



STOMATA AND  
WATER RELATIONS IN PLANTS

Israel Zelitch, Editor

**Stomata and Water Relations in Plants**



## Preface

We have the good fortune to have members of the Station staff in the fields of biochemistry, physiology, ecology, physics, and plant pathology who share a common research interest in stomata and the problem of water movement in plants. This Seminar was organized in order to share our experiences and views with others and thereby hasten progress in the important and pressing problems which have arisen in these related fields.

Stomata play a role in photosynthesis and in transpiration. At the present time the most urgent problem appears to be the control of their movements so as to diminish the loss of water from plant leaves. It seems likely that in many parts of the world people will encounter serious shortages of water even before shortages of food become evident. We hope that the present discussions will stimulate scientists to find new solutions to this old problem of providing more water.

The chapters in this Bulletin are edited versions of the lectures given by members of our staff and of the discussions that followed. The Seminar, which was held at The Connecticut Agricultural Experiment Station July 1 to August 9, 1963, also included experimental work, demonstrations, and research conducted by the visiting participants. We also had the privilege of hearing informal talks by the participants on their current research. Unfortunately, it is not possible to summarize these other activities of the Seminar, but I hope that the stimulation generated by these contacts will result in experiments that we can all read about in scientific journals in the future.

In these lectures, we include reviews as well as accounts of new research work that is being reported for the first time by the experimenter. Each lecturer was encouraged to discuss his subject from his special viewpoint and experience. We discussed the biogenesis of stomata, the mechanism of stomatal opening and closing, the comparative biochemistry and kinetics of solute and water transport in cells, the permeability of the root to water, the hydraulics of water movement into and through plants, the diffusion of gases into and from leaves, and the role of stomata in plant diseases.

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providing drought resistant and susceptible varieties of seed for use in laboratory experiments. I wish to thank Isabelle Namanworth for able technical assistance provided in the laboratory work, and Dr. Hubert Bradford Vickery for his help in the preparation of the manuscripts.

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## Introductory Remarks

By James G. Horsfall, *Director*

The Connecticut Agricultural Experiment Station

On behalf of my colleagues here at The Connecticut Agricultural Experiment Station, may I welcome you to the Advanced Seminar on the Physiology and Biochemistry of Leaf Stomata. We are delighted and excited that you have come to share experiences and experiments with us in the area of our mutual interest—stomata, their role in plants, and their chemical control.

For the first time we can chemically control the opening and closing of stomata. Sporadically in the past physiologists have observed that stomata are closed on plants treated with certain chemicals. During this conference we aim to discuss the possibilities of deliberately closing and opening stomata.

The prospects for using this new tool are stimulating indeed. Brown and Escombe's classical studies will surely be extended far as a result. The in and out movement of gases will now be under better experimental control and our knowledge of transpiration and photosynthesis should be improved thereby.

I hope you have a pleasant and productive conference, and please return again some day.

## Experimental Morphology of Stomata

By Milton Zucker

Epidermal surfaces of most land plants present major barriers to the escape of water vapor and to the exchange of gases with the atmosphere. The stomatal pores which penetrate the epidermis are major ports of exit and entry to the interior of the plant. Since these pores can be opened and closed by the very specialized epidermal guard cells which surround them, vapor and gas exchanges between the plant and its environment can be regulated. Thus stomata exert a profound influence on the physiology of the plant.

The movement of guard cells involves changes in their morphology. It seems advisable, therefore, to begin this monograph on the physiology and biochemistry of water relations in plants with a morphological orientation to the stomata. This chapter will review briefly the distribution of stomata in epidermal surfaces, and it will discuss the morphology and cytology of guard cells in relation to the opening and closing of stomatal pores. Some of the factors controlling the patterns of differentiation of stomata will also be described.

Many of the ideas and points emphasized here were first brought to the author's attention by participants in this Advanced Science Seminar. Thus, this chapter not only reviews the literature, but, in a sense, it is a review of the Stomatal Seminar insofar as morphological aspects were concerned.

### DISTRIBUTION

Stomata are usually considered in terms of the epidermis of flowering plants. They are characteristic of all angiosperms and gymnosperms with the exception of a few aquatic types (10). However, functional stomata are also distributed among the cycads (42), horsetails (15), and ferns (62). They are even found as epidermal structures in liverworts and mosses (14). Only the algae and fungi lack them. Functional stomata appear to be basically similar in structure regardless of the species (14). Few evolutionary trends can be discerned from comparative morphological studies of these ancient epidermal characters. The complexes of companion epidermal cells which often surround the stomata offer more fruitful material for comparative studies than the stomata themselves (52). Unfortunately there exist almost no comparative biochemical studies of stomata from lower forms of plant life. Wider application of modern biochemical techniques used success

fully to study angiosperm stomata may reveal more primitive functional types in other groups of plants.

Among some of the lower plant forms, stomata may be restricted to limited areas of the epidermis. In flowering plants, however, they are found on all epidermal surfaces with the exception of the tissue covering the roots (10). Stomata are most commonly associated with the leaf because they are most numerous and most important physiologically in this organ. Somewhat over 100 stomata per mm<sup>2</sup> of leaf area has been cited as an average figure for the stomatal density on leaves of common greenhouse plants (9). Assuming that the average pore diameter of an open stoma approaches 10  $\mu$ , the distance between stomata would be somewhat less than 10 pore diameters. Some leaves, particularly those of tetraploids, contain many fewer stomata per unit area, and gas exchange may be seriously limited by the sparse distribution (3).

Certain species of oak possess over 1000 stomata/mm<sup>2</sup> of leaf area (49). More than 50 per cent of the epidermal surface of these species is composed of guard cells. Most commonly, stomata are more numerous and larger on the underside of leaves and cotyledons. In some species, they may be present only on the under epidermis. The reverse situation has also been observed. Many workers have attempted to take advantage of the unequal distribution of stomata on the two surfaces of the leaf to determine the physiological importance of stomata.

Stomata are also distributed over the surface of most stem tissues, but far more sparsely than on leaves. The guard cells of stems may not contribute to the regulation of transpiration, but they may be factors in the penetration of fungi or chemicals applied to the stem. Some flower petals bear a few functional stomata, and stomata have even been observed as epidermal structures of anthers (20). Stomata occur also on the surface of fruits. Those of the grapefruit and orange have been examined in detail (37). Although present concern is chiefly with leaf stomata, these structures are characteristic of most epidermal surfaces.

#### MORPHOLOGY OF OPENING AND CLOSING

*Morphological Changes:* The morphological basis for the opening and closing of stomata was described in detail during the last century (10, 14, 16). The movement of guard cells was, at that time, correctly ascribed to changes in turgor of the cells, that is, to the uptake and loss of water from them. The light-induced water uptake of the guard cells causes them to swell and expand. Many guard cell walls contain locally thickened, rigid areas particularly around the stomatal pore. When the thinner, more elastic areas of the wall are extended by the

increased turgor pressure, these rigid, inelastic areas are apparently forced to bend, opening the stomatal pore. Dr. W. M. Dugger, Jr., suggested as a model a balloon with masking tape pasted along one side. Upon inflation, the rigid, taped wall of the balloon bends inward to accommodate the expansion over the rest of the surface. Where wall thickenings are not limited to, or do not occur around the stomatal pore, other models must be envisaged. Most closed stomata are roughly ellipsoidal in shape. When guard cells expand in the light and the stomatal pore is opened, the stomata assume a more nearly spherical, doughnut-like shape. This change is thermodynamically advantageous because it minimizes the energy required to stretch the walls of the guard cells upon inflation. That is, a sphere is the geometric form which encloses the greatest volume with the minimal amount of surface area. Scarth and Shaw (33) showed that the major axis of the elliptical, closed *Vicia faba* stoma does shorten as it opens. This decrease in stomatal length would compress the ends of the walls lining the stomatal pore, forcing the walls to bend and open the pore. Neither the balloon nor the doughnut model requires any stretching of the stomatal pore walls. In both models the perimeter of the stomatal pore would remain constant while the diameter increased or decreased.

When open stomata are transferred from light to darkness, water is lost from the guard cells, and the turgor pressure against the walls is relaxed. The distorted pore walls then spring back or are pulled back to their straightened form thereby closing the stomatal pore. The extensive localized wall thickenings may have an important function in returning the rounded, open stoma to its original elliptical form once turgidity is lost. Pressure from surrounding epidermal cells, as well as from the cuticular layer stretched over the stomata, may also aid the closing process. The rapidity with which stomata close compared to their rate of opening suggests some such mechanical pressure.

Although the wall thickenings of grass stomata are much more extensive and distinctive than those of other forms, grass guard cells function in a similar manner (11). In fact, the immature stomata of grass leaves are similar morphologically to those of other species (11, 29).

*Water Movement in Relation to Opening and Closing:* An actual increase in the volume of guard cells can be measured microscopically when stomata open (50, 51). Calculations based on the data of Scarth and Shaw (33) suggest that the light-induced opening of stomata in broad bean leaves is accompanied by an increase of 25 per cent in the volume of each guard cell. If the guard cell is assumed to be 1000  $\mu^3$  in size, then an uptake of something on the order of  $10^{-6}$   $\mu$ l of fluid is



required. Although this amount of liquid is minute in terms of laboratory manipulation, it could present quite a logistic problem for the individual guard cell. The transfer of water from one part of the cell to another with subsequent swelling of colloids might produce some expansion, but such mechanisms could not account entirely for the increase in guard cell volume. Water must move into the cell from an external source.

Adjacent epidermal cells have been postulated as the source of water for opening guard cells (16, 50). In some species each guard cell of the stoma is accompanied by a similar-sized companion epidermal cell. The companion cells often form a bridge over the sub-stomatal cavity connecting the suspended guard cell to the rest of the epidermal and mesophyll tissue of the leaf. Some plants are characterized by whole complexes of companion cells which completely surround the stoma (52). The fact that many species of plants completely lack companion cells has made it difficult to assign to these cells a general role in the opening and closing of stomata. Where companion cells do not occur, little morphological distinction can be found between epidermal cells adjacent to and distant from stomata. However, distinct biochemical differentiations have been observed, particularly in certain leguminous species. Scattered throughout the colorless areas of the epidermal tissues of sweet clover stipules there are highly pigmented, anthocyanin-containing cells. The microscope shows that the pigmented epidermal cells are only found adjacent to stomata. Aside from their pigmentation and proximity to guard cells, they cannot be distinguished from other epidermal cells morphologically. Goodwin *et al.* (13) observed in certain other leguminous species that each stoma is invariably accompanied by an epidermal cell in which a fluorescent porphyrin-containing body was found. In other respects the cell was indistinguishable from the rest of the epidermis. Thus there may be a biochemical if not a morphological basis for distinguishing epidermal cells adjacent to stomata from the rest of the epidermis.

The study of pigmented epidermal tissue of leaves or flowers containing functional stomata may provide a source of information on the water movement into and out of guard cells. In most instances where the epidermal cells contain large quantities of anthocyanin pigments dissolved in their vacuoles, the guard cells are colorless (19). Movement of water between guard cells and the adjacent pigmented epidermal cells may produce changes in anthocyanin concentration. Such changes in pigment concentration could be detected with the aid of a microspectrophotometer (6).

*Structure of the Cell Wall:* The highly localized wall thickenings found in guard cells are not generally characteristic of other epidermal cells.

However, the structure of guard cell walls appears to be similar to that of the primary walls surrounding most epidermal tissue. Detailed information on the ultrastructure of stomatal walls comes from electron microscopy of grass stomata (4, 39). These studies indicate that the walls are composed of rigid, inert, discontinuous cellulose microfibrils embedded in an amorphous matrix containing branched hemicelluloses, pectins, and other polysaccharides (39, 41). Water is the main component. The amorphous matrix, whose composition is ill-defined, appears to offer the best possibilities for the elastic properties of the walls. Electron photomicrographs of the thickened regions of the guard cell walls indicate that they are composed of layers of intertwining cellulose microfibrils alternating with layers of the amorphous matrix (39). Polarizing microscopy (68) suggests that the general orientation of the microfibrils in many types of guard cell walls is in a direction transverse to the long axis of the cell. Such orientation would allow the cells to swell most readily. However, electron photomicrographs of primary cell walls indicate that the orientation can vary from the inner to the outer part of the wall (40, 41).

Several studies of the *in situ* synthesis of cellulose microfibrils have been made with the aid of tritiated substrates and radioautography (40, 58). These studies show that the microfibrils are synthesized continually in all areas of the cell wall, including those most distant from the cytoplasmic boundary. Since plasmodesmata have not been observed in guard cell walls examined under the electron microscope (4, 39), this pattern of synthesis suggests that enzyme proteins must occur throughout the wall (40). Radioautography indicates that the synthesis of cellulose in guard cell walls occurs at a more rapid rate than in other epidermal walls (40). Apparently the stomatal walls are richly endowed with the enzyme proteins concerned with this function.

The cuticular layer which covers most of the outer epidermal surface of the plant extends over the guard cells. It may also extend into the stomatal cavity and line the walls of the pore. The cuticle is composed chiefly of esters of long chain fatty acids (34, 61, 63). As the plant ages, wax-like hydrocarbons impregnate the cuticular layer and are deposited as rods or platelets on its surface (48). The fact that the stomatal pore may be coated with a waxy layer has important consequences for the passage of aqueous solutions through the stomatal opening. Water and other liquids with a high surface tension would be unable to penetrate into a wax-lined capillary very readily. Any extensive flow of an aqueous solution through open pores could be obtained experimentally only by adding detergents which substantially decrease the contact angle of the solution (8, 26). Oil sprays which have a very low angle of contact on a waxy surface can penetrate

through the open pores readily. In fact, such fluids may even be sucked into the sub-stomatal chambers by capillary action (55).

The geometry and surface properties of stomatal cavities and pores may also influence the penetration of silicone rubber latex used to make stomatal impressions (67). Apparently the surface tension of the latex is sufficiently low to allow it to penetrate into the actual pore of a number of types of stomata. However, some caution is necessary in interpreting results if this technique is extended to a wide range of species.

Scott and co-workers (37, 38) have suggested that the cuticular layer covering the outer epidermis may also extend into the sub-stomatal cavity. A continuous cutinized or suberized layer is envisaged as lining the chamber. If such extensions of the cuticular surface into the sub-stomatal cavity are of general occurrence, then the actual evaporating surfaces of the leaf would be hydrophobic in nature. A cuticular membrane would be expected to have quite different properties from the moist cellulosic walls of mesophyll cells. In a succeeding chapter, Rawlins (31) suggests that, in addition to the stomata, there are evaporating surfaces in the leaf capable of regulating transpiration. Further examination of the surface layer of cells lining the sub-stomatal chamber is certainly indicated by both the morphological and physical data.

Numerous microscopic passages through the outer walls of guard cells and other epidermal tissues have been reported by German workers (12, 23, 47). These wall perforations called ectodesmata extend from the outer cytoplasmic membrane to the cuticular layer. They may function in the deposition of cutin or in foliar absorption through cuticle (61). The ectodesmata differ in two respects from plasmodesmata, which are permanent cytoplasmic penetrations through the side walls of most epidermal cells. Ectodesmata do not give cytoplasmic stain reactions and they are usually transitory in nature (12, 23). These outer wall penetrations appear when the plant is placed in the dark or at low temperatures, and disappear in the light and at higher temperatures. Such behavior would be intriguing in relation to turgor movements of guard cells if it were not for the fact that guard cell ectodesmata are stable, in contrast to those in surrounding epidermal cells.

The plasmodesmata which link most epidermal cells together are not always observed in guard cell walls. Both light (46) and electron microscopy (4) have failed to detect plasmodesmata passing through guard cell walls of a number of species, although the connections are readily observed between other epidermal cells.

The apparent morphological isolation of guard cells from other epidermal tissues is also accompanied by an obvious chemical isolation in some species. The characteristic lack of anthocyanin pigment in

guard cells surrounded by highly pigmented epidermal cells (19) has already been cited. Other plants have been described in which every cell of the leaf epidermis, with the exception of the guard cells, contains calcium oxalate crystals (59), or protein bodies (54). The guard cells in these plants completely lack such deposits. Sheffield (46) reported that all of the epidermal cells of tobacco leaves he infected with tobacco mosaic virus contained virus inclusions with the singular exception of the guard cells of the stomata. These sharply delimited chemical differences, coupled with the lack of cytological evidence for plasmodesmata, indicate that guard cells are isolated from other epidermal tissues. The metabolic activities and cytological structures unique to guard cells also emphasize their separation from other epidermal tissue.

*Cytology of Guard Cells:* The most outstanding cytological feature which differentiates stomata from other epidermal tissues is the presence of functional chloroplasts in the guard cells. The guard cell chloroplasts have long been of interest because they are considered to be the photoreceptors involved in the light-induced opening of stomata. Absorption spectra of individual stomatal chloroplasts, obtained with the aid of a microspectrophotometer (64), are typical of those given by mesophyll chloroplasts, and indicate the presence of both chlorophyll a and b. Chromatographic examination of extracts of epidermal strips of tulip leaves has confirmed the presence of both chlorophylls in guard cell plastids (45). However, the concentration of these pigments on a weight basis was found to be at least an order of magnitude lower than in mesophyll plastids. Some guard cells contain so little chlorophyll that it can be detected only by fluorescence microscopy (43). Shaw and Maclachlan (45) demonstrated with the aid of radioautography that the chloroplasts of guard cells are functional. They found that stomatal chloroplasts in washed epidermal strips fixed  $C^{14}O_2$  in the light at a rate of two to three times as great as that in the dark. However, the amount of carbon fixed was very low. A positive Molisch reaction involving the reduction of silver nitrate by chloroplasts in the light (43), as well as the light-induced reduction of tetrazolium salts by guard cell chloroplasts (7), is further evidence of photosynthetic activity.

Generally, when chlorophyll cannot be detected in stomatal plastids, the guard cells do not respond to light. Stomata of etiolated seedlings do not open in the light immediately after the plants have been removed from darkness. After two to three hours of exposure to light, during which time chlorophyll synthesis is initiated, a light-induced opening begins to appear (56). It might be of interest to repeat such experiments under conditions where chlorophyll synthesis is inhibited by chemical agents such as chloramphenicol or streptomycin.



Much work has also been done with stomata of albino leaves (44, 57). When considering such studies it should be borne in mind that the epidermis is derived from a tissue layer different from that which gives rise to the mesophyll. Consequently, in many albino leaves only the mesophyll is affected. The epidermal guard cells of albino leaves may often contain green chloroplasts and open in the light. Starch will also accumulate in such stomatal chloroplasts over albino areas of the leaf when exposed to light. Guard cells over green areas of the same leaf synthesize no starch if protected from the light (33). Where the stomatal plastids also lack chlorophyll, no opening occurs (57). The completely albino plant shows a very strong guttation instead.

Although guard cell chloroplasts are photosynthetically active, they obviously cannot compete photosynthetically with mesophyll chloroplasts which are several times larger and contain much more chlorophyll. Stomatal plastids also appear to differ from those of the mesophyll both in structure and metabolic activity. Electron microscopy of plastids from grass stomata indicates that few lamellae and no grana are present (4). Generally these guard cell plastids appear to be immature and undeveloped compared with their mesophyll relations. The structure of some dicotyledonous stomatal plastids has been reported to be more fully developed (17). However, no extensive comparisons are available at present.

Metabolically many guard cell plastids differ from those in the mesophyll in that they accumulate large quantities of starch or other polysaccharides. Often starch formation in stomatal plastids is not associated with photosynthetic activity. Starch may accumulate in guard cells at night and disappear during the day (24). The rate of starch hydrolysis in guard cells of many species is also extremely low (27). The lack of correlation between photosynthetic activity and active starch synthesis in some types of stomatal cells suggests that substrates are supplied by other tissues. Starch accumulation can be induced in stomatal plastids by supplying the guard cells with an exogenous source of glucose 1-phosphate (65), and other sugars (27). The rapid formation of starch under these conditions indicates that enzymes of synthesis (not necessarily phosphorylase) must be very active in these plastids. Even the proplastids of stomatal mother cells are capable of synthesizing large quantities of starch (24). Obviously in this case, exogenous substrates must be used.

Observations of guard cell chloroplasts in senescent leaves indicates that they are more stable than mesophyll chloroplasts. Under conditions where mesophyll plastids lose most of their chlorophyll, stomatal plastids may remain green. Dr. J. van Overbeek has pointed out that the retention of chlorophyll by cells in yellowing leaves is a typical kinetin-like

effect. Consequently, the difference in stability of the two types of plastids may reflect differences in hormonal composition between guard cells and mesophyll cells.

Starch-containing leucoplasts are found in many types of epidermal tissue. The epidermal cells of some species may even contain functional chloroplasts (62). In most instances, however, the chloroplasts of guard cells are quite distinct from particles found in surrounding epidermal cells. The difference between plastids in guard cells and other epidermal tissue is most striking in flower tissue of certain *Caltha* species. Epidermal cells of the flowers in such species contain numerous bright orange, carotenoid-filled chromoplasts. The guard cells, however, completely lack the orange chromoplasts. Instead, they possess green chloroplasts (35).

The exceptional ability of guard cell plastids to synthesize starch has been an important cornerstone of the osmotic theory of water movement into and out of guard cells (16, 21). However, the rapidity of the closing process suggests that starch-sugar interconversions cannot in themselves account for the movement of water in and out of guard cells. Recently Stocking and Ongun (53) found that many types of mesophyll chloroplasts contain large quantities of inorganic ions. In some species the amount of potassium, magnesium, and calcium in chloroplasts exceeded that in the vacuoles. If guard cell plastids also had the ability to concentrate ions, particularly in a bound, osmotically-inactive form, they would possess an additional mechanism by which to regulate the osmotic flow of water in guard cells. Transfer of salts between chloroplast and vacuole might have a substantial influence on the osmotic properties of the guard cells.

Chloroplasts are not the only organelles found in stomata. Mitochondria have been observed in electron photomicrographs of guard cells (4). Numerous small particles can also be observed in guard cells with a light microscope. With the aid of time-lapse photography, Dr. J. E. Pallas, Jr., has demonstrated their extreme rapidity of movement.

#### ORIGIN AND DIFFERENTIATION OF GUARD CELLS

The differentiation of stomata from epidermal cells follows a unique but simple pattern. The ability to control this pattern would provide some regulation of the water relations in plants. Where the lack of stomata limits the diffusion of CO<sub>2</sub> into the leaf and thus affects yields, a stimulation of differentiation would be beneficial.

Epidermal tissue from which stomata arise is one of the first to become differentiated in the plant. Consequently most stomata are formed in young leaf buds. However, differentiation may occur relatively late in

the development of the leaf as well. Epidermal tissues can be found in which relatively undifferentiated stomatal mother cells occur close to mature, functioning stomata. The division patterns of epidermal cells which result in the formation of stomata have been studied in many types of plants (5, 10, 11, 15, 29, 52). A similar sequence of events occurs regardless of the timing of the final appearance of the stomata.

The stomatal mother cells are developed directly from an embryonic epidermal cell by an asymmetrical, differential division. The two sister cells formed from the parent epidermal cell differ in size and density of cytoplasm. The larger, more vacuolate cell develops into a typical epidermal cell of the leaf. The smaller cell containing a more dense cytoplasm is the stomatal mother cell. After some growth, it undergoes a single symmetrical division producing two identical daughter cells which develop into the guard cells of the stoma. The plane of this symmetrical division appears to be determined by the underlying vascular tissue (49). Rarely one or both of the daughter cells undergoes further divisions and produces abnormal clusters of guard cells occasionally described in the literature (60).

The differential division which produces the stomatal mother cell is similar to the asymmetrical division leading to the formation of root hair initials from root epidermal cells. Avers (2) has examined such divisions under the electron microscope. The differential division which forms the stomatal mother cell produces a metabolic as well as a morphological differentiation. Biochemical characteristics of mature guard cells are apparently fixed in the stomatal mother cell at the time of its formation. For instance, this cell is capable of forming starch grains (25), and, if it is prevented from dividing, its proplastids will develop into green chloroplasts (32). The stomatal mother cell also appears to have the ability to induce surrounding epidermal cells to divide and form companion cells (5, 29, 52). Bünning (5) has postulated once a stomatal mother cell has been differentiated, it also establishes a sphere of influence around itself which prevents neighboring epidermal cells from producing stomatal mother cells. Epidermal cells surrounding the stomatal initial also mature rapidly (25). Such a hypothetical sphere of influence could explain the regular patterns of stomatal distribution found in many leaves.

