to parastrophe shows maximum sensitivity in the red, and is probably due to absorption by chlorophyll. The kinetics of these processes was studied in detail (Zurzycka, 1951) and later examined more closely by cinematographic techniques (Zurzycka and Zurzycki, 1957).

In strong light, chloroplast arrangement seems to have no effect on photosynthesis; the shift from epistrophe to parastrophe takes place in saturating intensities, but the photosynthetic rate is the same after the first minute as after 30 minutes, by which time the movement has been completed (Zurzycki, 1955a, c). No effect of polarized light on photosynthesis was detected in *L. trisulca* (Zurzycki, 1955b).

Few other direct studies of photosynthesis in Lemnaceae have been reported. The work of Lindeman (1951, 1952) on the effects of phosphate nutrition was mentioned above. Many of the papers in the "Interaction of Factors" series (e.g., Ashby and Oxley, 1935; White, 1939) provide indirect data in terms of net assimilation rate or increase in dry matter content. White (1937a) found that actual photosynthesis in *L. minor* increased linearly with light intensity from 50 to 300 foot-candles.

The optimal conditions of aeration and CO_2 supply for photosynthesis have received some attention. Ashby and Oxley (1935) found that increased aeration of their open, non-aseptic *L. minor* cultures did

not improve growth or assimilation rate. Gorham (1945), using *S. polyrrhiza* in aseptic culture, found that forced aeration of the medium with four percent CO_2 increased frond size and multiplication rate even in the presence of optimal levels of sucrose; the promotion was much greater at 300 than at 100 foot-candles of light. All of the multiplication rates obtained, however, were low. Steinberg (1946) reported that higher CO_2 levels increased growth in relatively crowded cultures where air diffusion was presumably limiting. Forced aeration with five percent CO_2 was used by Lindeman (1951, 1952) in growing *L. minor* in his photosynthetic experiments, but no unaerated controls were studied for comparison. Landolt (1957) detected no consistent effect of forced aeration on growth in his cultures, and concluded that it was probably unnecessary; the question remains open at present.

The photochemical activity (Hill reaction) of isolated chloroplasts from *L. minor* and *Wolffia punctata* was moderately high among a large number of plants studied by Clendenning and Gorham (1950), attaining one-fourth to one-half the values obtained with *Spinacia oleracea,* the favored organism for such work.

ORGANIC CARBON SOURCES; MAXIMUM GROWTH RATES. Many investigators have used sugars, chiefly glucose and sucrose, to increase growth under conditions of limiting light intensity; the optimum concentration of sucrose usually lies between 0.5 and 2.0 percent (Hopkins, 1931; Steinberg, 1941, 1946; Gorham, 1945; Hillman, 1954). Yoshimura (1944) tested several compounds as carbon sources for *Lemna* and *Spirodela* under very low light intensities: maltose was less effective than glucose, fructose or sucrose, while ethyl alcohol, glycerol, mannitol and inulin were not used. Also ineffective as carbon sources for *L. minor* are starch, lactose, arabinose, ribose, tartrate, succinate and acetate (Hillman, 1954). Landolt (1957) found that sucrose promoted growth of all Lemnaceae strains tested under low light intensities; the promotion fell off greatly under higher intensities. Sucrose raised the apparent temperature optima and maxima of many strains. *L. trisulca* required sucrose for growth even under continuous high light intensities (1000 foot-candles) when grown in Hutner's medium, although it would grow in tapwater without sucrose under these conditions; this phenomenon remains unexplained.

Growth with sugars may result in acidification of the medium (Henssen, 1954). It has also been observed that under certain conditions sugars cause the occurrence of large colonies of overlapping

fronds, presumably by preventing daughter-frond abscission (e.g., Yoshimura, 1944; Hillman, 1954). The effects of sucrose, glucose and fructose on growth and other processes often differ. Sucrose and fructose are more active than glucose in promoting anthocyanin formation and starch accumulation; their effects on turion production also differ. Some authors have reported glucose as superior to sucrose in promoting vegetative growth, while others record the opposite (cf. Thimann et al., 1951; Henssen, 1954).

Maximum multiplication rates observed by Landolt (1957) (continuous illumination of 1000 foot-candles at 24° with one percent sucrose) include values of 60 for *Wolffiella floridana,* ca. 150 for *Wolffia* species, ca. 190 for most *L. minor* strains, 200 to 230 for *S. polyrrhiza* strains, and 255-266 for *L. perpusilla.*

RESPIRATION. The effects of light intensity and mineral deficiencies on the respiration of whole *L. minor* plants and of roots were mentioned above. Culture age also affects respiration; according to Pirson and Göllner (1953b; also Pirson and Seidel, 1950), oxygen consumption by root tissue from 20-day-old cultures is considerably lower than that from 10-day-old cultures. The reasons for this are not clear, but culture age can affect inhibitor sensitivity as well (see below). The same authors have studied respiration in various regions of the root.

A study of *L. minor* respiration was undertaken by the author (Hillman, 1954, 1955) in an attempt to determine whether the marked formative effects caused by growth with benzimidazole were correlated with changes in respiration. The oxygen uptake under the manometric conditions employed (27° C.) frequently declined over a period of several hours; this decline could be prevented or changed to an increase by addition of sucrose or glucose (see also Pirson and Seidel, 1950; Pirson and Schaefer, 1957). Oxygen consumption was ca. 50 microliters per 100 plants (fronds plus roots) per hour; a respiratory quotient of 1.0 to 1.1 was recorded in the presence or absence of sucrose. If the plants were kept on distilled water for 24 hours previous to the experiment, addition of .05 M KCl or NaCl caused a 20 to 30 percent increase in oxygen uptake ("salt respiration").

Cyanide, azide, carbon monoxide and phenylthiourea all inhibited the oxygen consumption of normal plants, though never completely; azide inhibition was higher in plants from fresh cultures than in those from older cultures. The azide sensitivity of roots was the same as that of

the fronds. Inhibitions by 2,4-dinitrophenol and by iodoacetate were also observed. Cytochrome oxidase, polyphenolase, ascorbic acid oxidation, glycolic acid oxidase and catalase activities were detected in cell-free homogenates of normal plants.

The oxygen uptake of benzimidazole-modified plants was promoted by concentrations of cyanide, azide, carbon monoxide and phenylthiourea inhibitory to that of normal plants, and homogenates showed essentially no polyphenolase or "ascorbic acid oxidase" activities. The significance of these results for the normal metabolism of *L. minor* is not clear. They suggest the presence of certain oxidase systems which are promoted, or at least not inhibited by, the usual metal-enzyme inhibitors, a situation which is certainly not unique among higher plants. Daly and Brown (1954) mention that increased gas uptake in the presence of carbon monoxide was frequently observed in *Lemna* (species unnoted) as well as other plants.

Pirson and Schaefer (1957) report that hypotonic concentrations of polyethyleneglycols (ca. 0.2 M) have no effect on *L. minor* respiration, while plasmolysis with 0.6 M (or 0.6 M glucose) causes a sharp drop in oxygen uptake.

ROOT PHYSIOLOGY; PERMEABILITY AND PLASMOLYSIS. As a relatively simple organ consisting of a few cell layers and well-suited for microscopic examination, the *L. minor* root provides excellent material for studies of extension growth, plasmolysis and related processes. Some of the effects of external factors have already been mentioned. All of the recent work has come from the laboratories of A. Pirson in Germany, with plants grown under aseptic conditions.