The formation of the stomatal pore begins shortly after the guard cells have been formed by the symmetrical division of the stomatal mother cell. The first microscopic evidence of wall separation between the two daughter guard cells is a thickening of the middle lamella in the central region of the adjacent walls (37). Separation first occurs at this point and spreads toward the ends of the cells. Scott and Baker (37) attribute the thickening to the deposition of cutin. However, others

report that pectin synthesis is involved (10, 55). Further histochemical studies will be necessary to elucidate the exact nature of this important event. Subsequent growth of the guard cells leads to the thickening of the cellulose walls and to the development of functional plastids.

*Factors affecting stomatal differentiation:* Environmental conditions existing at the time of stomatal differentiation in the embryonic leaf bud greatly affect this process. Availability of water, light intensity, and temperature have all been shown to be important factors. Calculations based on the data of Penfound (28) indicate that leaves of sunflowers grown on a wet soil possess 20 times the number of stomata as corresponding leaves from plants grown under very dry conditions. Sun leaves of various species also had many fewer stomata than shade leaves. Since only the ratio of "stomata/mm<sup>2</sup> of leaf area" was used to measure the environmental influences, it is not possible to determine whether they affect specifically the asymmetrical divisions responsible for the formation of stomata or whether cell division in general is influenced. Some investigators (50) have employed the ratio of "stomata/epidermal cell" as a more specific measure of stomatal differentiation. This ratio eliminates differences in cell size produced by the imposed conditions of growth. Schuermann (36) was able with use of this ratio to establish that the effects of light intensity and water availability on sunflower stomata actually represent effects on stomatal differentiation. Adequate water supply during early leaf development increases the number of stomata differentiated from epidermal cells, while a shortage of water inhibits the asymmetrical stomatal divisions. High light intensity and increased duration of illumination favored the differentiation of stomata from epidermal cells. When light and water supply were kept constant during the growth of tomatoes, no effect of the level of leaf insertion on stomatal differentiation was found. In contrast to these environmental effects, changes in temperature appear to affect the development rather than differentiation of stomata. High growth temperature produces many abnormalities in stomatal morphology (1).

Nutritional conditions have also been shown to influence stomatal differentiation. Glucose is required for stomatal formation in cotyledons produced from pine embryos in tissue culture (30). Undoubtedly, the effects of environmental conditions on the general nutritional status of the plant are involved in their control of stomatal differentiation. Environmental factors may also regulate the degree of influence which stomatal mother cells exert on surrounding epidermal tissues. Factors which increase the sphere of influence would reduce the number of mother cells differentiated. Those which decreased the extent of influence would promote the differentiation of stomata from neighboring epi-



dermal cells. The effects of hormones on stomatal differentiation suggest that stomatal mother cells may produce these substances. Indoleacetic acid applied to seedlings of *Vicia faba* decreases the ratio of stomata to epidermal cells in the newly formed leaves (22). This would appear to be a direct effect on the differential stomatal division, for auxin, kinetin, or combinations of purines and pyrimidines have been reported to stimulate the subsequent division of stomatal mother cells once they have been differentiated from epidermal tissue (18). The opposite effect, an inhibition of the formation of guard cells from the stomatal mother cells in cotyledons, can be achieved by treating the seeds with colchicine (32).

The above studies of both environmental and chemical factors indicate that specific effects on stomatal differentiation can be obtained. This suggests the possibility of controlling stomatal distribution over the epidermal surface of the plant. Attempts to exert such control may have very practical results as well as to lead to fundamental information on the differentiation process itself. The ability to alter the polarity of the leaf, particularly in those species where stomata are formed from epidermal cells on only one surface, would have obvious importance in the regulation of water relations and gas exchange of the plant.

A number of specific types of cells are produced by differential, asymmetrical divisions. The mechanisms by which these divisions create new cell types raise some fundamental biological problems. The differential divisions producing stomatal mother cells are of particular biochemical interest. They involve the formation from a non-photosynthetic tissue of cells in which chloroplasts develop. The possibilities which modern histochemistry offers for the investigation of such problems make stomata intriguing structures. Even though they have been objects of intensive morphological study for at least a century, there is still much to learn.

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### Discussion

WITTWER: You mentioned the effect of light intensity on stomatal numbers. Do you have any information on the effects of different wave lengths of light? For example, does the action spectrum follow that of photosynthesis? If so, does the concentration of carbon dioxide in the atmosphere influence the number of stomata formed? If a red-far red light reaction is involved in stomatal differentiation, gibberellin, which in some instances will substitute for red light, might affect stomatal differentiation.

ZUCKER: Unfortunately, I do not know of any good data on the action spectrum of stomatal formation, nor can I give you information on the effect of carbon dioxide on stomatal numbers. I have mentioned that both indoleacetic acid and kinetin appear to influence one or the other of the two divisions in stomatal formation. Gibberellin might also be expected to have effects, but I cannot cite any specific experiments. Whatever the nature of the light effect, apparently there is no absolute light requirement, for stomata are differentiated in complete darkness.

KUIPER: I have made some comparisons of stomatal numbers in flowers and leaves which might bear on this point. In poinsettia there is a very marked decrease in the number of stomata per unit number of epidermal cells in the red bract-like leaves compared with the normal green leaves of the plant. This suggests that the same light-induced physiological processes which lead to flowering also influence the stomatal index.

ZELITCH: Dr. David A. Walker of Queen Mary College, who was with us last year, grew mungbean seedlings both in the dry atmosphere of the greenhouse in the winter time and in a very humid atmosphere under a plastic cover. Although the two environments did not affect the number of stomata formed, those in seedlings grown in high humidity were functional, while seedlings grown in the dry atmosphere



seemed to have incompletely formed stomata. Here is an effect of environment on numbers of apparent functional stomata.

MEUSEL: I have been growing grass (*Poa annua*) under different water levels and, with the aid of Mr. E. Stoddard of the Experiment Station, I have counted the number of stomata in relation to the epidermal cells. There is a great difference in the ratio of the number of stomata to epidermal cells in grass under very wet conditions, watered six times a week, compared to grass watered only once a week. In the heavily watered grass, there is approximately one stoma for every two epidermal cells, and in the grass watered once a week the ratio is one stoma to four or five epidermal cells. When we measured the number of stomata per mm<sup>2</sup> of leaf area, we found no differences. However, the heavily watered grass was first to wilt. I should like to know why this grass wilted more readily. Grass-wilting, you understand, is one of the scourges of the golf course.

KRAMER: I wonder whether the number of stomata is as important as other changes in structures of the epidermis, such as the amount of cutin produced. I would guess that the grass grown with 6 waterings per week would wilt even if all the stomata were tightly closed because the cuticular layer should be very thin and permeable.

I should like to come back to a point that was raised earlier in this talk, that is, the problem of water relations and where does the water come from when the guard cells change in volume. If we assume that there is a pretty good contact between the mesophyll cells and the epidermal cells, then to some extent the underlying mesophyll might serve as a source of water. Would this be likely?

ZUCKER: I think it is a good possibility. Apparently substrates for starch synthesis in guard cells can be obtained by them from the mesophyll. Why not water as well? Perhaps this is why no consistent cytological evidence for the role of surrounding epidermal cells in water transport is available.

PALLAS: I think you are correct in stating that the vacuole does increase in size as the guard cell opens. However, evidence from studies of what Stålfelt terms hydroactive and hydropassive phases of guard cell physiology suggests that the water moving into the guard cells does come from neighboring epidermal cells. In most sections of guard cells that I have observed there is no direct contact between the guard cells and the mesophyll. The path of water from the mesophyll through the epidermis to the guard cells would be similar to that proposed by Wylie (Wylie, R. B. 1943. The role of epidermis in foliar organization

and its relation to the minor venation. Am. J. Bot. 30: 273) in his work on vein-extensions.

KUIPER: In your lecture, and now in this discussion, a movement of water into and out of guard cells is proposed. Yet you have talked about the chemical isolation of guard cells. Would not this isolation also include water?

ZUCKER: No, not necessarily. Cellular membranes can respond quite differently to different species of chemical molecules. The blood-brain barrier in animals is a striking illustration of this. Certain dyes injected into the blood stream penetrate rapidly into most of the tissues of the body, but they are completely excluded from the brain cells. Yet, there is an extensive exchange of fluid between brain tissue and the blood stream. Many other instances of a very selective permeability could be cited.

KUIPER: I should like to offer another possibility to account for changes in turgor of guard cells upon opening and closing. Would it be possible perhaps that water is not transferred to and from the neighboring epidermal cells to the guard cells but from, let us say, the vacuole to the cytoplasm? There are some observations that on closing small vacuoles appear in the cytoplasm. There could be an exchange between water bound to proteins and free water in the vacuole.

ZUCKER: Perhaps there could be volume changes involved in the transfer of water from a free to a bound form within the guard cell. The hydration of colloids could lead to some swelling. However, I do not think such changes in volume could begin to account for the observed increases and decreases in size of guard cells as stomata open and close. There must also be some net movement of water into and out of guard cells under these conditions.

## The Control and Mechanisms of Stomatal Movement

By Israel Zelitch

Interest in this laboratory in the action of stomata was derived from biochemical studies on the role of glycolic acid metabolism in leaves. In the course of these investigations it was shown that  $\alpha$ -hydroxysulfonates, compounds with the structure R-CHOH-SO<sub>3</sub>Na, are highly effective competitive inhibitors of the oxidation of glycolate, CHOH-COONa, by purified glycolate oxidase (23). These early experiments *in vitro* were later extended to work with intact leaves. When excised tobacco leaves were placed with their bases in solutions of  $\alpha$ -hydroxysulfonates in sunlight, rapid and extensive accumulations of glycolic acid were produced (24). The concentration of glycolic acid increased as much as twenty-fold in 30 minutes (25). This *in vivo* demonstration of the inhibition of the metabolism of glycolic acid was observed only in light, when synthesis of the acid takes place, and when the leaf was returned to darkness the glycolic acid level quickly fell to its normal steady state concentration.

Accordingly, it was concluded that the metabolism of glycolic acid could account for a very significant portion of the carbon metabolism of leaves in sunlight. In experiments carried out with leaves placed in sunlight with their bases in  $\alpha$ -hydroxysulfonate in an atmosphere containing C<sup>14</sup>O<sub>2</sub>, leaves in the inhibitor were found to have 50 per cent of their fixed carbon in glycolic acid while control leaves kept in water had only about 5 per cent of the radioactivity in glycolic acid. Thus when the metabolism of glycolic acid was inhibited in the living leaf, a large part of the carbon that was normally channeled into carbohydrate accumulated instead as glycolic acid (25).

In experiments with excised leaves in strong sunlight, it was observed that the control leaves with their bases in water often wilted, while those in  $\alpha$ -hydroxysulfonate solution always remained fully turgid. It soon became apparent, by carrying out simple experiments with beakers of water and a balance, that the amount of liquid taken up by the leaves placed in water or in  $\alpha$ -hydroxysulfonate solution was the same. The water taken up from the control leaves was retained better by leaves in inhibitor, while large quantities were lost from the control leaves into the atmosphere (26). These first observations therefore suggested that in addition to their effects on the carbon metabolism of leaves in sunlight,  $\alpha$ -hydroxysulfonates also closed stomata under environmental conditions where they would normally be open.

While on sabbatic leave in the Botany Department at the University of Oxford, a possible method of rapidly and accurately measuring stomata was suggested to me by the then unpublished observations of Sampson (14). She had observed that excellent replicas could be obtained by the technique of making impressions of the leaf surface (negatives) in silicone rubber, followed by the preparation of replicas (positives) from the rubber casts with films of cellulose acetate. We found that the stomatal apertures could then be accurately measured on the films under the microscope (26). This method enables one to measure the stomata in large numbers of samples easily and without significant alteration of the leaf environment.

It seems clear that opening and closing of stomata involves the uptake and loss of water from guard cells (6). It is not too relevant for this discussion whether the water enters from outside the guard cells or is released from sources within these cells. Much confusion arises in the literature of water relations in plants because many workers have not considered the state of hydration of the plants being used (18). In order to overcome this objection, all of our work has been carried out under conditions where water could not be limiting. The experiments to be described were usually done with leaf disks which were floated on water or the solution to be tested. Thus when we refer to the mechanism of stomatal opening and closing, we can be certain that the responses occurred when the water available for all of the leaf cells, including the guard cells, was adequate. The stomatal closing that occurs in many species during the wilting of leaves may involve entirely different processes from the ones to be suggested here.

Leaf disks have also been used in our experiments in order to obtain samples of relatively uniform tissue. The results are usually expressed as the mean stomatal width, determined by measurement of 100 stomata on each pair of leaf disks which represented a sample. Sometimes the results are expressed as the "per cent of stomata open." An "open" stoma is one 2 $\mu$  or more, wide; "closed" stomata are arbitrarily those with apertures less than 2 $\mu$ .

### EFFECTS OF LIGHT

A standard disk assay for the measurement of stomatal opening was developed. Tobacco leaf disks provide especially useful tissue because this species has large stomata that open and close relatively uniformly in response to the usual environmental influences. The excised leaf is first kept in the dark for about one hour. Disks 1.6 cm in diameter are taken with a sharp punch and floated on water or the solution to be tested in light from tungsten bulbs that supply about 2,000 ft-c at



constant temperature. The amount of light necessary to obtain maximal stomatal opening is considerably less than the intensity necessary to achieve maximal rates of photosynthesis with this tissue. The stomata in such disks open to their maximal aperture when floated on water in about 90 minutes at 25° to 30° at a light intensity in excess of 250 ft-c (26).

Recently, Dr. Kuiper and I had the opportunity to carry out some preliminary experiments to determine the relationship between stomatal opening and the spectrum with Dr. Sterling B. Hendricks in his laboratory at the Plant Science Station, U.S.D.A., Beltsville, Maryland. At low light intensities it was possible to obtain rather narrow bands of light with his prism monochrometer. At the end of 60 minutes of exposure to various wavelengths of light, impressions were made in the usual manner on leaf disks. Stomata failed to open in the far-red region, they opened well in the red, were completely closed in green light, were open in the blue, and remained closed as the ultra-violet region was approached. There was a resemblance between the response of stomatal opening to various wavelengths of light and that for ATP synthesis by isolated chloroplasts (2). Somewhat similar conclusions about the relationship of stomatal opening to the spectrum have also recently been reported by Karve (9).

Dr. Kuiper has observed that the epidermis of the leaves of a succulent plant in the genus *Senecio* can be stripped off without any mesophyll tissue adhering. If such epidermal tissue is removed while the stomata are open, the stomata will remain open for about 30 minutes when the tissue is floated on water if light is provided. In the dark the stomata in the excised epidermis close rapidly. This tissue is thus able to carry out at least part of the normal stomatal activity, the maintenance of stomatal opening in continuous light. Dr. Kuiper found in preliminary experiments with Dr. Hendricks that the light spectrum for maintaining opening of stomata in *Senecio* epidermis was essentially identical to that for stomatal opening in tobacco leaf disks. It thus seems clear that this epidermal tissue, which is free from mesophyll, can carry out some of the reactions which are normally a part of stomatal movement. Presumably these partial reactions must take place in the guard cells themselves.

The stomata of leaf disks floating on water open in the light to their maximal aperture in about 90 minutes. When the disks are transferred to the dark, they close completely in 30 minutes, and when exposed to a second light period, they open at the initial rate (26). Such rates of opening and closing in light and in darkness are reminiscent of the rates and time scale for accumulation and disappearance of glycolic acid in the presence of  $\alpha$ -hydroxysulfonates (24). In the light, the sto-

matal opening reactions predominate, and in darkness, the closing processes are more significant.

In continuous light, stomata in leaf disks will remain fully open for as long as 8 hours, the longest period investigated. It thus seems clear that if leaves, or leaf disks, are maintained in a well hydrated condition, the so-called "midday closure" often observed in many species can be eliminated.

Other experiments that have a bearing on the mechanism of stomatal opening have been carried out with tobacco leaf disks that were floated on water while fitted with a shade so that half of the disk was in light and half in darkness (26). Stomata in the illuminated portion open normally, and stomata in the dark remain completely closed. The line dividing open and closed stomata on such disks is within one mm of the line which separates light from dark. This suggests that the light effect on opening must occur very near the guard cells that are being exposed.

Some observations have also been made with a variegated variety of tobacco (30). Disks were cut from these leaves so that half of a disk was green and the other half white. These were compared in a standard assay for stomatal opening with disks from the same leaf that were either completely green or all-white. In one experiment, at the end of 90 minutes, the mean stomatal aperture on the green half of the disk was 6.2  $\mu$ , and on the white half, 3.5  $\mu$ . The stomata in the all-green disk were opened about the same as in the green half, and the mean width of the stomata in the all-white disk was 3.9  $\mu$ . These results confirm the work of others that stomata in white portions of variegated leaves open to a lesser extent than stomata in the green portions. Although the white portions are almost devoid of chlorophyll in the mesophyll tissue, stomata still open significantly, again suggesting that the guard cells themselves are important in stomatal opening. As in the *Senecio* epidermis, this is another instance of stomatal opening in tissue lacking functional chloroplasts in the mesophyll tissue.

#### EFFECTS OF TEMPERATURE

During attempts to determine the effect of temperature on the rate of stomatal opening in leaf disks in this laboratory, Dr. D. A. Walker observed that stomata in disks maintained at 10° for long periods of time in continuous light failed to open widely (20). After 3.5 hours at 10°, the mean stomatal aperture was about 3  $\mu$  while at 30° the mean width was about 8  $\mu$ . Thus the steady state of stomatal opening at 30° is more than twice the width at 10°. Somewhat similar observations have been recently observed in leaves by Stålfelt (19). Since at lower tempera-

TABLE I

Effect of temperature on the rate of stomatal closing in leaf disks in the dark

	10°	30°
	Mean stomatal width, $\mu$	
10 minutes in dark	6.0°	5.9°
20 minutes in dark	4.7	3.2
30 minutes in dark	2.4	2.8

\*After 90 minutes in light at 30° the mean stomatal width was 7.5  $\mu$ . Leaf disks were floated in water in the light under conditions of the standard assay at 30°. After 90 minutes, samples of disks were transferred to darkness at the temperature shown and stomatal apertures were determined from impressions at the times indicated.

tures stomata do not open very well, this makes the determination of the effect of temperature on the rate of opening difficult to measure. When disks that were maintained for long periods at 10° were transferred to 30°, and disks kept initially at 30° were changed to 10°, the stomatal apertures quickly increased or decreased to the steady state dictated by the final temperature (20). The effect of temperature during continuous illumination, under conditions where water is not limiting, is therefore to shift the balance between the opening and closing processes in a freely reversible manner.

The effect of temperature on the extent of opening is primarily on the opening processes. Increased temperature stimulates the rate and extent of the opening reactions, as has been shown, but there is little difference between 10° and 30° on the rate of stomatal closing in the dark (Table I). Closing is presumably largely temperature independent, hence the great influence of temperature previously shown must have been brought about by temperature effects on metabolic reactions concerned primarily with the opening processes.

There is also an influence of the night temperature under which plants are grown on the extent of the stomatal opening at a constant temperature in the light. This was discovered when stomata in tobacco leaf disks in the standard assay for stomatal opening were sometimes found to open from 10 to 15  $\mu$  instead of the usual 6 to 8  $\mu$ . The excessive opening followed periods of unusually warm nights in the greenhouse environment where the tobacco plants were grown in sub-irrigated benches. The enhanced opening effect is also characterized by incomplete closing of the stomata in the same leaves in the dark. The marked influence of the night temperature under which the plants were grown on the subsequent opening of the stomata in the standard assay is shown in Table II. In these experiments, the day temperatures in the greenhouse were essentially identical, but the night temperatures were varied as indicated. Stomata from leaves grown on the warmer

TABLE II

Effect of night temperature of greenhouse on stomatal opening in tobacco leaf disks at 30°

	Mean stomatal width in disk assay, $\mu$	
	16°	27°
Plants kept 5 nights at		
Plant		
1	5.9	10.7
2	5.9	10.9
3	6.4	8.6
4	5.3	5.9
Plants kept 7 nights at	16°	27°
Plant		
1	5.9	9.0
2	5.5	10.0
3	5.9	6.9
4	5.8	7.3

Eight tobacco plants in each experiment were paired according to size and grown in subirrigated greenhouse benches at the night temperature shown and with essentially identical day temperatures. Disks were taken from the same leaf position in each plant and stomatal opening was determined under conditions of the standard disk assay.

night temperatures opened 50 per cent greater at a fixed temperature in the light. These results may have important ecological and agronomic significance. It seems likely that the inhibitions in photosynthesis that have often been noted after cold nights (12) may be caused by the reduced stomatal apertures that follow such low night temperatures. It is not yet certain how long this "memory" of a cold night is retained by the stomatal opening process, and we hope to investigate this problem further.

#### CHEMICAL INHIBITORS OF STOMATAL OPENING

The standard leaf disk assay has provided a useful method of testing the ability of various compounds to inhibit stomatal opening. With this assay a large number of metabolic inhibitors have been found to prevent stomatal opening. Some representative compounds that close stomata and their relative effectiveness in the disk assay are summarized in Table III. The most effective inhibitor to date is phenylmercuric acetate, with which some model studies on the relation between the effect of stomatal aperture and photosynthesis and transpiration in turgid leaves have already been carried out (15, 16, 28, 29). I assume that phenylmercuric acetate reacts with sulfhydryl groups in or near membranes of the guard cells and thereby alters the membrane permeability and prevents the guard cells from becoming turgid. Phenyl-



TABLE III

Summary of effectiveness of various compounds in standard disk assay

Compound	Concentration to produce 50% stomatal closure, M $\times 10^{-5}$
Phenylmercuric acetate	5
"Atrazine"	10
Carbonyl cyanide <i>m</i> -chlorophenylhydrazone	10
Na Azide (pH 4.5)	20
Na $\alpha$ -Hydroxydecanesulfonate	80
Na 1-Naphthaleneacetate	100
$\beta$ -Naphthoxyacetic acid	100
8-Hydroxyquinoline sulfate	100
Chlorogenic acid	100
Di- <i>n</i> -butyloxalate	100
K Iodoacetate	100
Na 2,4-Dichlorophenoxyacetate	200
$\alpha$ -Hydroxy-2-pyridinemethanesulfonic acid	300

mercuric chloride is less soluble in water, but is as effective as phenylmercuric acetate on a molar basis. Mercuric chloride and acetate, however, have little effect in the disk assay. Some mercaptides of phenylmercuric acetate have also been prepared, and on a molar basis these derivatives produce the same stomatal closure as the parent compound. Phenylmercuric acetate is toxic to some plants, especially at higher temperature, and when this substance is sprayed on leaves its concentration must be carefully controlled so that stomata are closed without producing toxic effects in these sensitive species. "Atrazine" (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine), an inhibitor of the Hill reaction of photosynthesis and a herbicide, was first reported to close stomata by Smith and Buchholtz (17). The ability of this compound to control stomatal movement at low concentrations has been confirmed by the disk assay.

Some of the members of the group of phenylhydrazones of carbonylcyanide that have been shown to be potent uncouplers of oxidative and photosynthetic phosphorylation (7) also inhibit stomatal opening at concentrations of about  $1 \times 10^{-4}$  M. Sodium azide is also very effective, and because of the interesting results obtained with this inhibitor it will be discussed in some detail later. Azide inhibits both stomatal opening and closing at appropriate concentrations.

Two of the compounds listed in Table III are representatives of the  $\alpha$ -hydroxysulfonates, compounds that inhibit glycolic acid metabolism. These compounds are not very effective for long periods when sprayed on leaves because they are apparently metabolized too rapidly by the tissues. Several growth regulators are also listed, and 8-hydroxyquino-

line, an effective chelator and fungicide, also closes stomata in the disk assay at about  $1 \times 10^{-3}$  M.

Several naturally occurring substances were also shown to inhibit stomatal opening. Chlorogenic acid, a phenolic substance, and esters of oxalic acid inhibit stomatal movement. Through the cooperation of Dr. Zucker and Dr. Hanson we have also obtained and tested some derivatives and isomers of chlorogenic acid (4). Some of these compounds appear to be more effective than chlorogenic acid itself. These observations may have a bearing on the mechanism of stomatal control in the normal leaf.

The compounds shown in Table III close stomata at relatively low concentrations, but similar results can be obtained by using an osmotically active substance at a high concentration. Sucrose, for example, at a concentration of 0.7 M will completely prevent stomatal opening in the disk assay. This is a concentration more than one thousand times higher than that of the effective inhibitors. The wide range of compounds that influence stomatal movement suggests that there are many biochemical sites at which the opening of stomata can be interfered with to cause closing.

The results of the disk assay have been found to correlate well with the ability of compounds to close stomata when sprayed on leaves. Phenylmercuric acetate, 8-hydroxyquinoline sulfate, and sodium  $\alpha$ -hydroxydecanesulfonate have caused similar stomatal closing when compared at the same concentration in the disk assay and when sprayed on leaves (28). Since the guard cells are located on or near the outermost surfaces of leaves, I assume that spraying will be the most efficient way of obtaining specific effects of stomatal control. If an effective substance is introduced through the roots, there would appear to be a greater possibility of introducing deleterious side effects on growth through actions of the compound with cells other than the guard cells.

When phenylmercuric acetate is sprayed on leaves, only the stomata on the surface sprayed are affected. Hence this substance is not translocated very readily even within a single leaf. The inability to be translocated would seem to be an advantage for any potentially useful compound, because this property would also make it more likely that only the biochemical reactions of the guard cells will be influenced.

With phenylmercuric acetate as a spray, we have carried out experiments on the effect of stomatal aperture on the rates of photosynthesis and of transpiration in excised leaves (28). It is clear from these studies and from those carried out at this Station with intact plants by Shimshi (15, 16) on several species, that closing the stomata reduces the transpiration significantly and inhibits photosynthesis to a lesser extent. Such results were predicted from the physical laws of diffusion, and by use of

reciprocal plots, linear regressions were obtained between transpiration or photosynthesis and the path length of stomatal diffusion as expected (28). When phenylmercuric acetate was sprayed on intact tobacco leaves in the greenhouse, transpiration was reduced more than 40 per cent over a period of 16 days, while the effect on growth varied with the environmental conditions (29). Under a drought environment the greater hydration of the leaf induced by chemical closure of stomata may increase the length of time during which photosynthesis can occur and more than compensate for the lower rate of photosynthesis. Thus stomatal closure may even result in enhanced plant growth as well as an increase in the survival time under drought environments.

The search for more effective and specific compounds with which to inhibit stomatal opening continues. At the same time, we are greatly concerned with the mechanism of stomatal opening and closing, and some of our attempts to clarify this problem will now be considered.

#### NATURE OF STOMATAL OPENING AND CLOSING PROCESSES

The classical description of stomatal opening given in most botany textbooks suggests that in the light carbon dioxide concentrations are reduced, and this results in a rise in the pH in the guard cells; the higher pH allegedly stimulates the conversion of starch to sugar so that an increase in the osmotic value of the guard cells occurs; water is accordingly taken up and the stomata open. In the dark, this process is reversed to bring about stomatal closure (11). This explanation may have pedagogic usefulness. Correct or not, however, it is certain that for stomata to open there must be an uptake of water and an increase in turgidity of the guard cells, and that stomatal closing involves a corresponding loss of water. Thus the explanation of the mechanism of stomatal action must ultimately account for the biochemical reactions that induce water movement across membranes.

Studies with the respiratory inhibitor azide have helped to separate the processes of water uptake and loss in the guard cells. In these experiments, variations of the standard assay have sometimes been used. One may start with stomata that are closed and permit them to open under optimal conditions of water, light, and temperature in the usual manner. The effect of substances on the closing of already opened stomata can also be evaluated. To study this, one allows the stomata in leaf disks in water to open in the light, and then transfers the disks to solutions of the compound to be tested in continuous light. Another variation of the standard assay has also sometimes been used. The stomata are opened in light as usual and the disks are then transferred to the solution to be tested and placed in the dark. In this way one can de-

termine whether the inhibitor will prevent the stomatal closing process in the dark.

With use of all of these techniques, the following conclusions can be supported by a variety of experiments: The opening and closing processes occur simultaneously in the light. In the light, the reactions which produce opening predominate, and in the dark, closing processes are more important. Most environmental and chemical inhibitors of stomatal movement exercise their effects on the opening processes. Opening and closing of stomata must occur by a different sequence of biochemical events, and not by a simple reversal of the same series of reactions. The synthesis and metabolism of glycolic acid is involved in the opening of stomata. Some speculations on still other possible factors that affect stomatal movement will also be made.

#### EXPERIMENTS WITH AZIDE ON OPENING AND CLOSING

The well known inhibitor of respiration and oxidative phosphorylation, sodium azide, at pH 4.5 inhibits the opening of stomata in the light and closing in darkness as shown in Table IV (20). At  $1 \times 10^{-4}$  M, azide completely prevents stomatal opening in the standard disk assay. At this same concentration, however, azide inhibits the dark closing of stomata only partially. When azide at  $5 \times 10^{-4}$  M is used, both the opening and the closing processes are blocked, and the stomata retain the aperture they had before treatment with azide. Although the re-

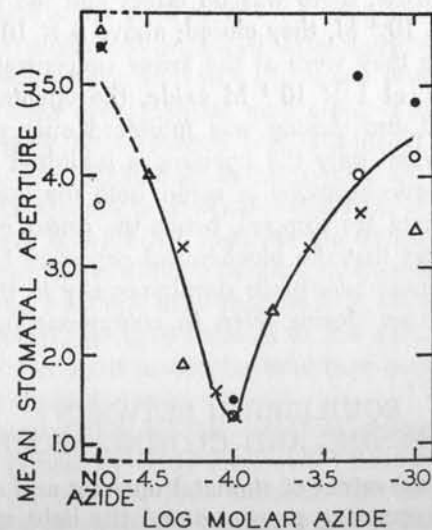


Fig. 1. The effect of azide concentration on stomatal aperture in continuous light.



TABLE IV

Effect of azide concentration on stomatal opening and closing in leaf disks

Azide, M	Opening in light, $\mu$	Closing in dark, $\mu$
0	4.9	1.2
$1 \times 10^{-5}$	5.4	1.3
$1 \times 10^{-4}$	1.5	2.2
$5 \times 10^{-4}$	1.4	6.4

To study opening in the light, disks were floated in the light on sodium azide in 0.01 M sodium tartrate buffer at pH 4.5, or on buffer alone as indicated for 90 minutes.

In the experiment on closing in the dark, disks were first floated on buffer for 120 minutes in the light. The stomata had a mean aperture of  $4.8 \mu$ . Sodium azide was added to make the final concentration indicated, and the disks were placed in the dark for 30 minutes before the stomatal apertures were determined.

Responses of opening and closing to azide concentration are similar, they are different, and this difference is best seen at the critical concentrations around  $1 \times 10^{-4}$  M.

Since azide affects both opening and closing of stomata, but with different concentrations required for maximal effect on each process, it should be possible to separate these processes. This was accomplished by allowing stomata to open in leaf disks and then transferring the disks to different concentrations of azide in continuous light. Such experiments are illustrated in Fig. 1 (20). Control stomata from disks in water were open about  $5 \mu$  in 90 to 120 minutes. Subsequently, at low concentrations of azide, there was no effect and the stomata remained open; at about  $1 \times 10^{-4}$  M, they closed; above  $5 \times 10^{-4}$  M, the stomata were again open as they were at the lower concentrations of azide. As shown in Table IV, at  $1 \times 10^{-4}$  M azide, the opening processes were completely blocked and closing was inhibited only partially. Thus in continuous light, when only the opening is inhibited by azide, closing occurs. At higher concentrations of azide, both the opening and closing movements of stomata are stopped, hence the *status quo* is maintained. These results suggest that the biochemical processes by which opening and closing occur must take place simultaneously in the light, and that azide can bring about closing even in continuous light by inhibiting opening.

#### EQUILIBRIUM BETWEEN THE OPENING AND CLOSING PROCESSES

If, as suggested, the extent of stomatal opening and closing represents a balance between opposing processes and the light merely accelerates the opening reactions, one should obtain the same endpoint of stomatal

TABLE V

Effectiveness of different compounds on final opening and closing of stomata in leaf disks

Compound	Concentration, M	Mean stomatal aperture, $\mu$		
		Opening in light, (A)	Closing in light, (B)	Closing in dark, (C)
Water control		5.8	5.7	1.1
Na azide in tartrate, pH 4.4	$5 \times 10^{-4}$	1.9	3.6	3.6
Phenylmercuric acetate	$3.3 \times 10^{-5}$	1.4	1.2	1.2
Atrazine	$1 \times 10^{-4}$	2.5	1.7	1.1
Chlorogenic acid	$1 \times 10^{-3}$	3.6	3.9	1.3
Na fluoride	$1 \times 10^{-3}$	4.2	3.9	1.3

(A). Opening in light was carried out under conditions of the standard disk assay. (B). Closing in light was studied by first permitting the stomata to open in light for 90 minutes; the disks were blotted and transferred to the solutions indicated for an additional 90 minute period in the light. (C). Closing in the dark was investigated by opening stomata in the light, and then transferring leaf disks to the solutions shown for a 30 minute dark period.

aperture in the light with inhibitors regardless of whether one starts with open or with closed stomata. More effective inhibitors of opening should merely shift the equilibrium more towards closing, and less effective substances should swing the steady state more towards opening irrespective of the direction in which the stomata are moving. Experiments of this kind have been carried out with a number of different inhibitors (19), and some examples are shown in Table V.

If columns A and B are compared, it is clear that, except for azide, all of the various inhibitors produced essentially the same final stomatal aperture whether one started with closed stomata and permitted them to open or with open stomata and induced closing in the light. Azide, as has already been pointed out, is unusual because at a concentration of  $5 \times 10^{-4}$  M, it inhibits both opening and closing. These results indicated that there is a balance between opening and closing, and these inhibitors operate by blocking the opening reactions. Azide also inhibits closing in the dark (column C), whereas none of the other inhibitors, including very effective ones such as phenylmercuric acetate and Atrazine, prevent closing of stomata in the dark. These results lead to the conclusion that most metabolic inhibitors presently known function solely on the opening processes.

The effect of temperature on the extent of stomatal opening in continuous light has previously been discussed. These results are also consistent with the view that the final stomatal aperture reflects the result of a shifting in the equilibrium. Temperature, in this instance, acts to change the steady state by affecting primarily the opening reactions.

### ANAEROBIC CONDITIONS IN RELATION TO STOMATAL OPENING AND CLOSING

With use of leaf disks, it was possible to conveniently test the effect of different gases in the atmosphere on stomatal movement. For these experiments the disks were floated on a thin layer of water in Erlenmeyer flasks capped with rubber serum stoppers. The moist gas was passed continuously through the flasks at rates of about 6 volume changes per minute by means of hypodermic needles inserted through the stoppers.

A requirement for oxygen for optimal stomatal opening in the light was shown by this method (20). Starting with stomata that had a mean aperture of  $1.0 \mu$ , disks were floated in the light in air, nitrogen containing 0.03 per cent  $\text{CO}_2$ , and in helium. At the end of 90 minutes the mean apertures were respectively  $4.5 \mu$ ,  $1.7 \mu$ , and  $1.5 \mu$ . Although there is an absolute requirement for oxygen for initiation of stomatal opening, once the stomata are open they will remain open in the light even under anaerobic conditions. Presumably there is a difference between the processes of initiation of opening and the maintenance of opening.

There was no requirement for oxygen for dark closing of stomata. Stomata were permitted to open in air in the usual way, and the disks were then transferred to atmospheres of air, nitrogen containing 0.03 per cent  $\text{CO}_2$ , or helium in the dark. The stomata closed completely within 30 minutes, and there was no effect of anaerobic conditions on the rate of closing (20.) Hence by changing the atmosphere surrounding leaf disks, one can also show that the processes of opening and closing are different and do not represent reversals of the same biochemical reactions.

### METABOLISM OF GLYCOLIC ACID AND STOMATAL OPENING

I. *Oxygen requirement for synthesis of glycolic acid.* The studies in this laboratory on the biochemistry of stomatal action arose from a previous interest in the role of glycolic acid metabolism in leaves. It seemed appropriate therefore to attempt to correlate further what we have learned about stomatal responses in terms of the biochemistry of glycolic acid. Evidence was found that factors that influence the synthesis and metabolism of glycolic acid are closely related to stomatal opening.

Experiments have been carried out with leaf disks floating in water to determine stomatal apertures, and with comparable leaf disks under the same conditions on 0.01 M  $\alpha$ -hydroxy-2-pyridinemethanesulfonic acid to determine the extent of glycolic acid accumulation in the tissue (24, 25). In one experiment disks in light in air in the presence of inhibitor

accumulated  $5.2 \mu\text{moles}$  of glycolic acid per g of leaf in 120 minutes, while disks in an atmosphere of nitrogen accumulated only  $2.2 \mu\text{moles}$  of glycolic acid. As was previously shown, there is a requirement for oxygen for optimal rates of stomatal opening, and oxygen is also needed for glycolic acid synthesis in the light by the same tissues.

II. *Effect of high concentrations of  $\text{CO}_2$  on stomatal opening and on synthesis of glycolic acid.* Another correlation between the opening processes and glycolic acid synthesis can be demonstrated with  $\text{CO}_2$ . The effect of high partial pressures of  $\text{CO}_2$  on bringing about stomatal closure has been well documented in the literature especially as a result of the work of Heath (5). With leaf disks, I have found that an atmosphere of 1.8 per cent  $\text{CO}_2$  in air is sufficient to close stomata completely or to prevent stomata from opening in the light. In an experiment in which glycolic acid accumulation was also measured in comparable tissue floated on a solution of an  $\alpha$ -hydroxysulfonate,  $6.2 \mu\text{moles}$  of glycolic acid were formed per g of tissue in 90 minutes in air, and only  $0.3 \mu\text{mole}$  in the presence of 1.8 per cent  $\text{CO}_2$  in air. Further experiments established that increased partial pressures of  $\text{CO}_2$  inhibit glycolic acid synthesis and stomatal opening to approximately the same extent.

Thus low concentrations of  $\text{CO}_2$  in light are necessary in order to obtain large stomatal widths, and low concentrations of  $\text{CO}_2$  also stimulate the synthesis of glycolic acid (22). These experiments also provide evidence that  $\alpha$ -hydroxysulfonates block the further metabolism of glycolic acid, as was suggested, and not its formation.

TABLE VI  
Stomatal closure by 0.5%  $\text{CO}_2$  and its reversal

Disks floated on	Mean stomatal width, $\mu$	
	Air 120 minutes	Air 120 minutes, 0.5% $\text{CO}_2$ in air 60 minutes
Water	5.1	1.5 } 1.6
	4.6 } 4.9	
0.0025 M K glycolate		4.1 } 3.9
		3.6 } 2.5
0.0025 M glucose		2.6 } 2.5
		2.3 } 2.0
0.0025 M K phosphate		2.0 } 2.0
		2.0 } 2.0

Pairs of disks were floated on the solutions indicated. The figures represent the mean stomatal widths in each of the pairs treated under a given experimental condition.



III. *Reversal of CO<sub>2</sub>-induced closing by glycolic acid.* If the hypothesis that high concentrations of CO<sub>2</sub> inhibit stomatal opening, because of interference with the synthesis of glycolic acid is correct, it should be possible to reverse closing brought about by CO<sub>2</sub> by providing leaf disks with glycolic acid.

Some results of a typical experiment are presented in Table VI. The mean apertures obtained in each disk of a pair are given to illustrate the agreement generally obtained with these methods. The disks were kept for two hours in the light in air, and for the next hour the disks were kept in an atmosphere of 0.5 per cent CO<sub>2</sub> in air. The higher partial pressure of CO<sub>2</sub> caused a significant closing of the stomata when the disks were floated on water or dilute phosphate. When the disks were floated on glycolate, and to some extent on glucose solution, the closing was largely reversed. Thus the closing caused by CO<sub>2</sub> in the light can be overcome by providing the tissues with glycolate or carbohydrate. In such experiments this effect of glycolate can invariably be demonstrated. Occasionally, in the disks on salt solution, the closing effect of CO<sub>2</sub> is also partially reversed. Some cations appear to be able to stimulate opening in the presence of CO<sub>2</sub> under certain experimental conditions. Experiments of this kind require still further detailed investigation.

IV. *Effect of concentration of α-hydroxysulfonate on stomatal closure and glycolic acid accumulation.* If the inhibitors of glycolic acid oxidase prevent stomatal opening by interfering with the metabolism of the substrate, one would anticipate that similar concentrations of inhibitor would be needed for maximal closing and for glycolic acid accumulation. In an experiment designed to test this hypothesis, it was found that α-hydroxy-2-pyridinemethanesulfonic acid at a concentration of  $3 \times 10^{-3}$  M inhibited stomatal opening in leaf disks about 50 per cent and also caused the accumulation of glycolic to about one-half its maximum. Complete stomatal closure and maximal accumulation of glycolic acid in the tissues occurred at a concentration of  $1 \times 10^{-2}$  M inhibitor. Thus the effect of α-hydroxysulfonates on inhibition of stomatal opening and on inhibition of glycolic acid metabolism are brought about at similar concentrations of inhibitor.

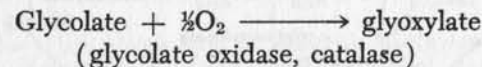
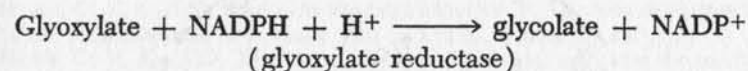
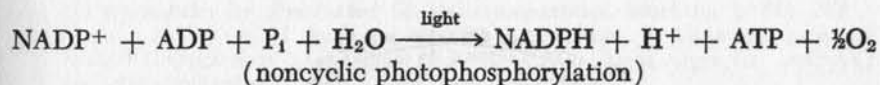
V. *Competitive inhibition of stomatal opening by α-hydroxysulfonate.* Some years ago it was shown with the purified enzyme from spinach leaves that α-hydroxysulfonates are competitive inhibitors of glycolic acid oxidase (23). Similar experiments have been carried out with leaf disks under conditions of the standard assay of stomatal opening. Leaf disks were floated on different concentrations of mixtures of α-hydroxy-decanesulfonate and glycolic acid, and stomatal apertures were deter-

mined after the system had reached a steady state. Graphical representation of the results obtained clearly demonstrated that the glycolic acid oxidase inhibitor was blocking stomatal opening in a manner that could be completely reversed by the added glycolic acid.

#### MECHANISM OF STOMATAL MOVEMENT

Certain generalizations can be made about the mechanism of stomatal movement as a result of the experiments described. Water is taken up by the guard cells to bring about opening in the light, and light serves to activate the "pump." It is possible that we are dealing with an osmotic pump in the guard cells, as in the classical theory, and that soluble carbohydrates increase in the guard cells in response to the light. The crucial role of glycolic acid could thus be to serve in the synthesis of carbohydrate. As has been shown in other plant tissues, carbohydrate can be produced from glycolic acid (8, 13, 21). Under certain conditions, significant amounts of carbon may be metabolized by such a pathway (25).

Another kind of "pump" that can be envisaged would be one that utilizes adenosine triphosphate (ATP) produced by photosynthetic phosphorylation in chloroplasts (1).



Recently, Butt (3) studied glucose uptake by algae under anaerobic conditions and showed that the metabolism of glycolic acid is involved in photophosphorylation reactions related to the uptake. The glyoxylate reductases in the tissues (27) may serve to oxidize the reduced pyridine nucleotides and thus enable the phosphorylation to proceed. The glycolic acid-glyoxylic acid shuttle would thus help to provide the energy-rich ATP needed to maintain the "pumping" of water into the guard cells.

Another factor on which only preliminary work has been done should be considered. This may be called the *natural inhibitor hypothesis*. As is shown in Table III, a number of diverse chemical substances will inhibit the opening of stomata. Two types of naturally

occurring compounds are known to be quite effective in the standard assay. These are derivatives of phenolic acids and oxalic acid. Oxalic acid is of special interest because it has already been shown to be derived enzymically from glyoxylic acid (10). If the synthesis of oxalic acid were favored in the dark in the guard cells, and the synthesis of carbohydrate predominated in the light, this mechanism would account for stomatal movement.

I have also attempted to isolate inhibitors of stomatal opening from epidermal tissue of leaves kept in the light and the dark. One can show that extracts of the epidermis from leaves in the dark contain material which inhibits stomatal opening, and the activity is considerably greater than extracts prepared from leaves kept in the light. The ultraviolet spectrum of such extracts appears to be typical of phenolic acids such as chlorogenic acid. The inhibition of opening with these extracts, however, was greater than would have resulted from the amount of chlorogenic acid they contained.

### SUMMARY

The effect of glycolic acid on stomatal movement is summarized in Fig. 2. The process of water uptake by the guard cells is stimulated by light, and by low concentrations of CO<sub>2</sub> and also requires oxygen. These

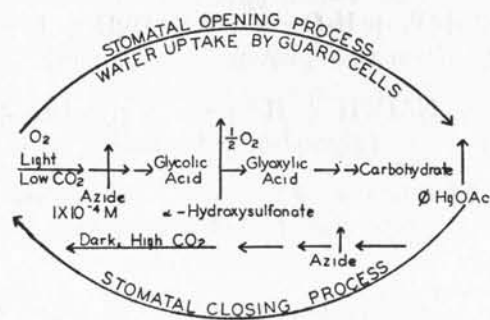


Fig. 2. Diagrammatic representation of biochemical reactions related to stomatal movement.

factors all stimulate glycolic acid synthesis. Low concentrations of azide probably inhibit opening prior to the synthesis of glycolic acid, because one can usually reverse these azide inhibitions by providing glycolic acid or carbohydrate to leaf disks. The second azide effect, at higher concentrations, is related to inhibition of closing. The  $\alpha$ -hydroxy-sulfonates inhibit the metabolism of glycolic acid, and they can be reversed by providing the substrate. Glycolic acid also overcomes the closing brought about by high concentrations of carbon dioxide. Phenyl-

mercuric acetate must function on or near the guard cell membrane which acts as the "check valve" of the "pump," because we have not been able to overcome the closure produced with this inhibitor by adding substrates. When mixtures of  $\alpha$ -hydroxysulfonate and phenylmercuric acetate are used in the standard assay, one obtains the same amount of stomatal closure as with phenylmercuric acetate alone. This also suggests that this inhibitor acts near the final step of water uptake.

Stomatal closing appears to represent the running down hill that occurs when the "pump" is slowed down or stopped. Only high concentrations of azide (and cyanide) are known to inhibit this process. The rate appears to be temperature independent.

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### Discussion

BLISS: I am concerned with the definition of assay as you have been using it. In assays of drugs or pesticides, as carried out a number of years ago, there was a tendency to report the results directly in units of the response, so that a lot of drug would be said to contain so many "mouse units" or "cat units" of the active agent. In many cases, however, this did not give a satisfactorily uniform result within the experimental error because of variation in the biological indicator. The problem was solved by turning the procedure into a comparative one in which a standard known preparation was tested concurrently under as nearly identical conditions as possible and the results were expressed in amounts of the test chemical which would give the same response as a unit quantity of the Standard. In the case of stomatal closing, you seem to be in this initial stage, since you are talking in terms of stoma-

tal response instead of in terms of the effectiveness of particular compounds. Would it not be a desirable next step to develop a series of standard materials? Stomatal movement would then be a biological indicator and relative potency expressed in equipotent amounts of chemical compounds.

In entomology, for example, insecticides are compared for a particular type of application, insect species, and set of experimental conditions at several dosage levels, measuring at each level the percent kill. The results are then comparable. On some of the slides shown here, the differences seemed to me far more variable than we would consider satisfactory in experiments on insecticides, fungicides, drugs or biologicals for the effective biological standardization of these materials.

ZELITCH: We have been able to use these assays routinely to determine whether compound A is better than compound B in closing stomata. The disk assay is carried out under standard and precisely defined conditions. The only thing that varies from day to day is our plant material. We find that the stomata of disks floated on water almost always have apertures between 6 and 8  $\mu$ . When the stomata are opening to this extent, I can very accurately predict how much closing will be produced by a given concentration of any compound we have ever tested. Yesterday, in the laboratory, for example, I made suggestions on the concentrations of phenylmercuric acetate our participants in this Seminar should use to get 50 per cent closure. I think those who completed the experiment obtained essentially the predicted result. What happens sometimes is that because of environmental factors under which the plants are grown, some of which have been discussed, the stomata open much wider under the standard conditions. If the stomata open to about 10  $\mu$ , that is 50 per cent greater than normal, you will need about twice the concentration of inhibitor to get 50 per cent closure.

If we wanted to be more precise we could always run a standard and compare a new compound with it as Dr. Bliss suggests. But in spite of these apparently crude methods, we have already been able to change our criteria for calling a compound effective. Originally, we were very happy to find compounds which closed stomata at concentrations of 10<sup>-2</sup> M, and now we usually begin to assay new substances at a concentration of 10<sup>-8</sup> M. We have now discovered compounds which give significant effects at concentrations around 10<sup>-5</sup> M. The assay is obviously useful in its present form even if the results are not precise in your terms.