Elongation of the root from one to at least 20 millimeters in length may take from 40 to 100 hours, depending upon the circumstances, and growth is more or less linear during this time (Pirson and Göllner, 1953a). The root shows a marked zonation into a meristematic region, region of extension growth, and mature zone; differing plasmolysis forms in the last two zones were first described by Strugger (1934). The permeability of the epidermal and subepidermal cells to various substances has been studied by a plasmolytic method; as expected, the permeability increased in more or less direct relation to the lipoid solubility of the substance in question (Marklund, 1936). Polyethylene glycols of molecular weight ca. 400 are excellent inert substances for such plasmolytic work (Pirson and Schaefer, 1957). At

the oldest end of the extension zone is a region of maximal plasmolysis time and minimal urea permeability (Pirson and Seidel, 1950).

Plasmolysis form and time depend on factors besides mineral nutrition and the region of the root observed. While attached and detached roots show similar phenomena in a qualitative sense, plasmolysis time declines rapidly from the moment of detachment; this effect cannot be prevented by supplying glucose, suggesting that the frond affects root cell properties by supplying other and unknown substances. In general, rapid root growth is associated with a long plasmolysis time and concave plasmolysis form, while slower growth has the reverse associations (see Schaefer, 1956). Temperature is also important: at 26° C. plasmolysis is rapid and convex, at 20° it is much slower and concave (e.g., Pirson and Schaefer, 1957).

Under certain conditions, plasmolysis time and protoplast-cell wall adhesion diminish in darkness and increase again in the light; such effects appear to be due entirely to the changes brought about by light in the partial pressure of CO_2. The action of CO_2, in turn, can be imitated by changes of pH in the external medium. Schaefer (1956) presents a complex summary scheme relating these and other findings on the plasmolytic susceptibility of *L. minor* root cells.

The *L. minor* root is probably of little importance for the uptake of materials from solution. Gorham (1941) found that application of lanolin to the undersides of fronds reduced multiplication rate and caused abnormal root elongation suggestive of nutrient deficiency. Applications of lanolin on the upper surface had no effect. Blackman and Robertson-Cuninghame (1955) reported that lanolin applications to the lower frond surface reduced 2,4-D toxicity, whether or not the roots were removed. Uptake of radioactive 2,3,5-triiodobenzoic acid by fronds appears to be unaffected by root removal as long as the root stump is greased to prevent "leaking" back into the medium. No uptake into the frond occurs when root stumps only are immersed in the solution (J. A. Sargent, personal communication). Uptake of dissolved substances can also take place through the upper frond surface. One can start a culture with a single colony lying on its upper surface, and normal growth will proceed at a reduced rate, though all the new fronds remain upside-down unless the culture is shaken; the roots do not elongate under these conditions. Such experiments are easier in an agar medium (Hillman, unpublished).

Heterotrophic and Non-photosynthetic Growth

Several species of Lemnaceae have been grown in darkness at the expense of organic substrates. Because of the growth-promotion caused by even brief exposures to light (see below), it would be well to make a distinction between true heterotrophic growth, in total darkness, and non-photosynthetic growth, in which occasional active light has been used but at levels insufficient to cause chlorophyll formation and photosynthesis. Heterotropic growth might be regarded as a subclass of non-photosynthetic growth. While this distinction is important in theory, it is difficult to make in actually considering the literature, since only two authors (Gorham, 1945, 1950; Hillman, 1957) have taken it into account. For this reason, meaningful descriptions of the conditions required for heterotrophic growth are few, though it is evident that various species differ in their requirements for non-photosynthetic growth in general (cf. Landolt, 1957).

The most detailed work concerns *L. minor,* which was first grown heterotrophically on a medium containing sucrose, casein hydrolysate and yeast extract in addition to minerals (Gorham, 1950). Sucrose alone would not support heterotrophic growth, nor would the other components in the absence of sucrose; all three organic supplements were required. The average steady multiplication rate under these conditions (at 25° C.) was about 27. The fronds were white or yellow but otherwise normal, and the root primordia did not elongate. Attempts to simplify the medium by feeding combinations of known amino acids, vitamins and other compounds were unsuccessful. Gorham (1950, 1945) also investigated the high rate of growth during the first few days of dark culture ("inoculum effect"). This effect was increased by pretreatment of the light-grown stocks with sucrose, higher light intensities and increased CO_2 supply, and decreased by aeration or by the growth acceleration induced by organic nitrogen sources. He concluded, however, that substances other than stored carbon sources were responsible for the inoculum effect.

Dim green safelights were used for the work above because short exposure to light increased subsequent growth in darkness, and green was the least effective color in this regard (Gorham, 1945). This observation was later extended by the report (Hillman, 1957) that non-photosynthetic growth at a mean multiplication rate ca. 40 can

be maintained indefinitely on sucrose alone by giving ten minutes of dim red light every three or four days. The multiplication rate rises rapidly for one or two days after each exposure and then drops off again. This action of red light is reversible by subsequent treatment with "far-red" (ca. 730 mμ) radiation, suggesting that it is mediated by the same pigment system which has been implicated in photoperiodism, seed germination and many other plant processes. Stated in other terms, low energies of red light can completely replace the complex organic supplements required in Gorham's work.

Further attempts to simplify the medium required for heterotrophic growth have shown that kinetin (6-furfurylamino purine) and certain related compounds will imitate the action of red light, at least in short-term experiments (Hillman, 1957).

The means by which Gorman's supplements, kinetin or red light act in this system are not known; thus, the nature of the growth inhibition by total darkness (other than its suppression of carbohydrate synthesis) is not understood. While Gorham (1950) stressed the inability of *L. minor* to utilize inorganic nitrogen in darkness, there is no direct evidence for such inability in this plant. The idea seems to depend entirely on the beneficial effects of supplements, such as casein hydrolysate, and on the fact that complete elimination of inorganic nitrogen did not reduce growth in the fully supplemented medium. Such results are not unequivocal evidence on the nature of the dark-induced block in growth, or, to state it in other terms, on the "non-photosynthetic light requirement". Note also that none of the non-photosynthetic growth conditions yet employed allows normal root elongation. This is not due simply to a low carbohydrate level, since the plants usually contain abundant starch.

Work with other Lemnaceae has been done under less clearly specified conditions. Hutner (1953) reported successful heterotrophic or at least non-photosynthetic culture of *S. oligorrhiza* on a medium supplemented with sucrose and glutamate, although Gorham (1945) succeeded in obtaining true heterotrophic growth only with his complete medium. Landolt (1957) studied a large number of species and strains. Weak incandescent light was apparently used in counting the fronds so that the exact degree of true heterotrophy is impossible to estimate; since, however, the inability of *L. minor* to grow without Gorham's supplements was confirmed, the conditions must have approximated total darkness at least during certain periods. Some species (e.g., *L.*

minor, L. perpusilla, L. trisulca) required yeast extract and casein hydrolysate in addition to sucrose; some (e.g., all species of *Wolffia,* and *Wolffiella floridana)* grew on sucrose alone but grew better with the supplements, while still others *(S. polyrrhiza, S. oligorrhiza, L. gibba)* grew well on sucrose alone and were either inhibited or unaffected by the supplements. In general, the "heterotrophic" multiplication rate of any strain was approximately equal to the increase in its rate which could be caused by addition of sucrose alone to cultures growing photosynthetically at low light intensities.