HORSFALL: As Dr. Bliss knows I am interested in the bioassay of compounds. It was my impression that the standard preparation is use-

ful primarily where one is dealing with variations in potencies of a given compound from day to day, from sample to sample, from batch to batch, or from company to company. The Food and Drug Administration, for example, would be concerned as to whether or not a given compound meets up to standard, whether or not a bottled drug contains what the manufacturer says it contains. If I understand the mathematics of this, the principle here is that the slopes of the dosage-response curves for a given substance and the standard are parallel. If so, the results can be expressed as the ratio of the  $ED_{50}$  values of the standard and the unknown. If the slopes are not parallel, the ratio of  $ED_{50}$  values is meaningless. It seems to me that if Dr. Zelitch were comparing the effect say of phenylmercuric acetate in the dark and in the light, in red and in green wavelengths, at high humidity and at low, with changes in carbon dioxide content or what you will, that perhaps a standard phenylmercuric acetate sample under standard conditions would be worthwhile because the slopes would probably be parallel. But if you are comparing phenylmercuric acetate with 8-hydroxyquinoline or with some of these  $\alpha$ -hydroxysulfonates, the slopes of the dosage-response slopes would probably not be parallel, and the use of the standard would be pointless. I once published a paper on an assay on fungicides in which I recommended a Bordeaux coefficient, which is what you are speaking of. The Bordeaux coefficient works quite well as a standard compound if you are comparing various copper-containing materials, or perhaps even other metals, but it certainly does not work if you are comparing Bordeaux mixture with an organic compound like nabam. Is that correct?

BLISS: That is one point that disturbs me on the slides that were shown. The dosage-response curves are not as detailed as one would need to establish the exact quantitative relationship. Given an all-or-none response, for example, the curve tends to be a sigmoid, and unless the doses are plotted on a logarithmic scale it will be an asymmetrical sigmoid curve. Consequently we transform both axes to units that plot linearly, so that we can make comparisons of slope. Compounds that differ chemically and differing test conditions can be compared directly when the slopes are parallel, as Dr. Horsfall has said. If the slopes are not parallel, then the form of the comparison is open to question, and the best manner in which they can be compared is still under investigation.

One needs to make every attempt to obtain leaf disks that are truly comparable. That kind of precaution is certainly necessary in testing insecticides. A colony of insects may be bred under standard controlled conditions and yet may differ ten-fold in its response to an insecticide

within a period of a month, as Neely Turner has shown, apparently without any other change. I would not want to assume *a priori* that plants or leaves would be uniformly consistent in their reaction.

ZELITCH: The slopes of the compounds we have investigated appear to be different (26) and this would be interpreted to mean that they act at different biochemical sites, which is not surprising. The stomata vary especially when the plants are grown at high night temperatures. When the night temperature is fairly constant, and the plants are grown in a subirrigated bench, the reproducibility of stomatal opening is remarkably good from day to day.

WITTWER: In the greenhouse experiments where you sprayed the tobacco plants with phenylmercuric acetate, did you spray both sides of the leaves or just one side?

ZELITCH: In that experiment both sides were sprayed. We have not carried out that type of experiment on intact plants with only one side of the leaf being treated.

WITTWER: There are several compounds in which I am very interested and I should like to know whether you have tested them for their effect on stomatal opening. What about the uncoupling agent dinitrophenol, and the plant growth substance that was introduced by Dr. N. E. Tolbert at Michigan State a number of years ago, (2-chloroethyl)trimethylammonium chloride, and related compounds. The latter has an effect on drought resistance, or at least drought plus heat resistance. In contrast to this, gibberellin seems to make plants more sensitive to moisture loss or creates some moisture stress. I'm wondering if any work has been done with these materials?

ZELITCH: We have tested dinitrophenol in a disk assay. At  $10^{-4}$  M, it has no effect, and that is about the upper limit of its solubility. We assume that it does not penetrate the guard cells. The other compounds you mentioned have not been investigated. I am aware of the recent literature on the effect of these choline derivatives on reduction of transpiration when watered on the roots, (Halevy, A. H., and B. Kessler. 1963. Increased tolerance of bean plants to soil drought by means of growth-retarding substances. *Nature* 197: 310), but no mention is made in this paper of any effect on stomata.

TING: We have been working with diffusion in model systems, and have calculations based on our data that indicate that the degree of stomatal opening would have very little effect on the diffusion until the stomata are nearly closed. This is a result of the decrease in interference



as the stomata close (Ting, I. P., and W. E. Loomis. 1963. Diffusion through stomates. *Amer. Bot.* 50: 866).

ZELITCH: In our diffusion experiments with leaves in a turbulent atmosphere (28), we find a good agreement between stomatal opening and transpiration and photosynthesis and the diffusion theory at all stomatal apertures.

GARDNER: In connection with that I would like to know how you decided that apertures  $2\mu$  or less were "closed." From a calculation of the resistance to diffusion,  $2\mu$  is wide open in my estimation.

ZELITCH: We arbitrarily chose this aperture because when leaves were kept in the dark, we never observed any stomata with apertures that were greater than  $2\mu$ . If you choose the right temperature for the disk assay, about  $25^\circ$ , then one can obtain a linear relation between the per cent of stomata that are "open" and the mean stomatal width.

PALLAS: Did you work with some other mercury compounds? What about mercuric chloride?

ZELITCH: Our original interest in phenylmercuric acetate arose from a paper published by Blandy (Blandy, R. V. 1957. The effect of certain fungicides on transpiration rates and crop yields. *Internatl. Cong. Crop Protect. Proc.* 4: 1513). He showed that in different areas of the world where fungicides were used, even in years where there was no fungus infection, the yields of potatoes were usually higher. He sprayed various fungicides on potato and tomato plants and he noted that when he sprayed phenylmercuric chloride on potatoes he obtained a significant reduction in transpiration, generally around 10 per cent. In his experiments he also obtained increased growth on the treated plants. The word "stomata" is not mentioned in his paper. When I read this report, we tested phenylmercuric chloride in our assay and found that it was the most effective compound that we had ever examined. Because it is so insoluble in water we preferred to use phenylmercuric acetate, but on a molar basis their activities are identical. We also examined mercuric chloride and mercuric acetate under the same conditions in the assay and even at  $10^{-2}$  M these compounds are completely without effect.

PALLAS: We have confirmed what you say about the effect of phenylmercuric chloride on stomata, and on other species also. But mercuric chloride has definitely reduced transpiration although it did not reduce the stomatal aperture.

DUGGER: I wanted Dr. Kramer to comment on the effects of temperature on stomata described by Wilson (Wilson, C. C. 1948. The

effect of some environmental factors on the movements of guard cells. *Plant Physiol.* 23: 5.), and those described by Dr. Zelitch.

KRAMER: I might point out that Wilson's data were arrived at by taking numerous observations and developing them mathematically to produce three-dimensional curves. He studied the interaction of light and temperature, and the relative humidity of the air in some cases. As to temperature, of course, there was a *general* effect of temperature. We did not attempt to separate opening and closing.

ZELITCH: Wilson's data show that with increase in temperature there was increase in stomatal opening, but a question always comes up as Dr. Kramer pointed out. You have to remember that our experiments were done with floating leaf disks, and water is never limiting, and that I think is a very critical point.

KRAMER: Certainly water was a limiting factor in some of these observations that Wilson made, especially at high temperatures where low relative humidity materially reduced stomatal opening.

I would like to raise another point, about the relationship between transpiration and photosynthesis. The fact that you are getting significant reductions in water loss from plants without any significant reduction in dry matter is an exceedingly important thing. I suppose in effect that you are doing this by eliminating the very strong mid-day drop in photosynthesis that is so often observed where plants transpire rapidly during the forenoon, develop a water deficit, the stomata close, and photosynthesis practically stops. To reiterate what you said, you are getting a relatively low level of photosynthesis but you are getting it all day, apparently. Do you have data that say that is actually what is happening?

ZELITCH: We have no direct experimental evidence. An additional favorable factor would be that photosynthesis is severely inhibited at lower soil moistures with the same stomatal aperture as shown by Shimshi (15, 16). Hence maintaining leaf hydration increases the relative photosynthesis.

van OVERBEEK: I would like to come back to Dr. Pallas' puzzle on transpiration reduction without apparent stomatal regulation. The other day I saw an electron micrograph dealing with plants that had been treated with mercury-containing fungicides. It was most amazing to see the electron-density of the membranes. The mercury seems to accumulate only in the membranes. Now just yesterday, Dr. Kramer and I had a little discussion on work that D. M. Stuart of the U.S.D.A. did on membrane permeability (paper at A.A.A.S.-Pacific 1963). He found

that ammonia gas tightens the membrane of root cells and the permeability is so much decreased that sugar beets in the fields wilt. At least this is the explanation. If you take a disk of sugar beet tissue and place it in hypertonic solution, then of course it loses water. If you follow the weight of the disk with time, then you find a considerable weight loss. Now if you treat this disk with any inhibitor of respiration including ammonia then you do not get such a weight loss. The weight of the tissue is maintained, showing that the membrane has been changed in such a way that it offers more resistance for water to pass through it. That, I think, accounts for Dr. Pallas' observations with mercuric chloride. This seems to be related with membrane permeability, a subject which comes up later for discussion.

Regarding Dr. Kramer's point about photosynthesis, I believe that when you close stomata you may have increased photosynthesis in the sense, first, that considerable internal  $\text{CO}_2$  is used. Secondly, you still get what Arnon calls photosynthetic phosphorylation. If  $\text{CO}_2$  is not present you do not have the Calvin cycle in the plant, then you have what Arnon calls cyclic photophosphorylation, and ATP is generated. Thus amino acids can be directly produced, phospholipids, and many other things, so you obtain weight increase in spite of the fact that the stomata are closed.

Electron micrographs show that the mercury ion is deposited in the cell membrane. Could it be that the effect of phenylmercuric acetate is not on the respiration of the stomata, but on the effect of the mercury atom on the permeability of the guard cell membrane?

ZELITCH: We do not know what metabolic affects are induced inside the guard cell by phenylmercuric acetate. An experiment with radioactive Hg in the molecule could test Dr. von Overbeek's suggestion.

## Active and Inactive Transport Across Cell Membranes

By Kenneth R. Hanson

The earlier papers in this series have been concerned directly with the behavior and morphology of stomata. In this lecture, phenomena of transport and non-transport across cell membranes will be considered with only passing reference to the guard cell. The study of transport processes has been so extensive that no hour-long presentation can be expected to summarize the material available. I shall, therefore, be more concerned to illustrate certain basic concepts than to provide the latest possible information about a rapidly developing field.

The reasons for including a problem in comparative cell physiology in this series of lectures will become clearer as we proceed. Some justification at this point is, however, desirable. It has long been postulated that the swelling of guard cells is an osmotic phenomenon dependent upon the semi-permeability of the plasma membrane. An increase in the number of osmotically active molecules within the cell produces an influx of water until equilibrium is again established. Such an increase could arise through depolymerization of a stored polymer (the traditional explanation), through the active transport of some metabolite into the cell from surrounding cells, or by active transport of some metabolite from a sub-cellular compartment surrounded by a semi-permeable membrane (e.g., a chloroplast or vacuole) into the main volume of the cell. Even if the swelling of guard cells is not directly linked to active transport, it is clearly desirable that more should be known about the compounds which enter and leave the guard cells, and how these compounds are transported across the various membranes of the cell. In designing experiments to answer these questions, it is useful to have as models the approaches made to the investigation of other systems.

*What compounds are transported across cell membranes?* A broad classification may be made on an intuitive basis: (1) water, (2) dissolved gases, (3) inorganic electrolytes, (4) organic molecules of low or at least moderate molecular weight. The fourth group may be subdivided into organic ions, such as the sugar phosphates or citric acid; compounds which have no net charge, such as the neutral amino acids; and uncharged organic compounds, such as the simple sugars. We exclude from consideration molecules or aggregates which are so large that the plasma membrane is disrupted and a new membrane forms when the molecule enters or leaves the cell.



The distinction made here between inorganic and organic molecules may be an arbitrary one. It is well known that enzymes are able to form complexes with both types of molecule in a highly specific manner, and that molecular size and shape play a critical part in this binding. Similar complex formation may play an important role in transport processes.

In most of this discussion the transport of water will be ignored. It should not be assumed, however, that because the cell exists in water, and because osmotic effects can be demonstrated, that cell membranes offer no resistance to the passage of water. Dr. Kuiper will describe models for the processes of water transport in the next lecture.

*In what manner are these compounds transported?* Two types of transport phenomena have been distinguished: inactive and active. Inactive transport has been further separated into simple diffusion and facilitated diffusion (3). Experimentally it is often difficult to decide which of these categories applies in a given case.

*Simple Diffusion:* The simple notion of a surface pierced with holes through which small molecules may wander is probably a poor model for biological membranes. Thus, lipid-soluble molecules may well pass into solution in the membrane during the transport process. For ionized molecules, the positive and negative charges associated with the membrane proteins would be expected to introduce ion exchange effects (12). For many systems, however, the rate of diffusion is proportional to the difference in concentration across the membrane. The energy for the diffusion processes (however simple or complex) is derived from the kinetic energy of the molecules of the solution and is not directly obtained from the processes of cell metabolism. For ions, energy for diffusion may also be derived from electrical potential differences. Ions move under the influence of electrochemical, rather than chemical, potential gradients. (9).

*Facilitated Diffusion:* Here again the energy for the transport process is derived from the kinetic energy of the molecules in the solution. The label is applied to passive transport systems which show a marked degree of stereo-specificity towards the substance transported, and for which the rate of transport approaches a limiting value as the external concentration is increased. Compounds structurally similar to the transported substance frequently act as competitive inhibitors of transport. In this context, a ferryboat (carrier) model is frequently invoked: The ferryboat picks up a passenger on one side of the river, transports him across the river, and leaves him on the farther bank. In the case of a membrane, some complex molecule, possibly a protein, is held to combine with the material to be transported on one side of the membrane,

and to release the material on the other side of the membrane. If the rate of carrier motion is constant, then a saturation effect will arise: it does not matter how many people are standing on the bank, only a certain number can cross in the boat.

Support for the ferryboat model is provided by the phenomena associated with "counterflow." Consider a suspension of cells in a solution of compound A. At equilibrium the rate of flow into the cells is equal to the rate of flow out of the cells. If the solution is diluted, then outflow will exceed inflow until equilibrium is again reached. At no time can the external concentration exceed its original value. If, however, a second substance B is present in the diluent, and if B is reversibly transported by the same carrier system, then A and B compete for the carrier. The rate of A outflow is initially unchanged but the rate of inflow is reduced. Under suitable conditions the external concentration of A may rise above the initial concentration, thus providing clear evidence for uphill transport of A. A and B may be chemically identical provided that one species is isotopically labeled (21).

*Active Transport:* When the expenditure of metabolic energy is required to bring about transport across a membrane, the process is termed "active." Active transport systems usually show the characteristics associated with facilitated diffusion, but, in addition, material may be transported against the concentration, or, for ions, electrochemical gradient. It must be remembered that an unequal distribution of a given ion may indicate a Donnan equilibrium situation or may be complementary to the active transport of some other ion. The amount of energy expended in maintaining a particular concentration difference for a neutral molecule is necessarily greater than the chemical potential difference (the osmotic work), since the system is not at thermodynamic equilibrium. The osmotic work is given by the expression

$$\Delta F = RT \log_e (a_2 / a_1)$$

$\Delta F$  is the amount of free energy required to transfer one mole of substance from side 1 to side 2 and from the activity  $a_1$  to the activity  $a_2$ . To a first approximation, molar concentrations may be used in place of  $a_1$  and  $a_2$ . For charged molecules the free energy change includes the electrical as well as the chemical potential difference (9).

#### SYSTEMS EMPLOYED IN TRANSPORT STUDIES

In this brief survey we shall move from the study of certain organs specializing in transport processes to the study of single cells and sub-cellular organelles (3, 14, 17, 23).

*Tissues of higher animals.* In the first part of the last century it was established that hydrochloric acid is excreted into the stomach. Chloride ions pass from the blood plasma to the stomach. Changes in blood pH are minimized through the buffering capacity of the blood. A somewhat similar process occurs in the salt glands of sea birds. By excreting sodium chloride, these glands maintain the constancy of the internal environment. The kidneys provide a whole series of transport phenomena for study (25). Ultrafiltration of the blood plasma takes place in the glomerulus. In the proximal tubule  $\text{Na}^+$  and  $\text{K}^+$  ions are actively absorbed, and an equivalent amount of anions is taken up passively. Some  $\text{Na}^+$  ions pass through to the distal tubules. In the distal tubules  $\text{Na}^+/\text{K}^+$  and  $\text{Na}^+/\text{H}^+$  exchange takes place. Normal blood level amounts of glucose are completely reabsorbed in the proximal tubules. This process is inhibited by phlorhizin (Fig. 1), a phenolic glucoside, which inhibits various other glucose transport systems. Urea is partly reabsorbed by passive diffusion, but creatinine is, for all practical purposes, not reabsorbed, the total amount passing to the urine.

Another organ frequently used in transport studies is the small intestine (1, 6, 7, 8, 18). Sugars, amino acids, and nucleotides are all actively transported from the lumen to the blood stream. It is fortunate that the intestines also have the ability not to absorb many undesirable compounds of plant origin. Transport across the placenta and transport into muscle tissue have also been extensively studied.

The blood-brain barrier may be mentioned as an example of a system which shows selective permeability (25). The system is permeable to

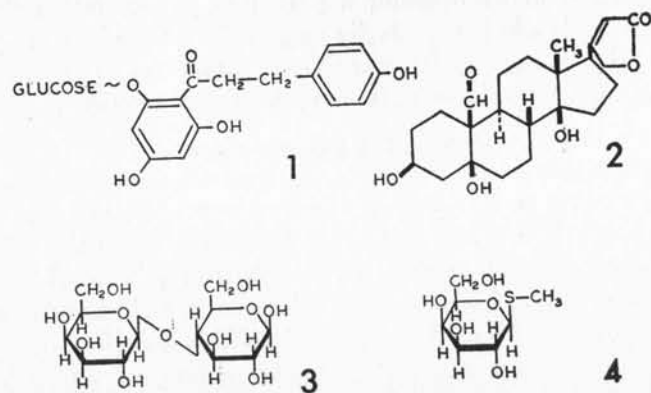


Fig. 1. (1) Phlorhizin: it does not appear to be known whether this compound is an  $\alpha$  or a  $\beta$  glucoside of the tetrahydroxydihydrochalcone. (2) Strophanthidin. (3) Lactose: 4- $O$ - $\beta$ -D-galactopyranosyl-D-glucopyranose (shown as  $\beta$  anomer). (4) Methyl 1-thio- $\beta$ -D-galactopyranoside; also referred to in the biochemical literature as methyl  $\beta$ -D-thiogalactoside and thiomethyl- $\beta$ -D-galactoside (TMG).

glucose and to glutamine, but is virtually impermeable to glutamic acid and various other compounds including many dyes.

*Single cells from higher animals.* The above examples all involve transport across groups of cells. The use of a single membrane is clearly much more desirable. When transport into single cells is studied this goal is approached, though, on occasion, allowance must be made for the effect of compartments within the cell. Red blood cells (erythrocytes) are particularly convenient because they are readily available and mutant strains in humans, sheep, etc. may be obtained (24). Considerable differences among species occur. Although glucose enters the erythrocyte with extreme rapidity it appears to enter by a facilitated diffusion process. The  $\text{Na}^+/\text{K}^+$  levels in the cells of sheep are genetically determined. The maintenance of these levels appears to be dependent upon an active transport system or systems, for removing  $\text{Na}^+$  from the cell and replacing it by  $\text{K}^+$ . The levels determine osmotically the volume of the cell. The cardioactive steroid strophanthidine (Fig. 1) inhibits the active transport process and leads to swelling of the erythrocytes. Ehrlich ascites tumor cells and the L-cell strain of fibroblasts have also been investigated. The relationship of  $\text{Na}^+/\text{K}^+$  transport to the establishment of electrical potentials and to nervous transmission has been studied in the giant nerve cells of the squid. The  $\text{Na}^+$  ions which constantly diffuse into the cell with the electrochemical potential gradient are actively pumped out of the cell. A second system, or possibly the same system acting in reverse, brings  $\text{K}^+$  ions into the cell (9).

*Microorganisms.* Most investigations of transport into microorganisms have been concerned with either *E. coli* or various yeasts. Penetration by inorganic ions has received considerable attention. The active transport of mono- and disaccharides, of amino acids, and of simple organic acids into *E. coli* has been demonstrated (4, 13). Active transport of  $\alpha$ -glucosides into yeasts has also been observed (3).

*Plants.* Since many of those present have firsthand experience with plant tissues, I will merely note that the interpretation of studies of ion uptake by roots and slices of storage tissues is rendered difficult by the morphological complexity of the tissue examined (9, 17). Simplification has been attained by studying single cells. Thus, for single cells of the algae *Nitellopsis obtuse* (5 cm  $\times$  0.5 mm), it seems probable that active  $\text{Na}^+$  transport from the cell sap takes place through the outer cytoplasmic membrane (or plasmalemma) with reverse  $\text{K}^+$  transport. Also, that active  $\text{Cl}^-$  transport into the vacuole through the vacuole membrane (or tonoplast) occurs (19). It is possible that in higher



plants organic acid anions, rather than  $\text{Cl}^-$  ions, are actively transported into the vacuole. Under such circumstances the vacuole would be maintained in a state of turgor (9).

*Compartments within the cell.* In addition to the vacuole in plant cells, such subcellular particles as chloroplasts, mitochondria and nuclei may be expected to function as separate compartments. I shall have more to say about mitochondria later in this lecture. The use of isolated vacuoles for transport studies presents an intriguing possibility (11).

### SOME DETAILED EXAMPLES

*Example 1: Absorption by the Small Intestines.* It has long been known that glucose is completely absorbed from the lumen of the small intestines. This takes place even if the concentration inside the intestine is very much less than that in the blood plasma. Although it would appear that active transport is involved, the interpretation of the experiment is complicated by the fact that absorbed sugars are swiftly metabolized in the intestinal epithelium. The uncertainty was largely removed when experiments were carried out with use of analogues of glucose compounds which are transported but are not themselves metabolized (8).

Experiments with isolated toad intestines have shown that sodium ions are essential for active transport. The section of intestine was placed in a physiological buffer solution with identical concentrations of the analogue 3-O-methyl-D-glucose on the inside and the outside. The increase in external concentration was then determined. With intestinal sections from various animals it has been established that the requirement of the active transport system for sodium ions applies only to the inner (mucosal) surface. Sodium cannot be replaced by lithium, potassium or magnesium. When the inner concentration is very much greater than the outer, then the sodium requirement disappears but phlorhizin is still effective as an inhibitor. It seems probable, therefore, that the system may be regarded as a facilitated diffusion system plus an additional sodium-dependent driving mechanism (8).

If, instead of studying transport across the intestinal tissue, transport into the tissue is examined, similar but more dramatic results are obtained. Thus the glucose analogue 6-deoxy-D-glucose, when applied to strips of small intestine of the hamster, is concentrated 17 fold. When phlorhizin or the uncoupling agent 2-methyl-4,6-dinitrophenol is present, or under anaerobic conditions, the internal concentration does not rise above the external concentration (6). In principle, the initial rate of entry into the tissue could be studied as a function of the external

concentration under aerobic and anaerobic conditions. Although this has not been done, the apparent rate of entry, estimated from the internal concentration after 10 minutes, has been studied for a number of sugars (aerobic conditions) as a function of the external concentration (7). Glucose uptake was studied under conditions where glucose utilization was 85 per cent inhibited. The apparent rate of entry approached a maximal value as the concentration was increased (Fig. 2A). A double reciprocal plot gave straight lines for which constants analogous to the Michaelis constant could be calculated. These  $K_m$  values are numerically equal to the external concentration required to bring about an apparent rate of entry of half the maximal (saturation) value. Typical values are: glucose,  $1.8 \times 10^{-3}$  M; galactose,  $2.2 \times 10^{-3}$  M; 1, 5-anhydro-D-glucitol,  $7.4 \times 10^{-3}$  M. Since these and various other sugars showed mutual inhibition effects, they are transported by the same system. Other sugars such as D-xylose, and D-ribose are not actively transported. In general it was found that D-pyranose sugars with  $-\text{CH}_3$  or  $-\text{CH}_2\text{OH}$  at C-5, and  $-\text{OH}$  in the glucose configuration at C-2 were transported. Phlorhizin acts as a true competitive inhibitor for the transport system (Fig. 2B):  $K_i$ , ca.  $6 \times 10^{-7}$  M (1). When D-galactose was allowed to accumulate in strips of hamster small intestine, it was found that the highest concentration occurred in the epithelial cell layer (the inner mucosal side) (18). It therefore seems reasonable that in the experiments with sections of intact intestine there is an active accumulation from the internal medium into the epithelial cells, followed by an inactive downhill passage to the external medium.

The analysis of this system has proceeded from the intact animal to the tissue section. The ultimate goal of such an analysis is to obtain a reconstructed system in which active transport across an oriented lipoprotein film is demonstrated. A possible step towards this goal is the study of the adenosinetriphosphatase isolated from epithelial tissue. This enzyme shows a marked sodium requirement and is inhibited by the various cardioactive steroids which also inhibit the intestinal active transport systems (8).

*Example 2: Active Transport in Escherichia coli.* Although certain of the transport phenomena exhibited by microorganisms resemble those of the small intestine, the route of scientific investigation has been a different one. One aspect of the story begins with the study of cryptic mutants. For example, various strains of yeast are known which are unable to use such disaccharides as maltose, cellobiose, or sucrose for growth. Each of these sugars will produce glucose on hydrolysis, and each strain will grow on glucose. Furthermore each yeast on lysis releases an enzyme capable of hydrolysing the disaccharide in question.



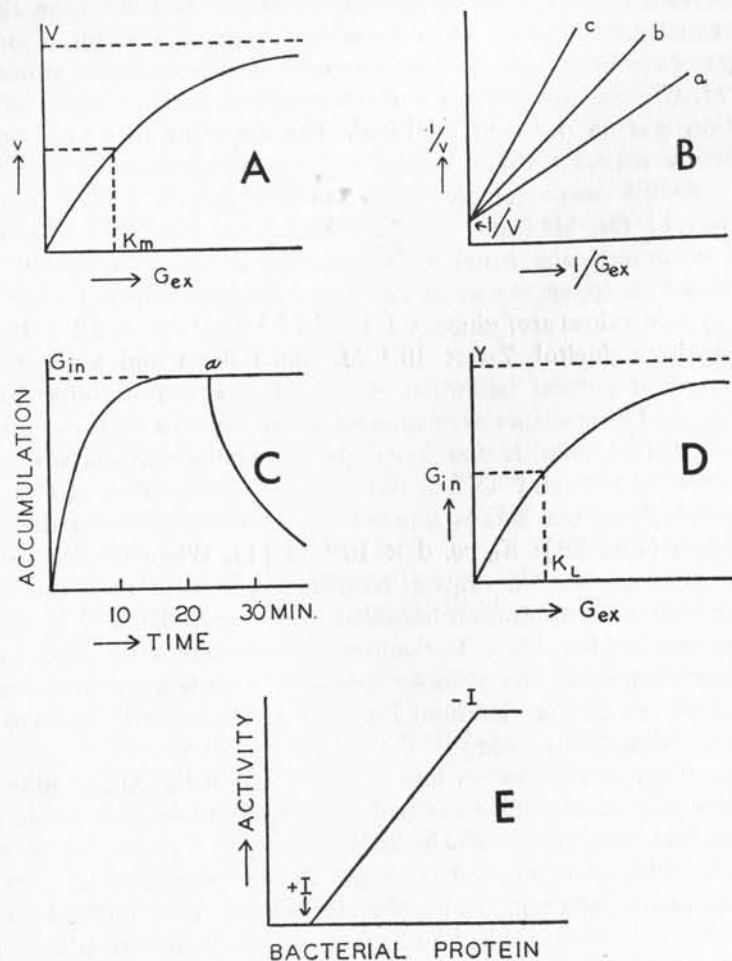


Fig. 2. Experiments with isolated strips of small intestine: A, Apparent initial velocity of entry ( $v$ ) as a function of the external concentration ( $G_{ex}$ ). Note resemblance to the Michaelis-Menten relationship. B, line (a): a double reciprocal plot of the curve shown in A. The effect of competitive inhibition by phlorhizin is represented by lines (b) and (c).

Experiments with *E. coli*: C, Accumulation of galactose or  $\beta$ -galactoside as a function of time. Note steady state value ( $G_{in}$ ) reached only after an interval of time. Adding 2:4-dinitrophenol at (a) leads to rapid loss of accumulated material. D, Steady state internal concentration ( $G_{in}$ ) as a function of the external concentration ( $G_{ex}$ ). Note resemblance to Langmuir absorption isotherm. A double reciprocal plot gives a straight line. E, Induction of  $\beta$ -galactosidase or permease as a function of bacterial mass under conditions of gratuity. Excess inducer (methyl 1-thio- $\beta$ -D-galactopyranoside) added at + I. Antagonist to inducer (phenyl 1-thio- $\beta$ -D-galactopyranoside) added at - I.

The notion was therefore introduced that these enzymes are hidden in some part of the cell (i.e. are cryptic), and are unable to attack the disaccharide. This seemed a more economical hypothesis than to suppose that the cell wall contained a multitude of stereospecific transport systems. When a mutant of *E. coli* was obtained that was unable to grow on glucose but could grow on maltose, the evidence pointed to a serious need for the study of cell permeability (10). Maltose enters the cell and is converted by amyloamylase to amylose and glucose. External glucose is unable to enter the cell, but the glucose formed inside the cell is phosphorylated by hexokinase and is further metabolized in the usual way. Similarly, in the yeast mutants the disaccharides are unable to enter, but glucose does enter.

In summary, microorganisms show great specificity in the compound they will permit to enter the cell. Selection occurs not only among classes of compounds but between compounds with minor stereochemical differences. Entry into the cell is a favor granted, not an inherent right.

The term "permease system" has been coined to label the complex responsible for admitting compounds to the cell (4). The characteristics of the permease systems for a number of compounds in different bacteria have been studied; however, the  $\beta$ -galactoside system of *E. coli* has received by far the most attention.

When lactose (Fig. 1) enters wild type *E. coli* it does so by way of the permease system. Once inside the cell it is hydrolyzed to galactose and glucose by  $\beta$ -galactosidase. For the expression of the permease gene and of the enzyme gene, the *E. coli* must be exposed to a suitable inducer in the absence of glucose. Lactose itself acts as an inducer of both permease and enzyme, but so do the  $\beta$ -methyl ethers of D-galactopyranose and 1-thio-D-galactopyranose (Fig. 1). The  $\beta$ -phenyl ethers of these compounds do not. All five compounds are taken into the cell by the permease system but the derivatives of thiogalactose are not hydrolyzed by the enzyme. This fortunate circumstance allows permeation (or the induction process) to be studied without having to allow for  $\beta$ -galactosidase activity. Thus the accumulation of methyl 1-thio- $\beta$ -D-galactopyranoside- $C^{14}$  in cells previously grown on lactose and in the absence of glucose may be readily studied. A similar use of analogues has already been mentioned in discussing the small intestine. An alternative method is to study the accumulation of lactose- $C^{14}$  in a mutant strain unable to synthesize  $\beta$ -galactosidase.

In a typical experiment with methyl 1-thio- $\beta$ -D-galactopyranoside- $C^{14}$  and induced cells, the internal concentration of radioactive material reaches a steady value in about 20 minutes (Fig. 2C). This steady value is dependent upon the external concentration. The relationship between the radioactivity per unit weight of cells at equilibrium and the ex-

ternal concentration is that of a Langmuir absorption isotherm (Fig. 2D). As the external concentration increases, the internal concentration approaches a limiting saturation value known as the capacity ( $Y$ ). A double reciprocal plot gives a straight line from which the constant  $K_L$  may be calculated.  $K_L$  is numerically equal to the external concentration required to give an internal concentration that is half the saturation value. The accumulation is an equilibrium process since the radioactive material is readily displaced from the cell by unlabeled compound. Although this phenomenon resembles an absorption process, the absorption explanation may be eliminated for the following reasons: (a) Too much material accumulates (e.g., 5 per cent of the dry weight of the cell). If absorption were involved, an enormous number of specific binding sites would have to be present within the cell. (b) The accumulation process depends upon the continuing metabolism of the cell. Sodium azide or 2,4-dinitrophenol both prevent accumulation or cause loss of already accumulated material. (c) Lactose accumulating in a mutant lacking  $\beta$ -galactosidase has been shown to be osmotically active. The cells were first rendered osmotically sensitive by treatment with lysozyme. Addition of lactose to the suspended cells caused extensive lysis; water passed into the cells to dilute the accumulated sugar until the cells burst (22). Presumably the swelling could be prevented by adding azide or 2,4-dinitrophenol. As indicated in the introduction, this type of explanation could account for the swelling of guard cells.

It is clear from the above that the permease system brings about the active transport of  $\beta$ -galactosides. If an additional reversible passive transport system is postulated, then the above experimental observations may be accounted for in mathematical terms (16): Let the net rate of entry into the cell be equal to the rate of entry by the action of the permease system, less the rate of exit by the passive route. Also, let the entry process obey Michaelis-Menton type kinetics (as discussed for the small intestine) and let the exit rate be proportional to the internal concentration (the correction for the external concentration may be ignored).

Equation 1 follows where  $G_{in}$  is the internal concentration (moles  $g^{-1}$ ), and  $G_{ex}$  is the external concentration of the transported substance (moles liters $^{-1}$ ), and  $y$  (moles  $g^{-1} \text{ min}^{-1}$ ) and  $c$  ( $\text{min}^{-1}$ ) are constants.

$$\frac{dG_{in}}{dt} = y \frac{G_{ex}}{G_{ex} + K_m} - c G_{in} \quad (1)$$

At equilibrium  $dG_{in}/dt = 0$ , hence

$$G_{in} = \frac{y}{c} \cdot \frac{G_{ex}}{G_{ex} + K_m} \quad (2)$$

Equation 2 correctly describes a Langmuir absorption isotherm where  $y/c = Y$ , the observed capacity of the cells (moles  $g^{-1}$ ), and  $K_m$ , the Michaelis type constant for the permease system, is identical to the observed constant  $K_L$ . For methyl 1-thio- $\beta$ -D-galactopyranoside,  $K_L = 4.3 \times 10^{-4}$  M.

This theory may be tested in another way (13). If  $G_{ex}$  is much greater than  $K_m$  then

$$dG_{in}/dt = y - c G_{in} \quad (3)$$

hence, integrating and substituting for  $y/c$

$$G_{in} = Y(1 - e^{-ct}) \quad (4)$$

$$\log_e \left( \frac{Y - G_{in}}{Y} \right) = -ct \quad (5)$$

Equations 4 and 5 describe the manner in which a substance is taken into the cell when the substance is present in saturating external concentration. When the experimental data are plotted according to equation 5, a straight line is obtained of slope  $-c$ . Once  $c$  is known,  $y$  may be calculated. A second estimate of  $c$  may be obtained by following the rate of exit of the compound when 2,4-dinitrophenol is added to cells already containing accumulated material. The galactose permease system of *E. coli*, which closely resembles the  $\beta$ -galactoside system ( $K_L = 7 \times 10^{-6}$  M), has been studied with a mutant constitutive for the permease and lacking galactokinase. Satisfactory agreement between the two estimates of  $c$  was obtained (13).

It seems probable that permease systems of the above type are irreversible. Thus wild type *E. coli* possesses a maltose permease system. A mutant strain has been obtained which appears to lack an exit system for maltose. Once maltose enters the cell it is unable to escape. Maltose in concentrations up to 25 per cent of the dry weight of the cell may be accumulated.

Various lines of evidence establish that a specific protein (termed a permease) is associated with a given system. The following may be noted: (a) The systems exhibit a high degree of specificity similar to that shown by enzymes. (b) The  $\beta$ -galactoside system is inducible in the same way that  $\beta$ -galactosidase is inducible. Both the permease system and the enzyme are induced together by the same compounds. If induction is studied under conditions where the permease system does not confer any growth advantages on the cells (conditions of grauity (5)), then the increase in the cell capacity  $Y$  is proportional to the cell mass. Exactly this type of behavior is shown for enzyme induction (Fig. 2E). (c) Induction does not take place if protein synthesis is prevented by adding chloromycetin, omitting an essential amino acid, or



adding an amino acid analogue which is incorporated to give abnormal proteins. (d) The permease system is inhibited by *p*-chloromercuribenzoate, and the inhibition is partially reversed by cysteine or glutathione. Compounds which are transported by the system protect the system from such attack. (e) The results of detailed genetic analysis show that the structural gene responsible for the synthesis of the permease *y*, and the structural gene responsible for  $\beta$ -galactosidase synthesis *z*, occupy adjacent regions on the chromosome (15). An operator gene *o* is adjacent to the *z* gene, and is under the influence of a regular gene *i*, a short distance away along the chromosome. Considering the gene at the chromosome level, there appears to be no qualitative difference between the *y* and *z* genes except their position relative to the operator. The fact that one yields a permease and the other an enzyme is quite secondary.

It is clearly desirable that the permease protein should be isolated and its properties studied. For this to be accomplished a satisfactory assay is required. An enzyme which brings about the acetylation of 1-thiogalactose has been purified. This enzyme appears to be genetically associated with the *o*, *z*, *y* region, but its role in the cell is not understood, and at present it seems unlikely that this enzyme is the permease appearing under another name.

If the permease system is irreversible, then the "exit" system is in all probability reversible. This assumption would account for the entry of inducer into non-induced cells, and the fact that, under conditions of lactose utilization, entry into the cell is only partly inhibited by concentrations of azide or of 2,4-dinitrophenol which totally inhibit active transport. Studies of the galactose system mentioned above suggest that this passive system also involves an inducible protein (13). Cells of the mutant form grown on succinate in the presence of galactose (i.e., gratuitly) show an increase in the exit constant *c* which is proportional to the increase in cell mass.

Before leaving the example of *E. coli*, it is perhaps worth emphasizing that the behavior of these few carbohydrate permease systems is not necessarily characteristic of all bacterial systems, let alone all microorganisms.

*Example 3: Some properties of mitochondria.* The behavior of mitochondria is of particular interest in the context of this Seminar, since the swelling of mitochondria is in some respects reminiscent of the swelling of guard cells (20). Isolated rat liver mitochondria undergo a slow swelling in potassium chloride solution (0.125 M) buffered at pH 7.4. This enlargement is greatly enhanced and accelerated in the presence of phosphate, both reduced and oxidized glutathione, the amino acid

hormone L-thyroxine, and oleate. Even after 40 minutes, the swelling still continues, although the rate is much diminished. Except in the case of oleate, the swelling is prevented by cyanide, various other respiratory inhibitors, and by anaerobiosis. The swollen mitochondria can be restored to their original size with ATP. This process is blocked by azide and other compounds. To say that mitochondria swell is equivalent to saying that water is taken into the organelle, and, since swelling is respiration-dependent, the transport of water has been termed active. It is however established that mitochondria can act as passive osmometers. Transfer from buffered isotonic sucrose to dilute buffer brings about swelling to a new steady state. The process is complete in less than 5 seconds and is not appreciably affected by cyanide or temperature. It follows that the mitochondrial membrane does not present a serious barrier to water entry, and that the "active" swelling is not an example of active transport in the accepted sense of this term. Presumably the non-osmotic swelling arises through changes in the physical properties of the various mitochondrial membranes.

Although the passage of water into mitochondria does not provide us with an example of active transport into a subcellular particle, evidence has been presented that phosphate can be so transported (2). The membrane appears to be freely permeable to magnesium, but the entry of phosphate is accompanied by a stoichiometric amount of magnesium. Cyanide, azide, 2,4-dinitrophenol, antimycin and ADP inhibit the process. Oxygen and a suitable substrate (succinate) are required.

*Concluding remarks:* Those familiar with the field will perhaps be surprised that I have said so little about the various models developed to explain active and inactive transport. In fact the only one cited has been the ferryboat model for facilitated diffusion. This hypothesis has the great virtue of simplicity. Nevertheless, the notion that a large carrier protein is scurrying back and forth inside a lipoprotein membrane is somewhat implausible. One would prefer to think of some specific structure mounted in the membrane and reaching from one surface to the other, but the properties required for such a structure are not self-evident. The ferryboat model must be regarded, even by the skeptical, as a very useful peg on which to hang certain facts. The manner in which metabolic energy is made available for active transport has been the subject of much speculation. If ATP is the mediator in certain cases, then the possibility of a conformational change induced by ATP hydrolysis presents itself. A change in protein conformation could bring about the release of a bound compound. By omitting any serious discussion of these matters, I do not wish to imply that such model building is not essential for progress in the field. Insofar as we



are concerned in this Seminar with the guard cells, however, speculation of this kind is premature. The initial problems are to discover what compounds are transported into the guard cells, and what transport takes place between the vacuole and the cytoplasm and between the chloroplasts and the cytoplasm. Are any of these transport processes active and critically linked to the changes in turgor of the guard cells?

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### Discussion

KRAMER: Are we justified in assuming that the only material of any significance that enters or leaves the guard cells during the changes in dimension is water? If one argues that considerable amounts of a substance, or substances, other than water enter the guard cells then what are these substances? Where do they come from? One certainly has to worry about their active transport. To start an argument I will say I doubt this postulate very seriously. The changes which occur must be changes within each guard cell rather than changes in amounts of solutes that are moved in and out.

ZUCKER: I would agree that it is not necessary to have movement of other materials into the cell. There could be exchange between various compartments of the cell. Mesophyll chloroplasts (I do not know about the guard cell chloroplasts) contain up to 75 per cent of the salt content of the cell. This salt could be excreted from the rigid chloroplast into the less rigid vacuole and thus bring about an uptake of water. This is similar to the problem of water uptake by potato disks. It was postulated in the 40's that there was movement of salt into the vacuole. No evidence was ever presented to support this suggestion.

KUIPER: Beyer (Beyer, A. F. 1929. Über Tropfenbildung in den Schlusszellen der Spaltöffnungen von *Tradescantia zebrina*. *Bot. Archiv.* 26: 227) has observed that during the closing of *Tradescantia* leaf stomata small vacuoles appear in the guard cells. The closing reaction is very rapid; about 5 minutes. In my opinion it is possible that protein-bound water is released and passes to the vacuoles. This transfer could change the volume of the guard cell and have an effect on the turgor pressure.

DUGGER: What volume changes are involved in the swelling of chloroplasts?

ZUCKER: About 25 per cent.

DUGGER: If water molecules packed around proteins are released into free solution by a change in the protein conformation then only a 14 per cent increase in volume would arise. This effect could not bring about such a large change.

MOSS: One substance that does move into the cell is  $\text{CO}_2$ .

WITTWER: Franke (Franke, W. 1961. Ectodesmata and foliar absorption. *Am. J. Bot.* 48: 683) has shown that large numbers of ectodesmata are associated with the external surface of the epidermal cell wall and I think there is evidence in Franke's photographs that the ectodesmata connect guard cells and epidermal cells. Ectodesmata may function in water and nutrient uptake and loss.

van OVERBEEK: Sargent at Oxford (Sargent, J. A., and G. E. Blackman. 1962. *J. Exptl. Biol.* 13: 248) found that radioactive 2,4-dichlorophenoxyacetic acid gets into guard cells from the outside (not through the stomata) more rapidly than into other epidermal cells.

## Some Considerations on Water Transport Across Living Cell Membranes

By P. J. C. Kuiper

It has been stated in previous lectures that the stomatal aperture is determined by the turgor pressure of the guard cells. Transport of water across cell membranes may cause changes in turgor pressure. It will not be considered for the moment whether this water moves across the cytoplasmic membrane of the guard cell to neighboring epidermal cells, or whether stomatal movement mainly depends on water transfer across the tonoplast to the vacuoles.

There is some evidence that water movement in the guard cells is of a non-osmotic nature. Changes in osmotic pressure in general are far too slow to account for the rapid changes in stomatal aperture frequently observed, especially in the closing process (14). It may be assumed that the energy required for the opening process is supplied by photosynthetic phosphorylation reactions in the guard cell chloroplasts, especially when the concentration of  $\text{CO}_2$  is low (8).

Since experimental data on this type of water movement in guard cells are difficult to obtain, some experiments have been conducted on the mechanism of water transport across cytoplasmic membranes of root cells. These membranes are located in the meristematic cells of the root tip and the endodermis of the upper root zone. It is evident that these membranes account for the main resistance to water transport in the root and stem, since, for example, damage done to the roots by keeping them at  $70^\circ \text{C}$ . for a short time greatly reduces the resistance to water transport in the stem.

In discussing the nature of the process of water movement through a cytoplasmic membrane, in general a distinction is made between diffusion (isotope effect), osmotic, and hydrostatic water flow (3, 7, 12). Fick's law, however, cannot adequately describe this type of water movement, since the molecular structure of the lipid layers of the cytoplasmic membrane should be considered. Such a lipid layer presents a potential energy barrier, which can only be crossed if the diffusing water molecule has sufficient kinetic energy (2). The height of such a barrier, the activation energy, can be determined from experiments on the effect of temperature on the rate of water transfer. Alternatively, one may speak of the  $Q_{10}$  of the process and the activation energy of passage of the water molecule (6). Some experiments on the effect of root temperature on the water uptake of intact bean plants

under conditions of high transpiration will be presented (9). It should be pointed out that essentially the same results were obtained in experiments on the water uptake by intact root systems, in which a constant suction tension of 50 cm Hg was applied to the cut end of the stem. In general, two kinds of temperature effects could be observed, one with a high and one with a low  $Q_{10}$  value of about 1.2 (Fig. 1). Above a certain critical temperature only the viscosity of water could be observed to limit water uptake, indicating the existence of permanent water-filled pores in the root cell membranes. The viscosity of water depends on the mutual forces between water molecules, that is on hydrogen bonds. These bonds must be broken before any movement of water can occur. The low energy required to break such a bond indicates that each hydrogen atom is bound to two or more oxygen atoms of neighboring water molecules. The number of those bonds increases slightly with temperature. The energy of one mole of hydrogen bonds is 6,000 cal (13).

Below this critical temperature the root cell membrane is characterized by a high potential energy barrier for water transport. The activation energy is about 25,000 cal per mole; the  $Q_{10}$  value is 4.0. In other plant species even higher  $Q_{10}$  values up to 8 were observed. When

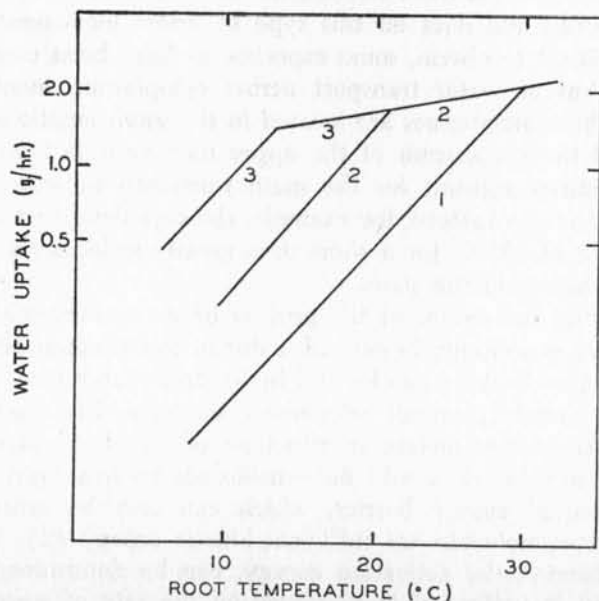


Fig. 1. Effect of root temperature on water uptake of young bean plants (schematic figure from Kuiper (9)). 1: Plants grown at high root temperature (25°) and favorable aeration conditions of the roots. 2 and 3: Plants grown at lower root temperatures or decreased aeration conditions or both.

there is a series of barriers and one barrier is much higher than the others, the rate of diffusion is determined very nearly by the height of the highest barrier (6). The highest barrier is probably associated with the lipid layers of the cell membranes, made up of long lipid molecules in a regular orientation (10). The energy required to break the hydrogen bonds between the diffusing water molecules and the surrounding water molecules will not contribute to the energy barrier, since the energy is required to separate the molecules of the lipid layer to form a pore (11). These pores may allow passage of a single string of water molecules. A pore may close again when the kinetic energy of the lipid molecules surrounding it exceeds the kinetic energy of the two hydrogen bonds between two successive water molecules. It is also clear that the rate of self-diffusion, across such a pore, as measured for example with tritiated water, should be much smaller than the rate of osmotic water movement and of hydrostatic water movement (3).

The critical temperature strongly depends on environmental conditions during growth. It was found to shift to lower values, for example, with decreasing root temperature and by decreasing aeration conditions during growth.