Probably the best way to conduct completely heterotrophic growth studies is to place a large number of replicate cultures in total darkness at the same time and then withdraw sets for counting at various intervals, discarding them after counting. This eliminates any exposure to light during the growth period. If the original number of fronds in each culture is known, the overall rate from start to each withdrawal can be calculated, and interval rates can be approximated from successive overall rates. Using this method, the ability of *S. polyrrhiza* to grow heterotrophically at multiplication rates of 70 or over with sucrose alone has been confirmed (Hillman, unpublished).

Chemical Growth Promotions, Inhibitions and Formative Effects

A diversity of substances has been tested for growth effects on Lemnaceae. Most of the work has used *L. minor* in crude or aseptic culture, but the control conditions employed have differed so widely that little comparison is possible between one report and another. The research on auximones has already been discussed, and many chemicals have been used in studying some of the specific physiological processes covered elsewhere in this review; only work not fitting into any other category will be considered here.

Some General Considerations. A growth promotion or inhibition is always relative to a control value, so that the basic conditions used are of real importance. Studies of inhibition and toxicity require only that the control conditions provide growth at a high enough rate so that various levels of inhibition can be easily distinguished. When growth promotions are reported, however, a more careful attention to the control conditions is in order.

Landolt (1957) considers it unlikely *a priori* that the growth of

intact green plants under optimal temperature, photosynthetic and nutritional conditions would be promoted by many organic substances. This seems reasonable, but unfortunately, little of the work to be discussed bears on this question at all; few attempts have been made to establish optimal or even near-optimal conditions before adding the substances tested. What little evidence is available tends to confirm the opinion cited. For example, growth promotion by sugars disappears under optimal light conditions (cf. Landolt, 1957). The increase in frond size often caused by auxins and by benzimidazole does not take place in a mineral medium which is already optimal for frond-expansion (Hillman, 1954, 1955). In addition, many of the growth promotions reported may also be due to non-specific metal-complexing effects, as discussed in the auximone section. The major recent investigations of chemical growth promotions (Nickell and Finlay, 1954; Nickell, 1955, 1956), to be considered later, have involved control conditions which can certainly not be described as optimal, judging from the photograph in the first paper cited. A basic medium devised for tissue-culture work was used in unmodified form for the growth of *L. minor;* promotions of over 500 per cent resulted from the addition of 50 mg/L. of the chelator EDTA to the medium. This sort of work is difficult to evaluate.

The action of weakly ionized compounds is strongly affected by pH, and great care must be taken to study pH effects on both controls and experimental treatments. In general, the effects of weak acids decrease with increasing pH above the pK of the compound. This phenomenon has been described in detail for *L. minor* with various substituted phenols (Simon et al, 1952; Simon and Blackman, 1953) and with 2,4-dichlorophenoxyacetic acid. The concentration of 2,4-D required to cause frond mortality increased tenfold (from 45 to 457 mg/L) as the pH increased from 4.6 to 6.1, while there was little effect of pH on growth under the control conditions used (Blackman and Robertson-Cuninghame, 1953). Similarly the weak base benzimidazole is much more effective at pH 6.2 than at pH 4.2 (Hillman, 1954, 1955).

Effects of pH on the availability of inorganic materials are also important. Clark (1926), for example, found that a soil solution with organic matter would produce good growth at a pH which was unfavorable under completely inorganic conditions.

The formative effects of a number of chemicals are described in the literature. In all cases so far studied, colonies showing the maximum effect will again give rise to completely normal cultures when placed

on control medium. Usually, several frond generations are required to achieve normal growth, probably reflecting the fact that the substance in question modifies growth at an early stage, before the fronds are visibily developed. Similarly, the full development of a formative effect may also take several frond generations (cf. Clark and Frahm, 1940b; Hillman, 1954, 1955; Sargent, 1957).

AUXINS AND RELATED COMPOUNDS. Clark and Frahm (1940a, b) studied the effects of indole-3-acetic acid (IAA), phenylacetic acid and phenylproprionic acid on *S. polyrrhiza* in aseptic culture. The compounds did not increase multiplication rate when kept in the medium at 0.01 mg/L. or lower concentrations; higher concentrations caused root inhibition and frond epinasty as well as an extreme development of the vascularized strands connecting daughters to mother fronds. Intermittent application of the growth substances, however—for example, contact for 30 minutes per day with solutions containing 0.1 to 1.0 mg./L.—caused small but significant and repeatable increases in multiplication rate. IAA was less active than the other two compounds. Gorham (1941) exposed *L. minor* in crude culture to IAA, indole-3-butyric acid and 1-naphthaleneacetic acid (NAA), and observed slight increases in multiplication rate and frond area. All the rates in the papers mentioned seem to be relatively low. A small but significant growth promotion by IAA was reported for *L. minor* in crude culture by Blackman and Robertson-Cuninghame (1954) who also noted frond epinasty and root inhibition by higher concentrations. Hillman (1954, 1955) reported similar formative effects for both NAA and IAA; the promotion of vascular strand development mentioned above was particularly marked in the presence of sucrose (aseptic culture). NAA and IAA both cause formation of an extra vein in *L. minor;* this vein, however, is normally present in a certain proportion of untreated plants (Sargent, 1957).

The action of 2,4-D on *L. minor* has been studied in crude culture under controlled light and temperature conditions. Levels of 2,4-D above 0.2 mg./L. depress growth and reduce the ratio of frond area to frond dry weight. These effects are partially overcome by IAA, which itself promotes growth and frond area increase in the absence of 2,4-D (Blackman and Robertson-Cuninghame, 1954). The growth inhibition increases with higher temperature and slightly increases with higher light intensities. When concentrations high enough to induce chlorosis are used, there is little or no effect of light intensity during treatment;

but the lower the intensity before treatment, the lower is the concentration of 2,4-D required for a given effect (Blackman and Robertson-Cuninghame, 1955). The mechanism of 2,4-D toxicity is not understood, but it is probably not due to simple auxin antagonism.

Of the compounds usually considered anti-auxins, only two have been used on *L. minor* to any extent—p-chlorophenoxyisobutyric acid (PCPIBA) and 2,3,5-triiodobenzoic acid (TIBA). PCPIBA reduces frond area and increases root length in *L. minor* (aseptic culture); in relatively high concentrations (ca. 20 mg./L.) it causes development of long slender vascular connections between fronds, and the fronds themselves may become peltate. PCPIBA partially reverses the formative effects of benzimidazole (see below); several other "antiauxins" have also been tested for interactions with benzimidazole (Hillman, 1954, 1955).

TIBA upsets the normal sequence of frond development in *L. minor* (crude culture), causing many fronds to develop both right and left daughters simultaneously, or even reversing the order characteristic of the clone. This effect may be analogous to an interference with apical dominance in plants with a more usual morphology, and is partially counteracted by addition of IAA to the medium. By inducing cells which would otherwise produce mesophyll to become vascularized, TIBA also causes occurrence of many extra veins (Wangermann and Lacey, 1953). Sargent (1957) has studied this vein-inducing action in great detail. The change takes place at an early stage in frond development, and increases with TIBA concentration up to ca. 10 mg./L. Cultures do not "adapt" to TIBA; the increased veining continues as long as the compound is present. The auxins NAA and IAA reduce the effect at relatively low and high TIBA levels but increase it in the middle range; this interaction is extremely complex. TIBA reduces the apparent extractable free auxin (IAA?) content and affects the level of growth inhibitor in the fronds. Sargent also tested many other halogenated benzoic acids and observed similar effects; he proposes a tentative hypothesis involving antagonism between such compounds and a naturally-occurring inhibitior of vascular development.