Most of the lipids of the cell membrane exist as esterified fatty acids. For a better understanding of the above experiments, the effect of temperature on the evaporation resistance of monolayers of stearic acid, oleic acid, and of mixtures of both compounds was studied. Experimental methods are described in the books by Davies and Rideal (7), and by La Mer (11). The evaporation resistance of a compressed monolayer of stearic acid showed a logarithmic decrease with temperature. The  $Q_{10}$  value was about 1.5 and the activation energy about 7,500 cal per mole. The strong cohesion between the hydrocarbon chains of this liquid condensed film probably is responsible for the height of this potential energy barrier. A similar experiment with a monolayer of oleic acid revealed no effect of temperature on the evaporation resistance. Evidently the oleic acid film is very expanded and hardly any cohesion between the molecules exists. The same result was obtained with a film composed of 99 parts of stearic acid to one part oleic acid. Some results of experiments on films composed of mixtures of stearic and oleic acids are shown in Fig. 2. All curves show a temperature-dependent part with a  $Q_{10}$  value of about 1.5. Above a certain critical temperature, depending on the amount of oleic acid added to the stearic acid, no effect of temperature on the evaporation resistance could be observed. It is thus clear that the film gradually changes from the liquid condensed phase to the "gaseous" phase with increasing temperature. At the site of an oleic acid molecule in the film, the regular orientation of the neighboring stearic acid molecules is decreased. This



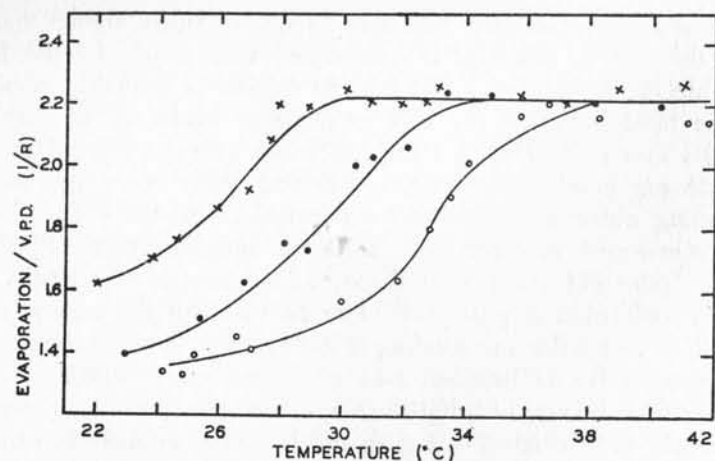


Fig. 2. Effect of temperature on the rate of evaporation through stearic acid-oleic acid monolayers expressed in  $\text{mg H}_2\text{O} \times \text{min}^{-1} \times 100 \text{ cm}^{-2} \times (\text{mm Hg})^{-1}$ .  $\circ$  —  $\circ$ , 0.1%;  $\bullet$  —  $\bullet$ , 0.2%;  $\times$  —  $\times$ , 0.5% oleic acid added to the stearic acid.

results in an increase in "free volume" in the fatty acid film, so that more free pores for water vapor transport are available. Because of a rise in temperature the kinetic energy of the fatty acid molecules increases to a point where the intermolecular attraction, as determined by the van der Waal's forces, is overcome, and permanent pores for water vapor transport are available.

The importance of this model for a better understanding of the behavior of water uptake by bean plants as affected by root temperature is clear. Incorporation of a specific molecule in the lipid layers of the cytoplasmic membranes, which can decrease the orientation of the neighboring hydrocarbon chains and which thus decreases the intermolecular attraction between the hydrocarbon chains, may explain the above effect of environmental conditions during growth on permeability of roots to water. Thanks to the cooperation of Dr. Zelitch I have already obtained some interesting compounds with which to test this hypothesis.

The squeezing of molecules out of a film is generally difficult to visualize (11). For this reason, it seems unlikely at present that reversible and rapid changes in the permeability of cytoplasmic membranes are caused by changes in the lipid layer of the membrane.

Experiments on the water uptake by bean roots, in which suction is applied to the cut end of the stem, suggest that the elasticity of the root cell membrane is determined by the protein layers of the membrane. The effect of hydrostatic suction on water uptake of the roots is different when proceeding in the direction from low suction to high

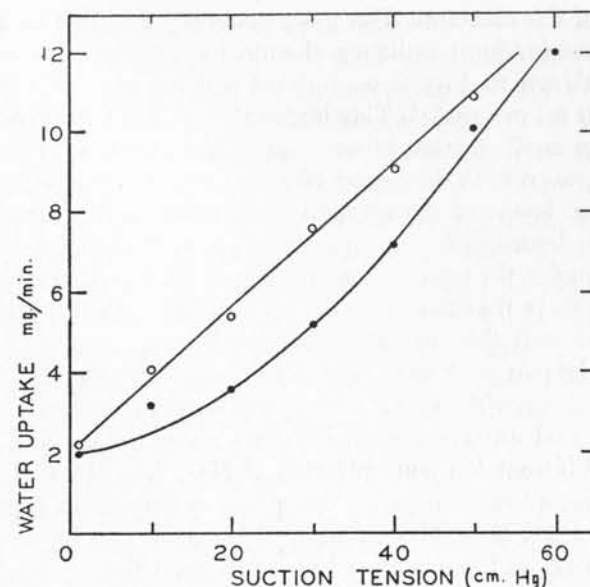


Fig. 3. Effect of suction tension (cm Hg) applied to the cut end of the stem on water uptake of bean roots.  $\bullet$  —  $\bullet$ , increase in suction in the direction of 0 to 60 cm Hg;  $\circ$  —  $\circ$ , decrease in suction starting with 60 down to 0 cm Hg. Experiment by R. L. M. Pierik, F. Kuiper, and P. J. C. Kuiper, unpublished, at Wageningen, Holland.

suction compared with a change from high suction to low suction. Increasing the suction resulted in a logarithmic relation between water absorption and suction, while a linear relation between water uptake and suction was found when moving from high values to low values of suction (Fig. 3). This linear relation is in accordance with Darcy's law for liquid flow through capillaries. Apparently in this case both the number of pores in the cytoplasmic membranes of the root cells and the area of the pores is constant at different suction tensions. The more than proportional increase in water uptake with increasing suction indicates an effect of hydrostatic suction on the number of pores. According to Johnson *et al.* (6), the reaction rates of most elementary processes show a logarithmic increase with increasing hydrostatic pressure as well as with temperature. One can calculate the activated volume change,  $\Delta V^\ddagger$ , from the slope of the curve of Fig. 3 from the relationship

$$k_p = k_0 e^{-\frac{p\Delta V^\ddagger}{RT}}$$

in which  $k_0$  and  $k_p$  are the reaction rates at zero pressure and at pressure  $p$ , which is the hydrostatic pressure in atmospheres. The gas constant,  $R$ , is 82 ml per mole,  $T$  is the temperature in  $K^\circ$ , and  $\Delta V^\ddagger$  is the volume of the activated molecular complex minus

the volume of the reactants. The  $\Delta V^\ddagger$  generally amounts to a few milliliters per mole for most ordinary chemical reactions. The value of the experiment shown in Fig. 3 was about 330 ml per mole per  $K^0$  (or about 100,000 ml per mole). This high value of  $\Delta V^\ddagger$  indicates a drastic change in the configuration of very large molecules, with the resultant exposure of groups with decreased affinity for water. Probably in stretching the protein layers of the cytoplasmic membrane as the suction is increased, more hydrophobic groups are exposed and so the free volume for water transfer through the membrane is increased. Preliminary experiments indicate that the value of  $\Delta V^\ddagger$  varied with the variety within a species and with the environmental conditions.

It is well known that ions may affect water uptake of plant roots. Some experiments with several strong monovalent cations and calcium will be presented on the relation between water uptake and suction as affected by different ion concentration of  $Na^+$ ,  $K^+$ ,  $Li^+$ ,  $Cs^+$ , and  $Ca^{++}$  ions. Fig. 4 shows some data on the effect of potassium ions on water uptake. In general two different suction processes can be distinguished, one with a high and one with a low activated volume change. An explanation of the lower one has already been given. The activated volume change below the critical suction (50 cm Hg for  $K^+$ ; 20 cm Hg for

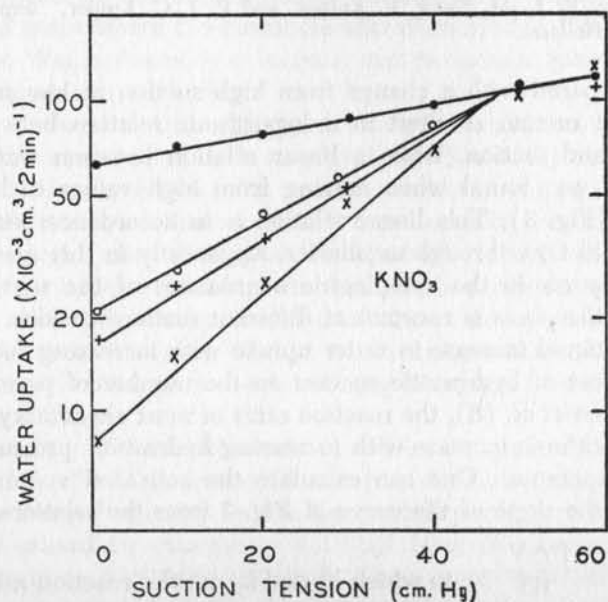


Fig. 4. Effect of suction on water uptake of bean roots in different  $KNO_3$  solutions. ● — ●, water; ○ — ○,  $1 \times 10^{-2}$  M; + — +,  $2 \times 10^{-2}$  M, x — x,  $5 \times 10^{-2}$  M  $KNO_3$  solution.

$Na^+$ ) is due to electrostriction as determined by the specific alkali ion and its concentration. In our experiments it ranged up to about 2000 ml per mole per  $K^0$ . Fig. 5 represents some results on the effect of ion concentration on the activated volume change,  $\Delta V^\ddagger$ , obtained from Fig. 4 and its analogues for  $Na^+$ ,  $Cs^+$ , and  $Li^+$ . The data are summarized in Table 1.

It is clear from Fig. 5, that  $Na^+$  and  $Li^+$  exert a much stronger effect on water uptake and activated volume change than does  $K^+$  and  $Cs^+$ . The specific effect of the cation on the permeability of the cell membrane depends on the degree of cross-linkage between the amino acid chains of the protein layers of the cytoplasmic membrane. In a membrane consisting of proteins with a low degree of cross-linkage one should expect an increase in elasticity and in activated volume change with an increase in hydration number of the ions and with an increase in hydrated ionic volume. In a membrane with a high degree of cross-linkage one should expect a relation between  $\Delta V^\ddagger$  and the ion size;  $Cs^+$  is the largest ion and  $Li^+$  the smallest. The partial reversal of the permeability as compared with the hydration number, indicates a moderate degree of cross-linkage between the amino acid chains of the protein layers of the cytoplasmic membrane, possibly in the form of covalent links. In agreement with this view is the observation that  $Ca^{++}$ , which is strongly hydrated, greatly influences the activated volume change.

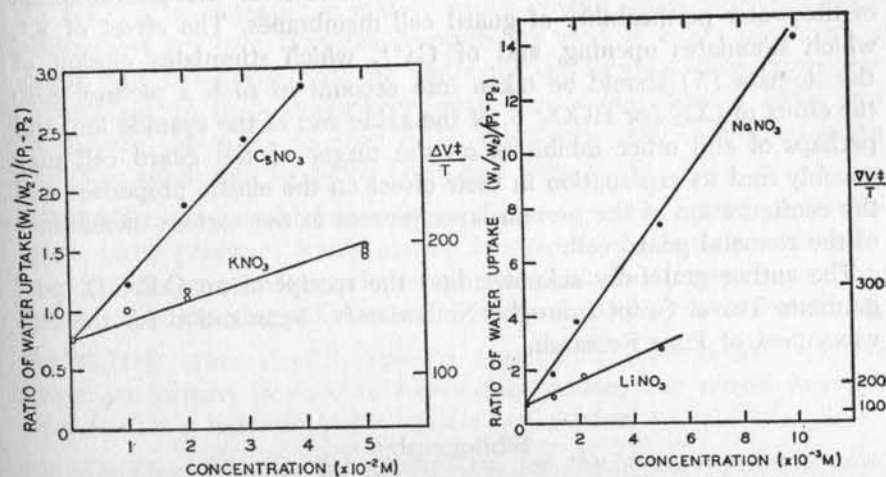


Fig. 5. The effect of  $Cs^+$ ,  $K^+$ ,  $Na^+$ , and  $Li^+$  ions on the ratio of water uptake per 10 cm Hg difference in suction tension (obtained from the curves of Fig. 4 and analogous experiments). The values on the ordinate are calculated from the slopes of the curves of Fig. 4, and from similar data obtained from experiments with the other cations.

TABLE 1

Effect of different cations on the water uptake versus suction curve of bean roots.

The hydration number (Column 2) has been obtained from Helfferich (4.)

The data on the effective ionic radius have been taken from Lange's

Handbook of Chemistry. Column 4 represents the ratio of water

uptake per 10 cm Hg difference in suction from a  $10^{-2}$  M

solution. Column 5 gives the molar concentration at

$\Delta V \ddagger = 200$  ml per mole per  $K^\circ$ .

1	2	3	4	5
Ion	Hydration number	Ionic radius	$(W_1/W_2)/(P_1-P_2)$	Molar concentration at $\Delta V \ddagger = 200$ ml
Li <sup>+</sup>	3.3	0.70	5	$2 \times 10^{-3}$
Na <sup>+</sup>	1.5	0.98	15	$0.6 \times 10^{-3}$
K <sup>+</sup>	0.6	1.33	0.9	$5 \times 10^{-3}$
Cs <sup>+</sup>	0	1.67	1.3	$14 \times 10^{-3}$
Ca <sup>++</sup>	5.2	1.00	6	$1.8 \times 10^{-3}$

In summary, permeability of cell membranes to water may be changed by incorporation of certain molecules into the lipid layers, by an introduced change in the configuration and elasticity of the protein layers of the membrane or by both mechanisms. The specific influence of any chemical applied to the membrane can be studied by measuring the effect of temperature and suction on water transfer through the cell membrane.

The above picture may also serve as a model for the determination of the water permeability of guard cell membranes. The effect of K<sup>+</sup>, which stimulates opening, and of Ca<sup>++</sup>, which stimulates closing of the stomata (5) should be taken into account in such a picture. Also the effect of CO<sub>2</sub> (or HCO<sub>3</sub><sup>-</sup>), of the azide and of the cyanide ion, and perhaps of still other inhibitors on the turgor of the guard cell may possibly find its explanation in their effect on the elastic properties and the configuration of the protein layer present in the various membranes of the stomatal guard cell.

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#### Discussion

GARDNER: Is the rate of water uptake in your experiments higher or lower than the normal transpiration rate and do you know how long the relation between water uptake and suction is logarithmic?

KUIPER: In general the rate of water uptake under suction of one atmosphere is lower than the transpiration rate of intact plants on a sunny day. In tomato roots the water uptake versus suction curve was logarithmic up to 3 atmospheres according to the data of Mees and Weatherly (Mees, G. C., and P. E. Weatherly. 1957. The mechanism of water absorption by roots. Proc. Roy. Soc. B 147: 367).

KRAMER: In Lopushinsky's experiments on water uptake of tomato roots under pressure, water uptake increased curvilinearly with pressure up to approximately 15 pounds per sq. inch. Above this pressure tension, a linear relation between water uptake and pressure was found.

RAWLINS: One should expect a logarithmic relation until all the pores are opened by suction. Above this suction, one should expect a linear relation between water uptake and suction.

GARDNER: What is the explanation for the water uptake at zero suction?

KUIPER: Probably its mechanism is osmotic. Ions probably decrease water uptake at zero suction by means of an effect on the osmotic activity of the root environment.



ZUCKER: In animal cells it is known that  $\text{Na}^+$  and  $\text{K}^+$  strongly influence the ATPase activity in the cell membranes.  $\text{Li}^+$  has a very specific effect on water uptake and on the respiratory pattern of potato disks. How far do ions affect water uptake more in a biochemical sense rather than in a biophysical sense such as you have presented for your experiments?

KUIPER: In my opinion it is not very useful to separate physical and chemical aspects of water uptake as affected by the permeability of the membrane. If one wishes, one can speak about the chemistry of weak bonds (hydrogen bonds).

## Resistance to Water Flow in the Transpiration Stream

By Stephen L. Rawlins

In 1948, van den Honert (5) stated:

It was Gradmann's idea [4] to apply an analogue of Ohm's law to . . . water transport as a whole. It may be applied here because it is applicable, not only to an electric current, but to any process where its velocity is directly proportional to a potential difference. This is the case in a current of water through capillaries as well as in a diffusion process.

Reasoning that for steady state the current of water would be the same through all segments of the transpiration stream, van den Honert proposed an equation similar to Ohm's law for water transport through the plant. Recently Slatyer (18) included water transport through the soil phase and expressed the steady state water current ( $v$ , gm/sec) through the whole soil-plant-atmosphere system by the equation

$$v = \frac{\Delta\psi_s}{R_s} = \frac{\Delta\psi_r}{R_r} = \frac{\Delta\psi_x}{R_x} = \frac{\Delta\psi_l}{R_l} = \frac{\Delta\psi_g}{R_g} . \quad (1)$$

Here  $R_s$ ,  $R_r$ ,  $R_x$ ,  $R_l$ , and  $R_g$ , are the resistances to water transport in the soil, root, xylem, leaf cells, and gaseous phases; and the  $\Delta\psi$  symbols represent the drop in water potential across each resistance. If, as van den Honert said, current is proportional to potential difference, then the  $R$ 's are constants. As used here,  $\psi$  is interchangeable in concept with diffusion pressure deficit in most instances where the latter is used. However, the two terms differ dimensionally and are opposite in sign. (For a precise definition of water potential see Taylor and Slatyer (20).)

Ray (14) pointed out that van den Honert's assumption that diffusion is directly proportional to potential difference is clearly in error. That this is true can be seen from the following. It is an experimental fact that vapor diffuses in air according to Fick's law, which can be written for one-dimensional diffusion as

$$v = -D^* \frac{\partial \theta}{\partial x} . \quad (2)$$

Here  $\theta$  is the water vapor concentration and  $D^*$  ( $\text{cm}^4/\text{sec}$ ) is the diffusivity of water vapor in air for the total cross-sectional area through which diffusion occurs. This change from the more commonly used  $D$  ( $\text{cm}^2/\text{sec}$ ) results from the use of current (gm/sec) on the left of equa-

tion 2 rather than the usual flux ( $\text{gm/sec cm}^2$ ). Equation 1 for the vapor phase (which can also be written in the form

$$v = -K_g \frac{\partial \psi}{\partial x}, \quad (3)$$

where  $K_g = l/R_g$  and  $l$  is the length of the path) could validly describe water transport only if  $\psi$  and  $\theta$  were linearly related. But in the vapor phase  $\psi$  and  $\theta$  are related non-linearly by

$$\psi = \frac{RT}{M} \ln \frac{\theta}{\theta_0}, \quad (4)$$

where  $R$  is the gas constant,  $T$  is the absolute temperature, and  $\theta_0$  is the vapor concentration at  $\psi = 0$ . Therefore, as it now stands, equation 1 is invalid for water transport in the vapor phase.

Ray (14) states:

The expectation of proportionality between  $S' [\psi]$  difference and transpiration rate is a clear example of confusion between rate and equilibrium parameters, which has serious consequences. It would mean, for example, that transpiration rate would increase without limit into perfectly dry air, whereas in fact it should never be more than about twice the rate found in air of 50 percent relative humidity (other conditions constant). ... A kinetically reasonable expression for transpiration rate must be based on diffusion coefficients and water vapor pressures not on  $S' [\psi]$ . Suction force of the air  $[\psi]$  is useful only in evaluating states of equilibrium, that is, of zero transpiration.

Slatyer (18), realized the failure of equation 1 in the vapor phase and stated:

It must be appreciated that the potential difference in the gaseous phase is in reality the vapour pressure gradient from the evaporating surface within the leaf to the external air. Because of the non-linearity of the water potential/vapor pressure relationship, the magnitude of the resistance in this phase is not usually as great as may appear.

In a later paper Slatyer and McIlroy (19) made this substitution of vapor pressure difference for water potential difference in the last part of equation 1. Because, as is seen from equation 4, differences in water potential do not equal differences in vapor concentration (or vapor pressure), vapor concentration can be substituted for water potential only if a new resistance term is also substituted for  $R_g$  in equation 1. Since this changes the equation to a diffusion equation (similar to equation 2), the new resistance term ( $R_g(\text{diff})$ ) must be a diffusion resistance having units of  $\text{sec/cm}^2$  rather than  $\text{cm}^2/\text{gm}\cdot\text{sec}$  as the remainder of the resistance terms in equation 1 have. Obviously these dimensionally different quantities should not be compared directly.

Therefore, if we wish to compare the magnitudes of the resistances in the different segments of the transpiration stream, we must express gradients in the same dimensions throughout. Yet, in view of Ray's (14) comments quoted above, this seems impossible. Although a diffusion equation can be used to describe water transport in the vapor phase, it cannot be used, for instance, for transport in full xylem vessels. The water-concentration gradient is zero in full vessels because water concentration is constant. Yet flow still occurs in response to pressure gradients. Therefore, the diffusion equation won't serve for any segments of the stream where saturated flow occurs.

Ray's objection to the use of a potential equation for rate processes applies where the resistance term in equation 1 is chosen to be the constant  $R_g$ . If this term is permitted to vary, the appropriate function,  $r_g$ , can often be determined experimentally, and then equation 1 can be employed for the rate process for which this function was determined. For the diffusion process the function  $r_g$  can be evaluated directly from the experimental result stated in Fick's law (equation 2). Since a unique relation between  $\psi$  and  $\theta$  exists (equation 4) for water vapor in air, by the chain rule for differentiation

$$\frac{\partial \theta}{\partial x} = \frac{\partial \theta}{\partial \psi} \frac{\partial \psi}{\partial x}. \quad (5)$$

Substituting for  $\partial\theta/\partial x$  in equation 2 from equation 5 yields equation 3 where  $k_g$ , the conductivity function, is given by

$$k_g = D^* \frac{\partial \theta}{\partial \psi}. \quad (6)$$

For diffusion of water vapor in air, the  $\partial\theta/\partial\psi$  term is evaluated from equation 4 to yield

$$k(\psi)_g = \frac{M\theta_0}{RT} e^{M\psi/RT}. \quad (7)$$

A more useful form of  $k$ , written as a function of  $\theta$  rather than  $\psi$ , is

$$k(\theta)_g = \frac{D^*M}{RT} \theta. \quad (8)$$

At any instant, the resistance for a length  $dx$  is  $dr_g = (1/k)dx$ . The total resistance over the segment of length  $l$  corresponding to the decrease in potential  $\Delta\psi_g$  is

$$r_g = \int_0^l \frac{dx}{k} = \frac{RT}{D^*M} \int_0^l \frac{dx}{\theta}. \quad (9)$$



Where  $\theta$  is a linear function of  $x$  of the form  $\theta = \theta_s + bx$ ,

$$r_g = \frac{RT}{D^{\circ}Mb} \ln \frac{\theta_s + bl}{\theta_s} \quad (10)$$

Here  $\theta_s$  is the vapor concentration at the source of the diffusing vapor and  $b$  is the slope of the vapor-concentration *vs.* distance line.

If  $r_g$  is used in equation 1 in place of the constant  $R_g$ , we obtain the same, correct water transport rates as are predicted by Fick's law. This, of course, has to be true because by using the  $r_g$  function, equation 1 reduces to Fick's law. The important advantage of using  $r_g$  and the potential difference rather than  $R_g$  ( $\text{diff} = 1/D^{\circ}$ ) and the concentration difference is that  $r_g$  is dimensionally equivalent to the other resistance terms in equation 1. Therefore, resistances can be compared throughout the entire transpiration stream.

The difficulty of the lack of proportionality between current and potential difference noted by Ray (14) is thus avoided by replacing the constant  $R_g$  in equation 1 with the function  $r_g$ . The equation no longer predicts infinite transpiration into dry air; rather it predicts transpiration rates identical to those obtained with the diffusion equation. Ray's (14) conclusion that water potential is not applicable to prediction of rate is true if resistance is assumed to be constant. Where the resistance function is known, water potential can predict rate.

An outstanding example of the use of water potential and a variable resistance in predicting a rate is that of unsaturated flow of soil water. It is well known that the conductivity of unsaturated soil decreases markedly as water potential decreases. There is also evidence that in some soils conductivity is not even a unique function of potential (13). This can be interpreted as a dependence of conductivity on the rate of flow as well as on potential. Therefore, it is clear that a constant  $R_s$  in equation 1 is inappropriate, and instead  $r_s$  depends upon  $\psi$  and in some cases on  $v$  as well. (This resistance function generally depends also upon temperature, salt content, etc. Also its value during wetting differs from that during drying. For purposes of this discussion these factors are considered to be held constant.) Where water flow through soil is treated by means of a potential equation having such a variable resistance function, as was the case for water transport in the vapor phase, it is not appropriate to state that water current is proportional to the difference in water potential through the soil. Current varies with potential in a more complicated manner, determined by this new resistance function. The effect of water potential on resistance is so great in most unsaturated soils that doubling the potential gradient by decreasing the potential on one side of a soil column usually does not increase flow. For instance, from the data of Moore (11) we see that

increasing the tension on an Oakley sand from 40 to 80 cm of water, increases the resistance to water flow about 30,000 times. Here doubling the water potential gradient by decreasing the potential on the dry end of the column not only fails to double the flow, but decreases it drastically. If, on the other hand, the potential gradient were doubled by raising the potential on the wet end of the soil column, the flow would have more than doubled because in addition to doubling the gradient the resistance would have decreased several thousand fold. Thus, even for first approximations it is not possible to state that changes in flux are proportional to changes in potential, because the change may be in the opposite direction.

Because  $\psi$  and  $\theta$  are related by the characteristic curve for a soil, by use of the transformation technique used above, flow of water in soil can also be expressed in terms of water concentration and diffusivity. In recent years this type equation has been used with greater frequency. However, rather than being constant (as is the  $D^{\circ}$  in equation 2) the diffusivity for unsaturated soil is a function of other equation variables, as is the conductivity or resistance.

It is possible, therefore, to predict water transport in both the soil and gaseous phases of the transpiration stream from either water potential gradients and a resistance function, or water concentration gradients and a different resistance function. Choice will depend upon one's objective. If the diffusion of water vapor from a leaf is the only process to be considered, equation 2 is the logical choice because the resistance function is constant. But if resistance to water transport in the gaseous phase is to be compared with resistances in other segments of the transpiration stream, equations must be written in terms of water potential (Eq. 3) throughout to preserve dimensional consistency.

Having seen that neither the resistance in the gaseous phase nor in the soil phase of the transpiration stream is constant, let us consider those resistances within the plant. Apparently, van den Honert (5) considered flow of water through the plant to be equivalent to flow through completely filled capillary tubes. If this were the case those parts of equation 1 dealing with the plant would not require modification because flow is essentially proportional to the potential gradient for such tubes. However, observations of variability in transport-resistance terms for the plant make it clear that this analogy is an over-simplification.

Slatyer (18) concludes that the natural factors that affect root permeability are root hydration, temperature, and aeration. Solutes in the soil water are also known to affect root permeability. In equation 1, temperature, aeration, and solutes can enter only as boundary conditions since they are independent variables and must be held constant for flow processes described in terms of  $\psi$ . However, root hydration

cannot be held constant for such processes because it is a function of water potential. Slatyer (18) points out that root permeability generally decreases with decreasing potential, but that there are exceptions. He further states that "... tensions across the root resulting from the influence of transpiration must be interpreted differently from the effect of a general decrease in the water potential of the root tissue associated with decreasing soil water potential." If this is the case, then not only is root resistance a function of water potential, but the function differs depending on how the root water potential arises.

In the preceding paper of this conference Kuiper presented data which not only showed the dependence of root resistance on water potential, but also indicated hysteresis in this resistance function. Obviously,  $R_r$  in equation 1 cannot be treated as a constant. Thus, for water flow through roots, as was true for water flow through the gas and the soil phases of the transpiration stream,  $R_r$  must be replaced by the function,  $r_r$ . Here again it is erroneous to assume that current is proportional to difference in water potential.

The resistance to water flow through the conducting elements of the xylem,  $R_x$ , is usually assumed to be constant. Provided water flows through continuous, rigid channels which remain filled with water at all values of  $\psi$  encountered, this assumption is probably valid. If, however, the xylem elements change geometry either by partially collapsing or filling with gas under stress,  $R_x$  would not be constant but would have to be replaced by the appropriate function of  $\psi$ . There is evidence that tree trunks decrease in water content during periods of high water stress, evidently as a result of emptying of some of the larger vessels. To the extent that this occurs,  $R_x$  also must be considered to be a function,  $r_x$ , of water potential.

The remaining resistance in equation 1, the resistance to water flow through the leaves ( $R_l$ ), is of special interest because of its relation to the subject of this conference. It is convenient to divide this resistance into two portions: (1) that which, in common with  $r_s$ ,  $r_r$ , and  $r_x$ , affects the supply of water to the leaf cells (supply resistance), and (2) that which affects the loss of water from these cells to the atmosphere (loss resistance). Implicit in this division is the assumption that all water passes through leaf cells.

#### SUPPLY RESISTANCE IN LEAVES<sup>1</sup>

The supply resistance in leaves (although given little attention in the literature) usually is considered to be negligible. Recently, how-

<sup>1</sup> Data in this section were originally presented before Div. I, Soil Science Society of America, Ithaca, N.Y., Aug. 22, 1962. Permission to publish them here has been granted.

ever, I have made some observations on tobacco plants which indicate there are circumstances where it cannot be ignored.

In one experiment a well-watered tobacco plant with all but two leaves removed was placed in a controlled environment chamber. (This chamber is described by Moss in the paper which follows.) To prevent transpiration, the bottom leaf was enclosed in a plastic bag and shaded from the lights. By lowering the relative humidity, water stress on the upper leaf was progressively increased until the leaf wilted. Samples of leaf tissue were then taken and water potential was determined by the thermocouple psychrometer technique of Richards and Ogata (15). Subsequently these same samples were quickly frozen in a dry-ice alcohol mixture, thawed, and the water potential of the exposed cell contents was again measured with the same psychrometer (Ehlig, 3). This is referred to as osmotic potential,  $\pi$ , since in the broken cells, solute concentration is assumed to be the only component of the water potential.

Figure 1 shows the position of the leaves on the stem and the areas from which samples were removed. The data in the inset show  $\psi$ , the water potential of the fresh tissue (equivalent to DPD);  $\pi$ , the osmotic potential of the killed tissue (equivalent to osmotic pressure); and TP, the theoretical turgor pressure (difference between  $\psi$  and  $\pi$ ). Since no water was moving into the lower leaf from the stem, the water potential in the stem of the plant at this point should have been the same as the potential of this leaf, i.e. about  $-3 \times 10^6$  ergs/gm. This is equivalent in magnitude to a tension (negative pressure) of 3 bars or about 3 atmos-

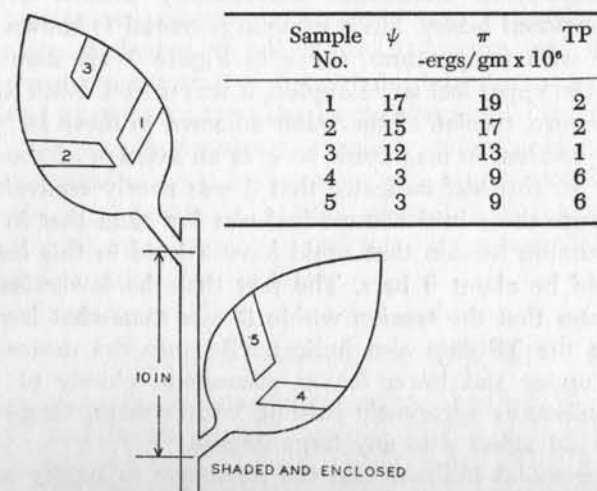


Fig. 1. Diagram of the tobacco plant used for a supply-resistant experiment. Water potential of the fresh tissue,  $\psi$ , osmotic potential,  $\pi$ , and the theoretical turgor pressure, TP, are tabulated for samples taken from the indicated areas on the leaves.



pheres. At the same time the potential in the upper leaf averaged about  $-15 \times 10^6$  ergs/gm. This drop in potential equivalent to about 12 bars between the stem of the plant, at the juncture of the lower leaf, and the upper leaf indicates that significant resistance to water flow exists between these points.

Subsequent observations indicate that a major part of this resistance occurs within the leaves. For instance, shaded leaves of tobacco commonly remain turgid, while adjacent leaves on the same plant exposed to direct sunlight wilt. Since adjacent leaves remain turgid, indicating that the water potential in the xylem was not low enough to cause wilting, a major part of the supply resistance to these wilted leaves occurs between the xylem and the leaf cells. Also, I have observed that one half of a tobacco leaf exposed to sunlight can be wilted while the shaded half of the same leaf remains turgid. Differences in water potential equivalent to several bars have been measured between the two halves of such leaves. Thus, the resistance to flow of water within these leaves was sufficient to prevent water potential equilibration.

Tobacco leaves commonly wilt at the margins before the entire leaf wilts. Measurements of stomatal aperture on partially wilted leaves almost always show stomata closed more in the wilted marginal areas than in the turgid areas. This observation also demonstrates supply resistance in the leaves and shows resistance to water flow to leaf margins to be greater than that to tissue closer to the midrib.

The following argument demonstrates that the  $\psi$  in Figure 1 are valid although temperature differences undoubtedly existed between the shaded and exposed leaves. Since osmotic potential is known to change only slightly with temperature, the  $\pi$  in Figure 1 are assumed to be valid. When the upper leaf was sampled, it was wilted. With this absence of turgor pressure, tension of the water adjacent to these leaf cells must have been equivalent in magnitude to  $\pi$ , or an average of about 16 bars. The low TP for this leaf indicates that  $\psi$  was nearly equivalent to this tension. Because the  $\pi$  in the lower leaf was less than that in the upper leaf, the maximum tension that could have existed in this leaf, even at wilting, would be about 9 bars. The fact that the lower leaf was not wilted indicates that the tension within it was somewhat less than this maximum, as the TP data also indicate. Because the measured potentials in the upper and lower leaves correspond closely to the actual hydrostatic pressures apparently existing within them, temperature apparently did not affect  $\psi$  to any large degree.

Other observations indicate that the resistance to supply of water to leaf cells is not constant. For example, leaves excised from plants which have a history of high sunlight and adequate water supply fail to wilt at transpiration rates far in excess of those required to wilt leaves from

plants that have either been in the dark or have been in a wilted condition prior to the experiment. Furthermore, when leaves of the latter type, which wilt at low transpiration rates, are kept for a day or two at high light intensity, but wilting is prevented by moist air, they regain their ability to remain turgid when the air humidity is again lowered and transpiration is rapid. Measurements of the osmotic potential of sap expressed from these leaves before and after this treatment show that the increase in  $\pi$  under the lights is far less than required to explain the differences in turgidity under the two conditions. Therefore, the resistance to water flow to the leaf cells apparently changes in response to some factor influenced by light. The fact that plants grown under wilting conditions in the light respond similarly to those kept in darkness suggests that some product of photosynthesis affects this resistance. Presumably, when the leaves are wilted, photosynthesis is diminished because of stomatal closure.

To some extent this change in supply resistance is not immediately reversible. For instance, many of these leaves failed to recover turgor when transpiration was reduced to about half that required to initiate wilting. Thus, wilting itself appears to increase the resistance. This increase in resistance lasted for several hours at least, and in some cases was observed to last for more than a day.

Intact tobacco plants have been found to behave the same as excised leaves. With intact plants, however, wilting could not be attributed specifically to supply resistance in the leaves because resistance in the soil or in the roots would have had the same effect. The similar behavior of intact plants and excised leaves, however, indicates that wilting which is often attributed to soil or root resistance may sometimes be caused by supply resistance in the leaves.

From these exploratory experiments it is impossible to state the exact nature of this supply resistance, even for tobacco leaves. Certainly, however, its magnitude can be large enough under some circumstances to contribute significantly to wilting. Also, it is not a constant, but, rather, appears to be a function of water potential as well as some other factor dependent upon photosynthesis.

#### LOSS RESISTANCE IN LEAVES

Considerable resistance to water transport exists between the vacuoles of leaf cells and the air outside the leaf. This is born out by the fact that leaf water potential of most plants, even when wilted, seldom drops far below  $-25 \times 10^6$  ergs/gm. If there were no resistance to water loss from the mesophyll cells, potential could remain this high only in an atmosphere exceeding 98% relative humidity at 20° C. The fact that



potential in the leaf is seldom less than  $-25 \times 10^6$  ergs/gm even in dry air proves that the loss resistance in leaves is high.

Assuming that the vapor in substomatal cavities is essentially in equilibrium with the surrounding cells, van den Honert (5) predicted that, under specified evaporative conditions, the stomata control the transpiration. The factors affecting stomatal aperture have been treated by Zelitch in a previous paper in this conference, and will be discussed further by Moss in the paper which follows. Here we need only recall that in addition to factors such as carbon dioxide concentration, temperature, and light intensity which can be held relatively constant during transpiration experiments, stomatal aperture is also markedly affected by water potential in the leaf. Thus, stomatal resistance cannot be considered constant.

The remainder of this section is devoted to non-stomatal loss resistance. Numerous investigators have questioned whether vapor is in equilibrium between substomatal cavities and the surrounding cells. Considering the large area of exposed cell wall in each of these cavities compared to the small diameter of the stomatal opening, equilibrium would be approached if these cell walls were moistened with solution in equilibrium with the vacuoles. Observations of several investigators cited by Kuiper (7, p. 33) as well as those made by Lewis (8, 9), however, indicate that not only are these cell walls not moist, but they appear to be hydrophobic, as though they were cutinized. These observations introduce the possibility of a major potential drop through cell walls.

At least three mechanisms could account for the required changes in the permeability of the mesophyll cell walls necessary to affect transpiration. One of these could be an actual chemical change of the cell wall similar to that postulated by Kuiper, in the previous paper, for root cell membranes. Another could be partial dehydration of the cell walls—the so-called “incipient drying” referred to in standard texts of plant physiology. Still another possibility was suggested by a series of experiments conducted by Boon-Long (2) on evaporation through membranes.

Boon-Long compared evaporation from pure water and 1.0 molar sugar solutions in open petri dishes and in dishes covered by collodian membranes in contact with the solutions. In the open dishes evaporation from the sugar solutions was less than that from pure water by 3.9%, about the amount that the sugar changed the vapor pressure difference between the solutions and the surrounding air. However, where evaporation took place through collodian membranes, the relative reduction in evaporation caused by the sugar solution was 10.6% in still air and 61.7% in moving air. Shreve (17) has reported similar results for evaporation through a parchment membrane. Boon-Long suggests that the large re-

duction in evaporation of the solution through membranes results from concentration of solute in the pores or at the surface of the membranes. Therefore, evaporation was actually taking place from a solution much more concentrated than 1 molar. I have repeated Boon-Long's experiments, and have verified the presence of high sugar concentrations at the membrane surface.

The equilibrium concentration of solute at the surface of such a membrane depends on the difference between the velocity of flow of solution to the surface and the rate of diffusion of solute back into the solution. High evaporation rates will increase surface concentration, as indeed Boon-Long's results show. Therefore, if this phenomenon occurs at the surface of mesophyll cells, the apparent resistance across mesophyll cell walls will increase as the transpiration rate increases. Because cell walls and collodian membranes have similar porosity the phenomenon observed by Boon-Long could occur in leaves.

Regardless of the mechanism, evidence does exist that transpiration varies independently of stomatal aperture at least under some circumstances (2, 5, 9, 10, 21). The major uncertainty in many experiments is the evaluation of the absolute stomatal resistance. Since stomatal aperture also varies with water potential, it is sometimes difficult to separate its effects from changes in mesophyll cell membrane permeability resulting from the same cause. By the use of phenylmercuric acetate as a leaf spray, Shimshi (16) closed stomata of potted corn seedlings to varying extents independently of water potential. The soil in the pots was brought to 24%, 18% (field capacity), 13%, and 10%. In this soil the

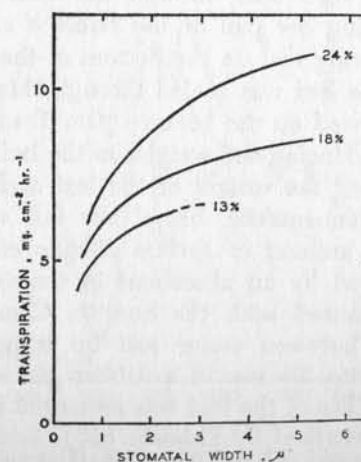


Fig. 2. Transpiration as a function of average stomatal width for corn seedlings at three soil moisture levels. Stomata were closed to varying extents with a phenylmercuric acetate leaf spray. (From Shimshi, 16.)

water content at 15 bars tension was 6.5%. By weighing, he then determined water loss under controlled conditions. Stomatal condition was determined by means of a porometer and silicone rubber impressions. His observations of transpiration rate as a function of stomatal aperture are shown in Figure 2. The curves in this figure show that transpiration was not a function of stomatal width alone, but varied with soil water content as well. Certainly something in addition to stomata affected transpiration. Presumably differences in soil water content caused differences in leaf water potential through changes in the unsaturated hydraulic conductivity of the soil or root resistance. This in turn affected some non-stomatal loss resistance. I determined osmotic potential of expressed leaf sap from some of these experimental plants and seldom found less than  $-10 \times 10^6$  ergs/gm. Osmotic potentials this high would not lower vapor pressure of the cells as a whole enough to affect transpiration significantly.

Shimshi clearly demonstrated non-stomatal control of transpiration because he closed stomata to different degrees independently of water potential and because he observed stomata directly. I attempted to investigate this same phenomenon by eliminating stomatal control entirely by maintaining stomata open rather than by closing them. This was done by conducting a transpiration experiment in carbon dioxide-free air. A large sugar beet leaf was supported by wire mesh in the controlled environment chamber and was provided with air-free water from a burette through a length of rubber tubing. The wire mesh was attached by a rod to a disk, which in turn rested on a balance below the chamber. An air-tight seal through the bottom of the chamber was achieved by raising the pan of the balance and forcing the disk upward against an O-ring seal on the bottom of the chamber. The tube supplying water to the leaf was sealed through this disk to the burette which was also supported on the balance pan. Transpiration was measured periodically by reducing the weight on the balance (thus lowering the disk) and recording the weight of the leaf and water supply. This opened the system momentarily, but if the fan was turned off previously, only a slight amount of carbon dioxide entered the chamber. This was soon removed by an absorbent in the system. Water-uptake by the leaf was measured with the burette. Changes in leaf weight were the difference between water lost by transpiration and water gained from the burette. By use of a rubber glove in the door of the chamber, air permeability of the leaf was measured inside with a porometer similar to that described by Shimshi (16). Leaf air permeability is expressed as the  $\mu$  moles of air passing through a  $\text{cm}^2$  of leaf in one second, for every mm Hg air pressure across the leaf.

Figure 3 shows transpiration rate (TR), relative water content (RWC),

and air permeability of the leaf (P) as a function of time. After 60 minutes in darkness, the leaf was illuminated at 5,000 foot-candles in order to open the stomata wider. Unexpectedly, for a brief period after this, both air permeability and transpiration rate were reduced significantly. At the same time the relative water content, which had been decreasing slowly, increased slightly because of the decreased water loss. At about 100 minutes, air permeability and transpiration increased abruptly indicating stomatal opening. As the leaf continued to lose water, both leaf air permeability and transpiration rate decreased. At 230 minutes, transpiration reached the rate that existed immediately prior to illumination; the air permeability of the leaf, however, was more than 20 times as great as it was previous to illumination. Thus, if air permeability of the leaf is a unique function of stomatal aperture, some factor in addition to stomatal aperture influenced transpiration. It is interesting to note that if we were to consider the curves after 100 minutes only, our obvious conclusion would be that transpiration is controlled by stomatal aperture, which in turn depends upon the water content of the leaf. However, since a different relation between air permeability and transpiration rate was found earlier, we must conclude that some other factor was involved.

The factor is not leaf temperature for the lights could only increase

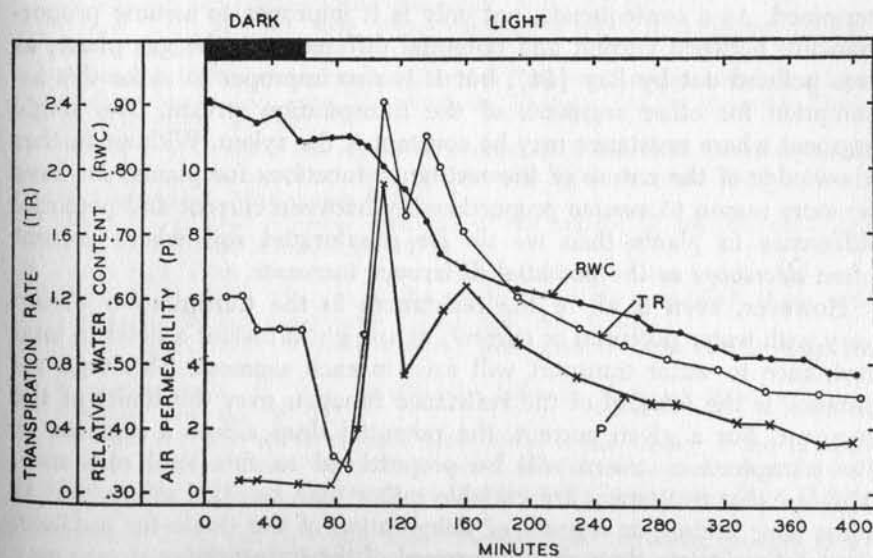


Fig. 3. Transpiration rate (TR,  $\text{gm min}^{-1} \text{mm Hg vapor pressure deficit}^{-1} \times 10^3$ ), relative water content (RWC, ratio of leaf water content to turgid leaf water content), and leaf air permeability (P,  $\mu \text{ moles sec}^{-1} \text{mm Hg air pressure difference}^{-1}$ ) as functions of time for an excised sugar beet leaf.



leaf temperature. This increase would increase the vapor pressure gradient from the leaf to the air since air temperature did not change. Therefore, the lights should increase transpiration at any given stomatal aperture, rather than decrease it. Neither could the direct effect of the lowering of vapor pressure inside the leaf cells account for this reduction in transpiration at fixed stomatal aperture. The water potential of this leaf was  $-25 \times 10^6$  ergs/gm at less than 50% relative water content, which would cause an insignificant vapor pressure lowering. The effect of water potential on the permeability of cell walls, or the solute concentration at the external surface of mesophyll cell walls appears to be a likely source of the resistance. Wherever this resistance is centered, it cannot be considered constant. It is a function of water potential at least, and may vary with transpiration rate as well.

Both supply resistance and loss resistance in leaves are variable. Therefore, here again the constant,  $R_1$ , in equation 1 should be replaced by an experimentally determined function,  $r_1$ .

### CONCLUSION

Evidence presented here demonstrates that most of the resistances in the van den Honert equation (equation 1) for the transpiration stream are not constant, but are functions that must be experimentally determined. As a consequence, not only is it improper to assume proportionality between current and potential difference in the gas phase, as was pointed out by Ray (14); but it is also improper to make this assumption for other segments of the transpiration stream. The single segment where resistance may be constant is the xylem. Without further knowledge of the nature of the resistance functions for plants, we have no more reason to assume proportionality between current and potential difference in plants than we do for unsaturated soil—where current often *decreases* as the potential difference *increases*.

However, even if all of the resistances in the transpiration stream vary with water potential or current, at any given instant a definite total resistance to water transport will exist in each segment. This total resistance is the integral of the resistance function over the limits of the segment. For a given current, the potential drop across a segment of the transpiration stream will be proportional to this total resistance. The fact that resistances are variable rather than constant means that at some later instant, as a result of dehydration of the tissue for instance, the total resistance through any segment of the transpiration stream may be drastically different from that presently existing. For the same current, however, this change in resistance merely results in a proportional change in potential difference across the segment. Thus, van den Hon-

ert's basic conclusion that since the greatest drop in water potential usually occurs across the gas phase this is the site of highest resistance, apparently remains intact. Conclusions to the contrary (1) are usually based on the inappropriate comparison of dimensionally different resistances.

The fact that resistance in the gas phase may be many times greater than all others combined does not mean that supply resistances are not important. Although resistance to water flow in roots, for instance, may not control transpiration directly, it could indirectly control transpiration by decreasing the water potential in the leaves, which in turn causes major increases in loss resistance. In this sense the plant may behave similarly to a high-gain amplifier circuit, with small changes in supply resistance amplified to large changes in loss resistance.

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### Discussion

BECKMAN: Why not the proposition that the stomata take over when the water supply drops to a certain dangerous level?

RAWLINS: In the experiment I presented, the relative water content of the leaves was down to 50 per cent and the stomata were evidently still open. This was, of course, in carbon dioxide-free air, and thus points out that stomatal aperture is a function of carbon dioxide concentration as well as leaf water content. At normal carbon dioxide concentration these stomata would have been closed.