ANTIBIOTICS, AMINO ACIDS, PURINES AND METABOLITE ANALOGS. According to Nickell and Finlay (1954), various antibiotics, including penicillin G and oxytetracycline, cause considerable growth promotions in aseptically-cultured *L. minor*. This work was carried out in the unsatisfactory medium mentioned earlier. Evidence is presented

that the action of oxytetracycline may be due, at least in part, to breakdown products formed in the absence of living tissue. Another possibility raised is that the antibiotics act by "detoxification" of inhibitory substances excreted into the medium.

Nickell and Finlay consider various hypotheses of antibiotic action, including the possibility of relatively non-specific complexing effects, but reach no definite conclusions. The effects of EDTA described earlier, the poor growth obtained in the controls and the fact that the data showing large promotions were obtained after long growth periods all suggest that the results may be due to the use of a medium so ill-chosen that almost any organic compound added might improve trace element availability.

Unfortunately at least two other investigations have been conducted under the same conditions. Nickell (1955, 1956) reported growth promotions by various compounds, including arginine, ornithine, citrulline, isoleucine, adenine and other purines. In addition, the growth inhibition caused by canavanine was reversible by arginine, ornithine or citrulline, and the inhibition caused by 2,6-diaminopurine was reversed by adenine. The data presented, however, do not need to be interpreted as specific reversals. While it is evident, for example (Nickell, 1955: Fig. 5), that growth with 2,6-diaminopurine is increased by addition of adenine, it is not increased to the maximum obtainable with adenine alone, a level far higher than the original control. The same relation holds in the canavanine-arginine interaction (Nickell, 1956).

A detailed analysis of these three papers is unfortunately impossible in the scale of this review, though they represent a major fraction of the literature on work of this kind. It is probably not excessive to conclude that, due to the techniques used, most of the data are virtually meaningless unless and until they can be confirmed under more reasonable conditions. Investigators using more favorable media have not observed any promotion of intact *L. minor* growth by adenine or related compounds (Wangermann and Lacey, 1953; Hillman, 1954).

Fromm and O'Donnell (1952) reported that 10^{-5} - 10^{-4} molar p-aminobenzoic acid (PABA) increased the growth of *L. minor* in crude culture. Later (1953) they found that it partially counteracted the inhibitory action of its analog sulfanilamide, but noted that growth was not brought up to the level attained with PABA alone. The inhibition caused by ammonium sulfamate was not affected by PABA. The

phytotoxic action of a number of other sulfonamide compounds, including sulfaguanidine and sulfathiazole, was also not antagonized by PABA (Fromm and O'Donnell, 1955).

The action of the purine analog benzimidazole (BZ) on aseptically-cultured *L. minor* was studied by Hillman (1954, 1955). In relatively high concentrations (ca. 3×10^{-3} M) BZ caused complete inhibition of root growth and about 50 percent frond area increase. These effects are similar to those of certain auxin levels, but, unlike them, allowed continued rapid growth. The increased frond size was apparently due entirely to cell expansion, not to increased cell number, and did not occur in more dilute medium, where frond size was already maximal. Attempts to reverse these effects with purines, or to imitate them with other purine analogs, were unsuccessful. One "antiauxin", PCPIBA (see above), partially counteracted the action of BZ.

The effects of BZ on respiration and oxidase activity have been mentioned in a previous section; these were attributed to its known copper-complexing characteristics. While BZ-modified plants contained no less copper than the normal, copper-enzyme activities were essentially abolished. However, a large number of other complexing agents failed to reproduce the formative effects caused by BZ, and no conclusion was reached on the mechanism of its overall action except that it did not appear to act as a purine antagonist.

TOXICITY ASSAYS; OTHER COMPOUNDS. *L. minor* has been used for toxicity and herbicide studies, mostly in crude culture, starting with Hessenland et al. (1932, 1933). Various methods have been outlined by Offord (1946), Fromm (1946, 1951) and most critically by G. E. Blackman and collaborators (Blackman, 1952; Sampford, 1952; Simon and Blackman, 1952; Simon et al., 1952). It is important to note that the toxic effects of certain compounds are cumulative, while others simply cause a constant depression in relative growth rate (Blackman, 1952). In addition, the pH factor appears to be important in the *Lemna* assay only because of the high ratio of solution to tissue; it becomes negligible when the results are to be interpreted in terms of spray effects (Simon et al., 1952).

Besides the work already covered, toxicity studies have been reported with the following compounds: chlorates, bromates and iodates (Hessenland et al., 1932, 1933); Methoxone (Kar, 1947); substituted acrylic acids (Fromm, 1948, 1955); maleic hydrazide (Bertossi, 1950);

sulfonamides (Fromm and O'Donnell, 1951; Fromm and Pace, 1957); various nitrophenols (Simon and Blackman, 1953); phospho-organic insecticides (Capozzi, 1953); pentachlorophenol (Yui and Koike, 1955; Yui, 1958); ammonium sulfamate (Fromm, 1943); and methylcoumarins (Ciferri, 1947). Slight promotions at low levels were reported in the last two papers.

A few other growth promotions have been reported. Tomiyama et al. (1951) found that "solubilized fish" extracts promoted *L. paucicostata* growth; the effect was due to unidentified organic compounds. Yone and Tomiyama (1953) have reported that the growth-promoting property of purified liver fractions was not due to folic acid or vitamin B_{12}, though the latter slightly promoted growth at 0.02 mg./L. under the conditions employed.

Gibberellins have been tested on *L. paucicostata* by Yabuta and Hayashi (1939) and by Kato (1943). The former observed a slight promotion by 350 mg./L. of crude gibberellin, while the latter found an optimum of 0.5 mg./L. for the purified substance. The scanty data available are inconclusive, and the subject awaits further investigation.

Anthocyanin Formation

Anthocyanin production by *Spirodela oligorrhiza* in aseptic culture has been investigated in detail by K. V. Thimann and collaborators. Their five papers that have appeared, the first one in 1949, not only represent a major contribution to the study of anthocyanin synthesis but also constitute the outstanding examples to date of the use of Lemnaceae for biochemical investigations.

Preliminary work showed that light is essential to continued pigment formation in a mineral or mineral-sugar medium. Addition of sucrose greatly increased pigment formation; glucose did not, while fructose was intermediate. Since the rate of pigment production was relatively independent of growth, conditions which reduced growth—for example, low boron, zinc or molybdenum, or excess copper—resulted in increased anthocyanin content on a fresh weight basis (Thimann and Edmondson, 1949). This work was carried out in growing cultures with complete nutrient solutions; later, "non-growing" cultures of plants transferred to distilled water or distilled water plus various substrates were used. This technique permits relatively rapid (4-5 day) experiments

and largely eliminates complications due to "dilution" of newly formed pigment by new growth (e.g., Thimann, Edmondson and Radner, 1951).

A specific role of copper in anthocyanin formation was established by the use of copper-complexing agents. Several of these inhibited pigment production but inhibited growth also. Phenylthiocarbamide (PTC: phenylthiourea), however, inhibited it without affecting growth; this inhibition took place also in non-growing cultures, suggesting that PTC interfered with the action of copper within the plant, not merely copper uptake. Addition of copper partially reversed the action of PTC. The absorption spectrum of the pigment extract obtained from PTC-treated plants was different from that of normal plants, indicating a change in the pigment or an accumulation of intermediates (Edmondson and Thimann, 1950).

Thimann, Edmondson and Radner (1951) examined the relationship between sugars and anthocyanin production more closely. In growing cultures, glucose promoted growth and not pigment accumulation, while the opposite was true of sucrose. In non-growing cultures, however, sucrose, glucose and fructose all promoted anthocyanin production equally. Paradoxically, anthocyanin content was closely related to the reducing sugar content of the plant, not to the sucrose content. The authors concluded that all three sugars could be utilized directly in anthocyanin synthesis but that glucose was preferentially used for growth. A number of glycolytic intermediates failed to promote anthocyanin formation, and phosphate level had little effect on the process. Henssen (1954) found that sucrose and glucose were both used by *S. polyrrhiza* for non-photosynthetic growth but that sucrose promoted both anthocyanin formation and turion development, while glucose promoted neither.

Further studies with non-growing cultures (Thimann and Radner, 1955a) showed that a number of sulfur-containing compounds, particularly ethionine, methionine, sulfadiazine and thiouracil, were effective inhibitors of anthocyanin formation. The inhibitory action of these compounds could not be ascribed to any single property or chemical grouping. Ethionine was the most active compound studied. While it did not prevent the small amount of pigment synthesis which occurs in darkness, it did inhibit subsequent formation when present during pre-illumination treatments which would otherwise promote the proc-

ess. This result was interpreted as the blocking of a light-reaction whose products can be converted to anthocyanin in darkness. The action of thiouracil led to an investigation of other pyrimidine and purine analogs (Thimann and Radner, 1955b). Benzimidazole, 2,6-diaminopurine, quinine, aza-adenine and azaguainine inhibited anthocyanin formation to various degrees; the inhibitions were at least partially reversible with purines or pyrimidines except in the case of benzimidazole. Thiouracil itself, like ethionine, inhibited only in the light. This inhibition was completely reversed by copper ions or by uracil or thymine, but uracil supplied in the dark, after thiouracil treatment, was ineffective. The authors concluded that the light reaction must involve participation of a copper enzyme and probably synthesis of purines, pyrimidines or nucleotides as well.

The most recent paper in this series (Thimann and Radner, 1958) draws together a large body of evidence that the light reaction in question is primarily concerned with the synthesis of riboflavin. The effects of almost all the inhibitors previously used can be completely reversed by riboflavin, and the amount of riboflavin required remains more or less constant, irrespective of inhibitor concentration. Anthocyanin production in darkness (with sucrose) is raised by the addition of riboflavin to levels approximating normal light yields. Determinations of riboflavin content show that it varies parallel with anthocyanin content, and that the effects of the inhibitors studied are primarily effects on riboflavin synthesis. The riboflavin probably acts not as a photoreceptor but as a dark catalyst in the production of anthocyanin from sugars, with each mole of riboflavin leading to the production of 30 to 60 moles of pigment. Riboflavin synthesis is thus an important limiting step in anthocyanin formation; the remaining dark reactions and the details of the various biosyntheses involved are still to be identified.

The major *Spirodela* anthocyanin was first characterized as a diglycoside of cyanidin (Thimann and Edmondson, 1949). However, Geissman and Jurd (1955) have re-examined it by more sensitive techniques and consider this impossible on the basis of their results. While the structure of the pigment remains unknown, it is closely related to the flavonoid substances of the plant; seven of these have recently been isolated by paper chromatography, and those studied appear to be "glycosides of or closely related to vitexin, a derivative of apigenin" (Jurd et al., 1957). The 7-glucoside (saponarin) and a new 4′-glucoside (isosaponarin) have been identified.

DEVELOPMENTAL PHYSIOLOGY

TURION FORMATION AND DORMANCY. Landolt (1957) has reported turion formation in *S. polyrrhiza,* certain strains of *L. minor* and three *Wolffia* species under controlled aseptic conditions, but did not study the process specifically. Two detailed investigations, however, have been reported, using *S. polyrrhiza,* the plant in which turions are most prominent.

Jacobs (1947) studied both growth and turion formation in crude cultures under many combinations of controlled temperature, light intensity and light duration. He concluded that turions were produced under any condition which would maintain photosynthesis at levels considerably in excess of carbohydrate utilization for growth and respiration. Thus increased CO_2 levels strongly favored turion formation, and turions occurred even at relatively low light intensities and high temperatures when growth was reduced by nitrogen deficiency. Turions were produced experimentally at all temperatures between 10° and 35° C. Turion primordia were indistinguishable from normal frond primordia until they were at least 0.4 mm long, but their "destiny" was determined at half that length. Turions never produced turions; at least two vegetative generations had to intervene between turion germination and new turion formation.

The rapidity of germination depended upon the temperature used. Turions produced at 25° C. would remain dormant for six months if kept at that temperature, but germinated rapidly after being held at 10° for two weeks. Turions produced at relatively low temperatures, on the other hand, germinated quickly without further low temperature treatments. The minimum temperature for germination was 15° C. Light was required for germination, but intensities of 5 to 10 footcandles were sufficient. Actual germination was preceded by formation of a gas bubble in the tissue, which served to float the turion to the surface.

These results have been largely confirmed, and extended, by Henssen (1954) who worked with aseptic cultures. Under the apparently constant stock conditions used, *S. polyrrhiza* formed turions spontaneously from August through April, with a maximum in the winter months. Turions were produced at any time, however, under conditions of moderate mineral deficiency or addition of various sugars. Sucrose caused turion fomation in both light and darkness, but glucose was effective

only in the light. Fructose and maltose also caused turion formation. Henssen followed starch formation, amylase activity and pH changes, but was unable to establish any relation between these factors and turion production.

The dormancy of naturally-occurring "winter" turions, gathered outdoors, was different in some respects from that of experimental, sugar-induced turions. Temperature effects like those mentioned above were observed with the winter turions. In addition, brief treatments with potassium cyanide or 2,4-dinitrophenol would break dormancy, but, while "winter" turions germinated in both light and darkness following KCN treatment, sugar turions required light. Unfortunately Henssen appears to have been unaware of Jacobs' earlier work, so that he made no attempt to perform exactly comparable experiments on the light requirement.

Dark germination of the "winter turions" was associated with a moderate rate of starch degradation and high amylase activity, and light germination with a higher rate of starch degradation but very low amylase activity. Another enzyme, presumably phosphorylase, degraded starch under these conditions. The inhibition of amylase by light was reversible by subsequent darkness, and was observed also in normal vegetative plants.

SENESCENCE AND REJUVENATION. Vegetative reproduction in *L. minor* exhibits cycles of senescence and rejuvenation under constant external conditions, and thus provides excellent material in which to study the fundamental problem of meristem aging. These cycles were first described by Ashby and collaborators in Britain (Ashby and Wangermann, 1949; Ashby, Wangermann and Winter, 1949; Ashby, 1950; Wangermann and Ashby, 1950). The basic observations on senescence are that any given frond has a definite life span, during which it produces a definite number of daughter fronds; and that each of these daughters is smaller in area than the one preceding it. This reduction in area of successive daughter fronds is not due to a change in cell size but to the presence of fewer cells in late than in early daughters. Late daughters not only are smaller but produce fewer daughters than the earlier. For example, with the clone and (crude) culture conditions used, the mean life of first daughter fronds was 33.3 days, during which they produced a mean of 3.8 daughters; mean mature area was 5.13 square millimeters. The mean life span of fourth daughter fronds was 18.7 days, during which time 1.2 daughters were produced,

and the mean mature area was 1.04 square millimeters (Wangermann and Ashby, 1950).

These results seem to imply that a *Lemna* clone produces ever smaller fronds with shorter life spans and finally disappears. No such disaster occurs, of course; in fact, the mean area of fronds in a clone does not change at all under constant conditions. The reason is that the aging phenomenon described is balanced by that of rejuvenation—the small, short-lived daughter fronds produce first daughters which are much larger than themselves. This process continues in succeeding generations of first daughter fronds until the maximum size is reached, thus maintaining the average area of the clone. For this reason the data above, and those in all other experiments, were derived by starting with third-generation first daughters (that is, first daughters of first daughters of first daughters), in which area, life span and capacity for frond production are maximal.

This senescence-rejuvenation cycle is of interest not only in itself but also for the effects it may have on studies utilizing measurements of individuals or small numbers of plants, rather than average values derived from large numbers. This was recognized by Pirson and Göllner (1953a) who confirmed the existence of such cycles in their clone of *L. minor,* and thus found it necessary to select plants carefully for their studies on root growth. These authors found that root length provided a convenient measure of frond age; the chief value of their work on this question, however, is simply in their demonstration of the cycle under better conditions. The use of crude cultures by Ashby's group leaves open the possibility that at least part of the "cycle" might be due to progressive bacterial growth within the mother frond pockets. While absolute asepsis was not maintained even by Pirson and Göllner, particularly when fronds were marked with india ink, both stock cultures and nutrient solutions were aseptic at least at the start of each experiment.

The senescence-rejuvenation cycle can be modified by environmental conditions. Increased temperatures decrease the life-span of each individual frond, and the rate of daughter-frond production increases proportionately, so that about the same number of daughters is produced before death. On the other hand, increased light-intensity increases the rate of frond production but has little effect on the life-span. The amount of rejuvenation—that is, the increase in area of a first-daughter

frond over the area of its mother—is not affected by either temperature or light intensity, but is inversely related to the area of the mother frond (Wangermann and Ashby, 1951).

Working towards an interpretation in terms of growth substances, Ashby and Wangermann (1951) showed that the growth of daughter fronds was promoted by some material received from the mother; a small fragment of the mother was sufficient for this promotion, which was not dependent upon light intensity. From such data and from an analysis of individual frond growth rates it appeared reasonable to postulate that the cycle was controlled by the levels of two substances or groups of substances, A and B. In this scheme, final frond area depends on the amount of A in the mother; A decreases as the mother frond ages, and may act on the daughter frond by controlling the size and cell-number of its initial. The rate of diminution of A is also assumed to determine life-span, since life-span in the mother and the rate of decrease in area of successive daughters are always closely and inversely correlated. Rate of A diminution is thus "rate of aging". The second substance, B, must reach a threshold value before a daughter frond enlarges; thus the rate of B production is reflected in the rate of daughter frond production.

Further experiments in this series were directed at defining A and B more closely. Wangermann (1952) showed that rate of aging (decline in "A" level) was unaffected by length of day or by removing daughter fronds; rate of frond production ("B" level) was affected by both factors. Adenine had no effect on rate of aging. In certain concentrations it increased the cell size of daughter fronds prematurely removed from their mothers, but it did not increase cell number. 2,3,5-triiodobenzoic acid (TIBA) did not affect rate of aging, in spite of evident effects on the auxin status. The rate of aging was significantly increased by ultraviolet radiation. This increase was not affected by the presence of indoleacetic acid, and the overall conclusion was reached that neither adenine nor auxin levels were closely related to the postulated changes in "A" levels (Wangermann and Lacey, 1953).

More recently, the same authors (1955) reported that relatively low nitrogen levels decreased the rate of aging; the respiration rate of low-nitrogen fronds was lower, on an area or dry weight basis, than that of the high-nitrogen plants. This suggestion of a direct relation between "A" decrease and respiration rate was at least partially confirmed by

the observation that the life-span of fronds kept under nitrogen atmospheres during the dark period was longer than that of those allowed to respire at the higher normal rate.

The inverse relation between nitrogen level and life span was not confirmed by Böszörményi and Böszörményi (1957) who suggested that this effect was probably dependent upon the nitrogen/phosphorous ratio and perhaps on the interaction of other nutrient components as well. It is evident that the biochemical basis of the senescence-rejuvenation cycle requires much further study and represents an important challenge to investigators of growth and differentiation. Aseptic techniques would simplify the testing of organic substances and should be employed in future work on this problem.

FORMATIVE EFFECTS OF LIGHT. Although incidental observations are recorded in several other papers, only two investigations have dealt primarily with the morphogenetic action of light. Both have used *L. trisulca,* in which the more or less submerged fronds remain connected by vascularized petiole-like processes. When the plants are grown in crude culture, each frond produces only one or two daughters; in low light intensities, only the "plus" daughter develops, giving rise to "spiral" colonies, while both "plus" and "minus" daughters elongate under higher intensities, so that the colony branches dichotomously (Goebel, 1921) This is probably an effect of carbohydrate status, since Zurzycki (1957b), using aseptic culture techniques, found that sucrose causes the production of two or more daughters per frond even in dim light.

When dim light of selected spectral regions is used, marked elongations of frond "lamina" and "petiole" occur under far-red light, and slight elongations may occur under blue. Elongation is also considerable (but less than under far-red) in darkness, while growth in red, yellow or green is similar to that in white light. If high intensities of light are used in the absence of sucrose, elongation takes place under both red and far-red but not under blue light. There are also differences in the numbers of daughter fronds formed in the various spectral regions. Zurzycki considered the hypothesis that these light effects are due to changes in auxin levels, but did not test for interactions with added growth substances.

EXPERIMENTAL CONTROL OF FLOWERING. Some of the environmental factors believed to be associated with flowering have already been discussed. So far, most of the attempts to control it by various

combinations of temperature, photoperiod and changed nutrient media have been unsuccessful (e.g., Hicks, 1932a; Brooks, 1940; Pirson and Seidel, 1950; Landolt, 1957). Hicks (1932a) obtained flowering *L. minor, L. trisulca, L. minima, L. valdiviana* and *Wolffia columbiana* by treating crude cultures with ultraviolet radiations. The first two species also flowered following treatments with near-toxic levels of sodium hydroxide. Unfortunately no attempt was made to see whether the original plants used, or the controls, already had flower primordia. No attempt to repeat this work has been reported, although ultraviolet treatments did not cause *L. minor* flowering for Wangermann and Lacey (1952, 1953), and high pH (although not specifically sodium hydroxide injury) has been used by many investigators without inducing flowering.

The first unequivocal control of flowering in the *Lemnaceae* was established by Kandeler (1955, 1956) who showed that several strains of *L. gibba* flowered as long-day plants in aseptic culture. The critical daylength of strain Gl lay between 12 and 14 hours of light per day at 30° C. under mixed fluorescent and incandescent light. Flowering was studied by dissection in terms of five arbitrarily assigned stages of primordium development. When only fluorescent light was used, flowering occurred only in "aged" medium (old cultures) and did not do so if the medium was changed frequently. Under incandescent light, however, flowering took place even in fresh (frequently changed) medium within three weeks. Experiments with weak supplementary light to extend the day-length showed that far-red light (7200-7600 Å) promoted flowering more than red (6000-7000 Å) or other wavelengths; red itself was inhibitory at high intensities. Interruption of the long nights with far-red (7000-9000 Å) promoted flowering, but with a relatively high energy requirement. This was lowest (ca. 400 kilo-ergs/cm^2) at the start of the dark period, and rose steadily during that period. The observation that far-red promotes flowering while red inhibits, though somewhat unusual for long-day plants, probably accounts for the more rapid flowering observed under incandescent light, where the far-red/red ratio is relatively high. The aged medium effect, however, remains unexplained and may bear on the suggestions mentioned earlier that flowering depends on the composition of the pond water.

In the course of experiments described previously, Landolt (1957) observed flowering in various strains of *L. minor, L. gibba* and *L. perpusilla.* Two strains of *L. perpusilla* were observed flowering in most

of the conditions used, including darkness; these will be discussed further below. In all the rest, flowering occurred only under long photoperiods at relatively high light intensities and temperatures and in old, crowded cultures (Hutner's medium was used). Sucrose promoted flowering in some cases, and inhibited in others. Flowering intensity varied greatly from strain to strain within a single species. Several attempts were made to induce flowering in recalcitrant strains by growing them in mixed cultures with flowering strains. These were unsuccessful except apparently in the case of one *L. gibba* strain which flowered in mixed culture with a flowering *L. minor* but not under the same conditions alone. However, it did flower alone under the same conditions except for slightly higher light intensity. Neither this effect nor other factors affecting flowering were investigated in any detail, and no dissections for flower primordia were performed.

In contrast to the long-day flowering response of *L. gibba,* at least one strain of *L. perpusilla* flowers as a short-day plant under certain conditions. This is Landolt's strain 6746, one of the two mentioned previously as flowering under all conditions. While it does eventually flower in old (Hutner's medium) cultures under all photoperiods, it flowers rapidly (7-9 days to full anthesis at ca. 26° C.) in fresh medium under short photoperiods. The critical daylength lies between 13 and 15 hours, and as little as 15 kiloergs/cm^2 of red fluorescent light given in the middle of each flower-promoting long night can completely inhibit flowering. Flower primordia can easily be detected after six days under an eight-hour photoperiod. Cultures maintained under long days in fresh medium do not flower at all (Hillman, 1958).

Little can be said in summary of the experimental control of flowering in Lemnaceae, since the subject has barely been opened. At present, flowering can be definitely controlled only in a few strains of *L. gibba* and *L. perpusilla.* Further work may uncover some basic mechanism common to the family as a whole, but the presence of both long- and short-day responses within the genus *Lemna* itself suggests that flowering in the Lemnaceae is probably controlled by factors as various as those affecting this process in plants in general. The rapidity of flowering, together with all the other experimental advantages of the Lemnaceae make them perhaps the most favorable organisms for future studies on the physiology and biochemistry of flowering. In addition, the fact that flowering strains of both *L. gibba* and *L. perpusilla* set abundant seed, opens the way to the production of biochemical

and other mutants, and possibly to their use in genetic investigations as well.

MISCELLANEOUS

AMINO ACID CONTENT. *L. minor* in aseptic culture, and *S. polyrrhiza,* were used by Keser (1955) in a general investigation of free and bound amino acids in a number of higher plants as studied by various extraction techniques followed by paper partition chromatography. Most of the known amino acids were identified. *S. polyrrhiza* starved in darkness (no carbohydrate source) had a higher free amino acid level than normal light-grown plants; under these conditions, methionine appeared as a free amino acid and taurine disappeared from the protein fraction. Similar studies were carried out with *L. minor* grown in a complete medium or with deficiencies of nitrogen, phosphorous or potassium. The last two deficiencies caused no major changes in amino acid content. Nitrogen-deficient plants were very low in asparagine and free amino acids, while their protein fraction appeared to lack the methionine and two other sulfur-containing compounds normally present.

GROWTH SUBSTANCE CONTENT. Thimann and Skoog (1940) and Thimann et al. (1942) used a plant ("Lemna minor") later identified as *S. oligorrhiza* (cf. Thimann and Edmondson, 1949) for tests of auxin extraction methods. Extracts were assayed by the *Avena* curvature test. Ether extractions continued to yield activity over many months. The auxin yield was increased by pretreatment of the tissue with trypsin, chymotrypsin or ficin, but no extraction was possible with water, or alkaline autoclaving.

Sargent (1957) used both long- and short-term extractions with ether or water, followed by paper partition chromatography and *Avena* coleoptile section tests, to assay the growth-active components in *L. minor.* Four growth-promoting substances and one inhibitor were found, their proportions depending upon the extraction techniques used. The major promoter was tentatively identified as indoleacetic acid on the basis of its Rf value in the solvent system used.

ACTION OF IONIZING RADIATIONS. As mentioned earlier, Hicks (1932a) reported that flowering in a number of Lemnaceae followed treatment with ultraviolet radiations from a quartz mercury vapor source. Plants receiving treatments of ten or more minutes at 105 ergs

per square centimeter per second were visibly burned or browned, and most of the upper cells were killed with longer exposures. Radiation ca. 2500 Å was used by Wangermann and Lacey (1952, 1953) on *L. minor*. No energy data are reported, but evidence is given that much of the damage caused is due to the action of ozone, so that care must be taken to exclude the latter if UV effects alone are to be studied.

The effects of X-rays on *L. minor* were studied by Johnson (1941). Doses in the range 1000 to 2500 r caused continued production of a high proportion of deformed, "etiolated" fronds and greatly reduced the multiplication rate during several weeks after treatment. These injuries were less apparent under greenhouse tank conditions in fall and winter than in the spring.

SUMMARY: TOPICS FOR FUTURE INVESTIGATION

This paper is intended as a guide to work on the Lemnaceae, and calls particular attention to their valuable characteristics as organisms for developmental, physiological and biochemical investigations. These characteristics include small size, rapid clonal growth and adaptability to aseptic culture techniques. For the subjects covered, see the outline. The paragraphs below summarize some topics which, in the opinion of the writer, either represent unsolved questions of considerable interest or are of general significance for any experimental work to be conducted with these plants.

1. Recent embryological evidence strongly supports the traditional hypothesis of a close relationship between the Lemnaceae and the Aroids, but the taxonomic status of genera and species is not entirely clear. The chromosome numbers reported for at least two *Lemna* species suggest no very close relationship between them. A revision of the family by the most modern systematic methods, including collections of living material, chromosome counts and the use of standard environmental conditions for the characterization of races or varieties, is highly desirable.

2. Precise repetition of experiments depends on the use of the same clone, medium, stock conditions and experimental period, among other factors. Trace metal nutrition, pH and other medium variables change with time in culture, may interact in a complex fashion, and should not be left entirely to untested assumptions in a given series of experiments; plants from older cultures may differ from those in younger

cultures in many respects. Increase in frond number is exponential under good culture conditions, but is not analogous to the growth of microorganisms by simple division. Annual cycles in frond multiplication rate and other growth values under constant light and temperature have been reported, but require confirmation under conditions controlling as many other variables (e.g., air pollution) as possible.

3. Certain species are able to grow in darkness if supplied with minerals and sucrose; others will not do so unless given additional supplements or low doses of red light. Roots do not elongate except under normal light conditions. The nature of these dark-induced blocks in growth is unknown.

4. Growth in aseptic culture is often promoted by the addition of organic compounds to the basic mineral medium. Sugars promote growth only under suboptimal light supply. Many other compounds may promote growth by improving trace-element availability as complexes, or buffering pH changes. The question whether such compounds would promote growth under optimal conditions of light, temperature and inorganic nutrition remains unanswered.

5. Anthocyanin formation, the only biochemical process so far studied in any detail, appears to depend largely on riboflavin synthesis. Studies of chemical formative effects and of respiratory processes have also been reported, but little is known concerning the mechanisms involved.

6. The cycle of "senescence and rejuvenation" in vegetative reproduction, and its modification by external factors, offer an excellent model system for the study of aging. It is also an important consideration in any other study in which small numbers of individuals are used.

7. Flowering can be controlled in a few strains of two *Lemna* species, but the control of flowering in general is not understood. Factors such as photoperiod, temperature and medium composition have recently been investigated. In nature, crowding and changes in pond-water composition have been suggested as important factors. The flowering strains mentioned set abundant seed, inviting the development of techniques for the production, crossing and biochemical study of mutant strains.

ACKNOWLEDGMENTS

I am indebted to Dr. A. W. Galston for encouraging the writing of this review; to Dr. O. Tippo and Dr. W. L. Stern for reading and

criticizing the manuscript; to Mr. M. Furuya for translations from the Japanese; and to Dr. A. W. Naylor, in whose laboratory I was introduced to the experimental possibilities of the Lemnaceae.

The National Science Foundation supported this work under grant NSF G-4433 to A. W. Galston.

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SUPPLEMENT

The preceding review was completed in September, 1958. Since then, more papers have appeared, and the writer has belatedly come to know about several others. These are briefly noted below.

Natural History

Various species of *Lemna,* together with the water ferns *Azolla* and *Salvinia,* often completely cover mediterranean rice fields by the end of summer (De Bolós and Masclans, 1955). Dore (1957) has reported on the ranges and some other aspects of *Wolffia* species in Canada. *Lemna aequinoctialis,* previously known only in Africa, has now been found in many western hemisphere locations and in the Philippines (Giardelli, 1959).

Normal Vegetative Growth

Ikusima (1955) and co-workers (Ikusima et al., 1955; Ikusima and Kira, 1958) have studied the growth of *L. minor* and *S. polyrrhiza,* and of mixed cultures, under non-aseptic but controlled light and temperature conditions. They concluded that mutual shading plays a relatively small part in the growth reduction at high frond densities. They also used various transformations and logistic equations to analyze the growth rates mathematically.

Boron toxicity at concentrations as low as 0.1 mg./L. has been reported for *L. minor* grown on clay suspensions; increased calcium levels partly overcame it. The roots appeared to be particularly sensitive indicators of calcium-boron interactions (Fox and Albrecht, 1958). Eyster et al. (1958) found that the manganese requirement of *L. minor* is much lower for heterotrophic growth (short-term) than for autotrophic growth and optimum Hill reaction activity.

Heterotrophic and Non-photosynthetic Growth

The ability of Lemnaceae to grow non-photosynthetically under suitable conditions suggests the possibility of obtaining permanently chlorophyll-free strains, as has been done with *Euglena.* However, Scher and Aaronson (1958) were totally unsuccessful in their attempts to do so, whether the "bleaching agent" used was darkness, elevated temperature, streptomycin or 3-amino-1, 2, 4-triazole. Temporarily "bleached" cultures resumed the production of normally green fronds when returned to normal conditions, if they survived at all.

Further experiments by the writer indicate that kinetin may not maintain long-term heterotrophic growth of *L. minor* on sucrose alone, in contrast to the effects of small red light doses and to the short-term

effects of kinetin itself. However, definitive experiments are still required.

Chemical Growth Promotions, Inhibitions and Formative Effects

Ono (1952) reported strong growth promotions by IAA and NAA in *S. polyrrhiza* and *Lemna* sp., particularly on the roots. The medium contained no added iron or other trace elements. A marked negative phototropism of the roots was observed, but the observation that plants failed to grow when illuminated exclusively from beneath does not accord with this writer's experience.

Blackman et al. (1959) followed the uptake of C^{14}-carboxyl-labeled 2,4-D and related compounds by *L. minor*. The rate of 2,4-D uptake was maximal for the first 20 minutes and fell to zero after an hour or two, to be followed by a net loss during the succeeding 24 hours. Phenoxyacetic acid was accumulated steadily, without loss; compounds intermediate between 2,4-D and the unsubstituted parent compound in growth-regulating activity showed intermediate patterns of uptake and loss. The initial rate of 2,4-D uptake was dependent upon pH, being closely although not completely correlated (positively) with the concentration of undissociated molecules. Loss was relatively unaffected by pH. There was no consistent relationship between 2,4-D treatments resulting in net loss and their effects on subsequent growth as measured by dry weight increase.

Blackman and Sargent (1959) have conducted a detailed study of the uptake by *L. minor* of TIBA labeled with iodine-131. The mechanism of uptake appears to have several features in common with that for 2,4-D. In addition, the results suggest that TIBA interferes with cellular transport mechanisms. A paper by Sargent and Wangermann (1959) on the effects of TIBA is largely based on the work of Sargent (1957) cited in the review.

Deysson (1959) has reported growth promotion of light-grown *L. minor* by 10^{-8} molar kinetin or thiokinetin. The control growth appears to have been poor, and the data are derived from small frond numbers. Breaking of turion dormancy by these compounds was also observed. Growth promotion by kinetin of *Wolffia columbiana* grown in the light has also been studied by the writer (Hillman, 1960a); it was not observed when the medium was optimally dilute; it probably repre-

sented a moderation of unidentified toxic effects of excessively concentrated medium.

Gibberellic acid causes a small but statistically significant increase in the MR of *L. perpusilla* under a variety of conditions. Frond size is greatly decreased by this substance (Hillman, 1960b).

Kojic acid at concentrations between 10^{-6} and 10^{-3} M was reported by Yokota and Shimada (1958) to promote the growth of *S. polyrrhiza* in Knop's solution. EDTA also promoted growth under these conditions, and the kojic acid promotion was interpreted as a similar chelation effect. Oxine was toxic above 10^{-4} M, and had no effect at lower levels.

Todd et al. (1956) used *Lemna* sp. as a test organism for the effects of "synthetic smog" (ozonated hexene) and found that this substance was considerably more effective in inhibiting photosynthesis than ozone alone.

Developmental Physiology

According to Yoshimura (1943), molybdenum deficiency may cause flowering in several species, including *S. polyrrhiza.*

The writer has further investigated flowering in *L. perpusilla* strain 6746 and a strain of *L. gibba* (Hillman, 1959a, 1959b, 1961). The short-day response of the former depends on the presence in the medium of high levels of chelating agents such as EDTA (as in Hutner's medium). At moderate temperatures (22-28° C.) in a Hoagland's-type medium it is daylength-indifferent, although at high temperatures (ca. 29° C.) it shows a short-day response even in this medium. *L. gibba,* on the other hand, fails to flower, irrespective of daylength, in the Hoagland's-type medium, at least under fluorescent light. It flowers rapidly as a long-day plant in "aged" Hoagland's-type medium, as previously shown by Kandeler. EDTA replaces the "aged" medium effect.

Thus EDTA, and other chelators as well, convert *L. perpusilla* 6746 from a daylength-indifferent to a short-day plant, and the strain of *L. gibba* from a plant unable to flower to a long-day plant. This may have important implications for the understanding of photoperiodism.

Flowering of *L. perpusilla* is strongly inhibited, in both long and short days, by levels of gibberellic acid that promote MR (Hillman, 1960b).

MISCELLANEOUS

Chechenkin (1955) reported that *L. minor* contained no high saturated fatty acids in its oils. Linoleic acid was the most unsaturated acid present.

L. minor, like several other angiosperms and some bacteria, but unlike certain fungi, uses aspartic acid in lysine synthesis through the diaminopimelic acid pathway (Vogel, 1959).

Posner and Hillman (1960) investigated some effects of X-rays on *L. perpusilla* under carefully controlled conditions. The sensitivity to radiation was not changed by the presence of yeast extract and casein hydrolysate. A permanent shift in the "handedness" (asymmetry) of many of the clones derived from irradiated cultures was observed.

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