GARDNER: I am reluctant to give up the role of stomata. It seems to me that in your data, as well as those of Shimshi, the boundary layer resistance is at least as large as that of the stomata. This is evident in Fig. 3 of Shimshi's paper because the curves do not come into the origin, but have a positive intercept. Modest differences in this boundary layer due to differences in treatment would be enough to shift the curves. It is not necessary to invoke anything other than the resistance of the boundary layer to explain the differences you get.

RAWLINS: I suspect the boundary layer resistance was smaller in my experiment than in Shimshi's because of the much more rapid air exchange. In my system sufficient air was passed through the chamber to completely exchange it 100 times a minute. I have no measure of boundary layer resistance, however. In my analysis I assumed constant evaporation conditions from the leaf, which, of course, would be in error if this boundary resistance were not constant. To explain my data, and those of Shimshi, one would have to show this boundary layer resistance to be a function of leaf water potential.

GARDNER: This does not disturb me.

RAWLINS: If this can be shown, then it is, of course, a possible explanation.

van OVERBEEK: Old-fashioned plant physiologists grew up with the idea of cuticular transpiration and stomatal transpiration. This factor X, where you find that you can eliminate stomatal transpiration, is per-

haps cuticular transpiration. All that remains is either the membrane under the cuticle or the cuticle itself. The cuticle is very responsive to water content. The wax platelets are "floating" in a hydrophylic matrix. When this matrix dries out, the wax platelets come together and form a barrier.

RAWLINS: My data certainly would not eliminate this possibility.

WITWER: We have evidence that the surfaces of mesophyll cells in the inner stomatal cavities are covered by a cuticular-like membrane. Factors which affect the permeability of that membrane will affect transpiration losses. It goes back to the old idea we have had concerning foliar absorption. Whether it is from the innerstomatal cavities or from the external leaf surfaces, practically all absorption is cuticular. There is a good deal of evidence that the permeability of these membranes is independent of stomatal apertures. There is evidence that penetration is dependent to some extent on the mechanism Dr. van Overbeek mentioned. There are also a great number of other factors that affect this penetration, however.

## The Effect of Environment on Gas Exchange of Leaves

By Dale N. Moss

The Fick equation for diffusion has been used to describe the exchange of gases between a leaf and its surrounding atmosphere. Although a leaf-air system usually contains turbulent as well as still air, the result of turbulent transfer is in effect to reduce the length of the diffusion path between given concentrations of the diffusing gas in the leaf and in the atmosphere. Thus, the diffusion equation fits data for systems with both turbulent and still air (1, 3).

A schematic representation of a leaf-atmosphere system is shown in Fig. 1. The symbols chl, I, and A refer to positions where the conditions for the equation for transpiration  $T$  and photosynthesis  $P$  are found. Chl refers to the chloroplast, the site of  $\text{CO}_2$  utilization. It is the sink for  $\text{CO}_2$  during photosynthesis. The symbol I refers to the position of an air-water interface inside the leaf, where the concentration of water vapor is usually taken as the saturation vapor concentration for the temperature of the leaf. The symbol A refers to the atmosphere or the position where the fixed concentrations of  $\text{CO}_2$  and  $\text{H}_2\text{O}$  are found.

In this discussion, we will be concerned only with conditions where water is lost from the leaf (positive  $T$  in Equation 1), and where carbon dioxide is absorbed by the leaf (positive  $P$  in Equation 2). Under special conditions, of course, either of these quantities could be negative.

Transpiration,  $T$ , is directly proportional to the difference in concentration between the atmosphere and the air-water interface in the

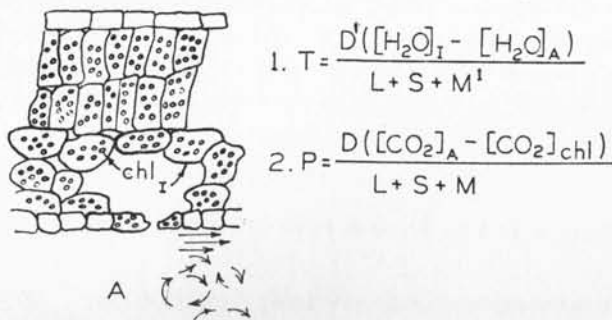


Fig. 1. The diffusion equations for transpiration and photosynthesis and a schematic drawing of a leaf-atmosphere system. For a definition of symbols see the text.

leaf, and inversely proportional to the quantity  $L$  plus  $S$  plus  $M'$ . As discussed later, for calculations in this paper,  $M'$  is assumed to be zero.  $D'$  is the diffusion coefficient for water vapor in air. In the form in which Equation 1 is written  $L$  and  $S$  represent the effective diffusion path lengths through the air and through the stomata respectively. They do not represent any real quantities, but  $L/D'$  is a measure of the resistance of the air to water vapor flow and  $S/D'$  the resistance of the stomata. When discussing the movement of carbon dioxide and water vapor which have different diffusion coefficients, however, this form is convenient because the numerical values of  $L$  and  $S$  are then the same for Equations 1 and 2.

The process may be visualized by following the path of a water molecule from I to A in Fig. 1. Water vapor moves from I toward A by diffusion along a concentration gradient through the still air of the substomatal cavity, diffuses through the stomatal opening, and passes across a laminar flow layer of air near the leaf surface represented by the straight arrows. Then by turbulent transfer the vapor moves through the turbulent air further from the leaf out into the atmosphere. This part of the process is summed in the quantity  $L$ , the distance by which I and A would be separated for the same flux to occur in still air and unobstructed path.  $S$  is a measure of the obstruction caused by the epidermal layer of the leaf. The value of  $S$  can be changed by varying the size of the stomatal openings. Thus  $S$  can be evaluated independently of  $L$ .

The same path must be followed in the opposite direction by carbon dioxide entering the leaf. Thus,  $S$  and  $L$  are the same for both processes  $P$  and  $T$ . However,  $\text{CO}_2$  must also dissolve in the water at the cell surface and diffuse through the cell solution to the site of utilization. The magnitude of this additional barrier is summed in the quantity  $M$ .

$T$ ,  $P$ ,  $[\text{H}_2\text{O}]_A$  and  $[\text{CO}_2]_A$  can be measured directly.  $D'$  and  $D$  are known.  $L$  has been estimated for model systems as a function of wind speed and leaf geometry.  $S$  has been estimated as a function of the size of the stomatal apertures.  $[\text{H}_2\text{O}]_I$  is commonly taken as the saturation vapor concentration at the temperature of the leaf. (Gaastra (p 45) discusses in detail the validity of this assumption. For many experiments, the assumption is probably valid. However, Rawlins' (7) experiments show that serious errors would result under certain conditions. In that case, Equation 1 should contain another factor  $M'$  in the denominator corresponding to  $M$  in Equation 2 and representing the apparent pathlength for vapor through the cell walls and cell membranes. In our discussion we will assume any factor  $M'$  to be zero.)

Neither  $M$  nor  $[\text{CO}_2]_{chl}$  can be measured directly. Therefore, it is not possible to place an absolute numerical value on either  $M$  or  $[\text{CO}_2]_{chl}$



separately.  $M$  is really a residual quantity representing the effect of all unknown resistances to  $\text{CO}_2$  flux inside the cell. To be strictly correct Equation 2 should contain a coefficient of diffusion for  $\text{CO}_2$  in water as well as  $D$  in air. However, since we do not know the rate at which  $\text{CO}_2$  dissolves in the cell solution, the pathlength of  $\text{CO}_2$  diffusion inside the cell, or the concentration of  $\text{CO}_2$  in solution at the end of the path, it is not realistic to attempt to describe the process inside the cell in detail. Rather we use the term  $M$  to indicate that resistance to flow of  $\text{CO}_2$  inside the leaf does occur although we are unable to determine the exact nature of the resistance.

$[\text{CO}_2]_{\text{chl}}$  has been equated with  $[\text{CO}_2]_A$  in a closed system in which the plants have removed all the  $\text{CO}_2$  possible. Since under these circumstances there is no net flux of  $\text{CO}_2$  into the leaf, it has been postulated that the  $\text{CO}_2$  concentration at the chloroplast is then the same as the concentration in the atmosphere. This would be true if there were no sources of  $\text{CO}_2$  inside the cell. However, cell respiration produces  $\text{CO}_2$  and the respiratory sites, for example, mitochondria, are not necessarily the same as the sites of  $\text{CO}_2$  utilization. Therefore, there must be a net flow of  $\text{CO}_2$  inside the cell from source to sink, even though there is no net flow of  $\text{CO}_2$  from the atmosphere to the sink. The situation is analogous to differences of electrical potential in a D.C. circuit as illustrated in Fig. 2. The concentration of  $\text{CO}_2$  in the atmosphere in the closed system at equilibrium corresponds to the electrical potential at point A and  $[\text{CO}_2]_{\text{chl}}$  to the potential at chl or the sink. The resistance  $R_3$  between A and chl corresponds to  $M$  in Equation 2. The potential

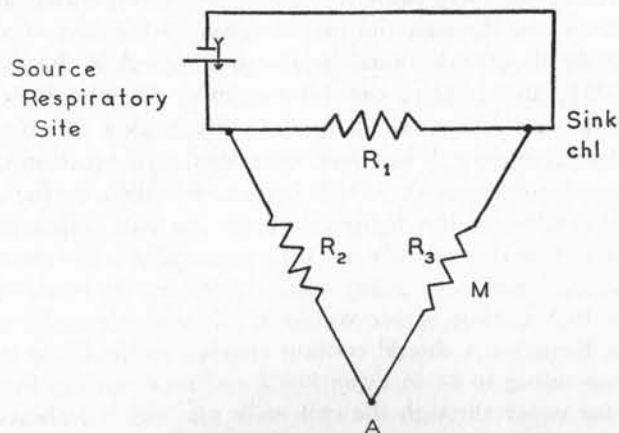


Fig. 2. A D.C. circuit analogous to the  $\text{CO}_2$  path between a respiratory source and photosynthetic sink inside a leaf cell.

at A depends both on the potential difference between the source and sink and on the relative values of the resistances  $R_1$ ,  $R_2$ , and  $R_3$ . The analogy to carbon dioxide concentrations in the leaf and atmosphere is obvious. Thus, even at equilibrium in the closed system we cannot equate  $[\text{CO}_2]_{\text{chl}}$  with  $[\text{CO}_2]_A$ .

Although numerical values for all of the quantities in Equations 1 and 2 cannot be assigned with certainty, it is useful to examine the carbon dioxide and water vapor fluxes as physical processes which can be described by the equations. First, for transpiration,  $T$  can be changed by varying the water vapor concentration gradient. In a properly controlled system  $[\text{H}_2\text{O}]_A$  can be set at any desired value corresponding to a relative humidity from 0 to near 100 per cent.  $[\text{H}_2\text{O}]_I$  can be varied by changing the temperature of the leaf. That is all that can be done with the top of Equation 1. The only other way of changing  $T$  is to change  $L$  (turbulence) or  $S$  (stomatal opening). (This is true as long as the assumption  $[\text{H}_2\text{O}]_I =$  saturation vapor concentration for the leaf temperature is valid. Again, Rawlins (7) discusses conditions where the assumption may not be valid.) Likewise, for  $P$  we can change  $L$ ,  $S$ , or  $[\text{CO}_2]_A$  and  $M$  or  $[\text{CO}_2]_{\text{chl}}$ . Thus, environmental changes which affect  $T$  or  $P$  can be evaluated in terms of their effect on factors of Equations 1 or 2.

The value derived from trying to describe gas flux by a simple equation depends, in part, on the significance of the terms which are used in the equations. For example, we do not have any terms in Equations 1 or 2 for cuticular gas flow. If a treatment, changing the temperature for instance, affected the permeability of the cuticle to water and thereby markedly changed  $T$ , the effect might erroneously be attributed to some other factor of the equation. Thus, the validity of each factor in the equations must be justified by experimental evidence of its importance to the process. As stated above,  $[\text{CO}_2]_{\text{chl}}$  and  $M$  cannot be evaluated independently nor do we have a valid basis for assuming any particular value for either of them. However, it is still useful to separate them in the equation to emphasize a difference in the diffusion path for  $\text{CO}_2$  and  $\text{H}_2\text{O}$  vapor. Since the diffusion paths are somewhat different for  $\text{CO}_2$  and  $\text{H}_2\text{O}$  vapor, the possibility exists that measures might be taken to reduce water loss from plants which would have little effect on the rate of photosynthesis. Zelitch and Waggoner (11) have discussed this possibility in some detail. They also give numerical estimates of  $M$  after making the assumption that  $[\text{CO}_2]_{\text{chl}}$  was constant. Shimshi (9) tested that possibility by closing stomata of potted corn plants with phenylmercuric acetate and succeeded in reducing transpiration relatively more than photosynthesis.



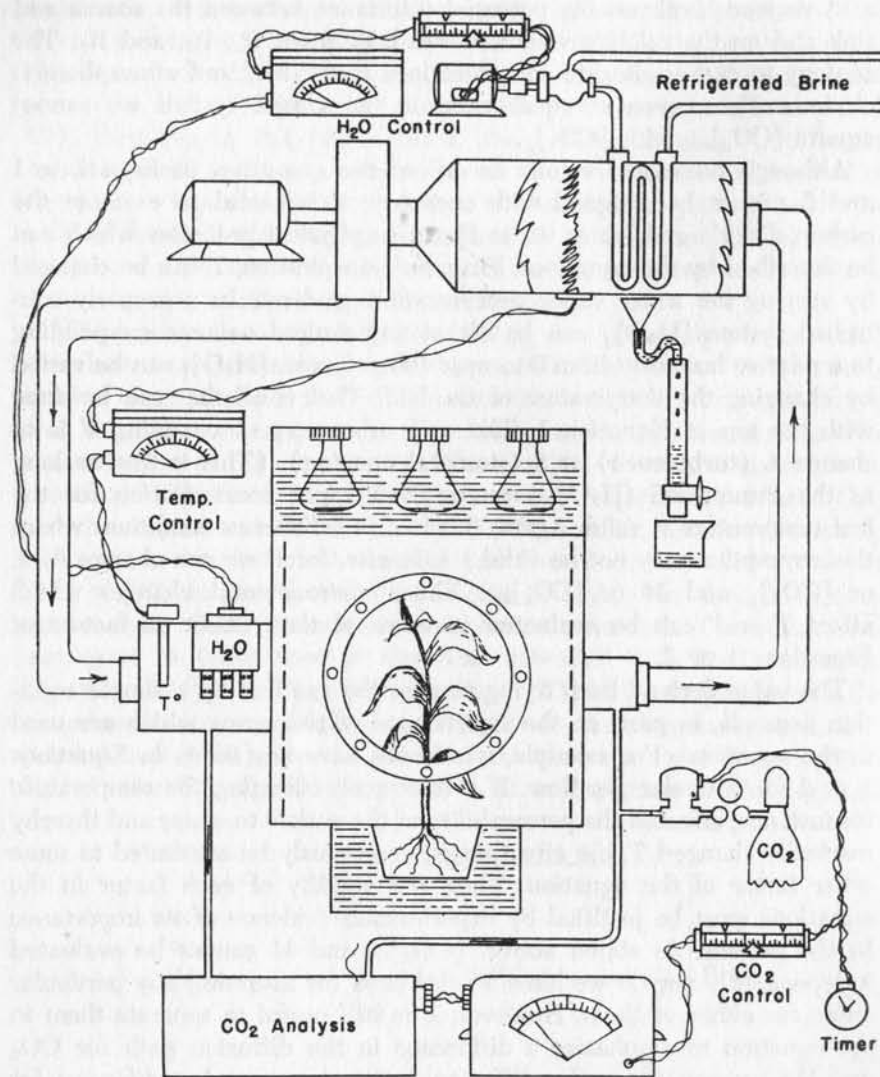


Fig. 3. A controlled environment system for measuring photosynthesis and transpiration.

The plant chamber (center) is 40 cm square and 50 cm in height and is constructed of 0.6 cm acrylic sheet. A 20 cm diameter circular opening permits easy access to the chamber. The cover to this port (not shown) is fitted with a rubber glove which allows manipulation inside without opening the chamber to the atmosphere. The entire system is carefully sealed so that no exchange with the atmosphere occurs after the port cover is in place. Plants are grown in plastic pots which fit snugly into the pot holder in the chamber floor. The temperature of the roots can be controlled by controlling the temperature of the water in the jacket surrounding the holder.

A centrifugal fan (top left) rapidly recirculates the air through the plant chamber and through the air conditioner (top right). Baffle plates on either side assure uniform dispersal of the air as it passes through the plant chamber. The air in the chamber is exchanged 100 times per minute.

The chamber is lighted with seven 300-watt reflector spot lamps immersed in water to control heat and to provide a 5 cm water filter between the lamp and the chamber (8). Light intensity measured with a Weston illumination meter is 6,000 ft-c 10 cm above the floor of the chamber and 10,000 ft-c 40 cm above the floor. Energy of wavelength shorter than 3 microns is  $1.6 \text{ cal cm}^{-2} \text{ min}^{-1}$  10 cm above the chamber floor and  $2.4 \text{ cal cm}^{-2} \text{ min}^{-1}$  40 cm above the floor. Screens are used to reduce light intensity.

The pressure differential across the plant chamber causes a continuous flow of air (Fig. 3—Bottom) through the  $\text{CO}_2$  analyzer (Beckman Model 15A). The output from the analyzer is fed into an electronic control meter (International Instruments Model 2547-2) which, by means of a solenoid valve, controls the flow of  $\text{CO}_2$  from a compressed supply, through a pressure reduction valve, and through a calibrated capillary. A timer indicates how long the solenoid valve has been open. From this the amount of  $\text{CO}_2$  added to the system can be computed. Variation is  $\pm 5$  ppm of the desired  $\text{CO}_2$  concentration at concentrations up to 300 ppm  $\text{CO}_2$  in the plant chamber and  $\pm 30$  ppm at 1,000 ppm  $\text{CO}_2$ .

The control of humidity in the chamber (Fig. 3—Top) is accomplished by cooling the air stream and condensing the excess water. The temperature of the condensing coils is controlled by regulating the flow of refrigerated brine through the condenser. A lithium chloride resistance humidity detector (American Instruments Co. Model 15-3000) continually determines the relative humidity in the air stream entering the plant chamber (left). The output from the hygrometer is fed into another electronic control meter which regulates, by means of a reversible motor, the opening or closing of a valve which determines the flow of refrigerated brine through the condensing coils. If the humidity rises in the plant chamber, the valve will open, the coils will get cooler and condense more water from the air. To prevent over-shooting, a time delay is built into the circuit so only very small adjustments of the valve are made with a minute delay between successive adjustments. The temperature of the refrigerated brine is controlled and is adjusted so that brine flows continuously through the coils when the desired humidity is reached. Once equilibrium is attained the humidity remains at the desired level  $\pm 0.01\%$  relative humidity.

The water condensed on the humidity control coils is collected and, since the plant is the only source of water in the system, gives a direct measure of transpiration. A water trap keeps the system closed.

Temperature in the chamber is controlled by re-heating the air which has been cooled by the condensing coils. A temperature probe monitors the temperature of the air as it enters the plant chamber. A proportional controller (Technical Instruments Co., Electron-O-Therm Model 148) then regulates the voltage applied to a nichrome heater in the air conditioner. The heating circuitry is illustrated immediately above and to the left of the plant chamber. The temperature of the leaves is monitored with small thermocouples (#40 wire).

Plants are prepared for measurements by sealing the soil surface with a mixture of equal parts petroleum jelly and paraffin. A porometer is mounted through the chamber wall (not shown) and can be used to make measurements of relative stomatal opening without opening the chamber. Silicone rubber impressions can be made from materials previously put in the chamber by means of the rubber glove access to the inside.

## FACTORS AFFECTING CARBON DIOXIDE UPTAKE

## Equipment for Environmental Control

For precise measurements of the effect of environmental factors on the gas exchange in leaves, it is desirable to be able to control the environment and to be able to change and control the factors independently. For instance, to do meaningful experiments on the effect of  $[\text{CO}_2]_A$  on gas exchange, it was necessary to be able to measure stomatal apertures and water loss as well as photosynthesis. This could not be done in simple leaf chambers. In addition, Dr. Stephen Rawlins, in his work on water relations of plants which is discussed in the preceding paper, found need for a carefully controlled and reproducible environment. Therefore, through our joint efforts a climate control chamber was constructed in which photosynthesis and transpiration could be measured simultaneously. A diagrammatic sketch is shown and a description of the system is given on pages 90 and 91.

## Effect of Light

The most obvious factor of the environment affecting the carbon dioxide flux is the light intensity. One of the effects of light is to cause the stomata to open, reducing  $S$  in Equation 2. In many experiments the effect on  $S$  is seen as an initial lag in  $P$  and  $T$  when a leaf is placed in the light, then a gradual increase in both processes as the stomata open. However, measurements of  $P$  as a function of  $S$  are difficult to make if  $S$  is changing because the measurements take a finite amount of time. However,  $P$  or  $T$  as a function of  $S$  can be obtained by changing  $S$  in other ways. This will be discussed later.

The effect of light on factors other than  $S$  in Equation 2 can be shown by measuring  $P$  after the leaves have been in the light and the stomata are open. The response of photosynthesis by leaves of several different species is shown in Figure 4 [adapted from Hesketh (4, 5)]. These measurements were made by enclosing attached leaves in transparent envelopes, passing air over the leaves at a known rate and measuring the difference in carbon dioxide concentration of the incoming and exhaust streams. Several interesting facts are apparent in Fig. 4. First, not all species respond alike to light. Photosynthesis in some species continued to increase as light increased. In Fig. 4 the curve for maize illustrates this type of response. In parenthesis are other species, sugar cane and sorghum, which had a similar response to light. Other species respond to light as intensity increased up to an intensity equivalent to about  $\frac{1}{3}$  sunlight. At higher intensities no further increase in photosynthesis was found. These species, represented by orchard grass in Figure 4 but typical of many other species, are said to be light satu-

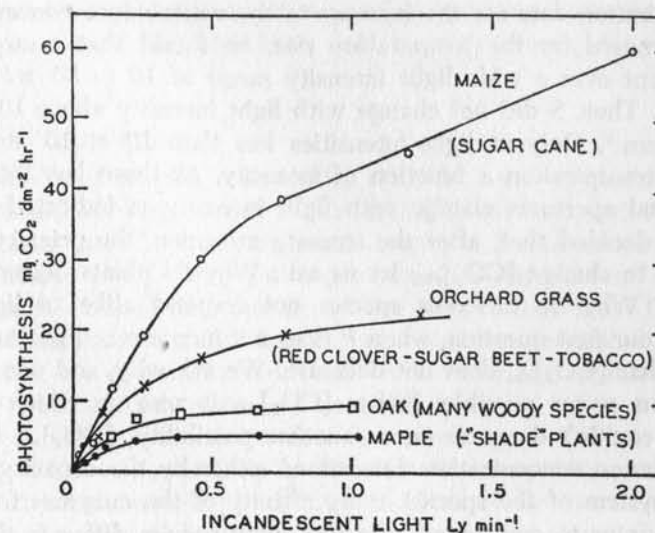


Fig. 4. The photosynthetic response of different species to varying light intensity.

rated at the higher light intensities. Still other species respond to light only at very low intensities, light saturation occurring at  $\frac{1}{10}$  that of full sun. Many tree species and shade plants were found to be in the third class. Thus, the response of many species could be classed in three categories, those that show no light saturation, those that are light saturated at about  $\frac{1}{3}$  full sun and those that are saturated at about  $\frac{1}{10}$  full sun.

In what way can Equation 2 be useful in understanding the response of these species to light? The answer lies in finding how changing light affects the physical process of diffusion. The air temperature in the leaf enclosures was maintained essentially constant by cooling the air at the higher light intensities. Although transpiration was not measured in the particular experiments from which Fig. 4 was taken, in many similar experiments transpiration did not change with light intensity if the leaf temperature was maintained constant. Turbulence was constant in the system. It seems unlikely that light would affect any physical barriers to  $\text{CO}_2$  flow represented by  $M$ . Thus, the probable effect of light is to reduce  $(\text{CO}_2)_{chl}$ . This seems reasonable because the light provides energy to the enzyme systems which fix  $\text{CO}_2$  and, thereby, reduces the concentration at the chloroplast.

Further evidence that the effect of light can be evaluated independently of its effect on  $S$  is shown in the work by Kuiper (6). He studied the effect of light intensity on transpiration and found a linear increase in transpiration as the light intensity increased. However, the leaf temperature increased as the light became more intense. When he corrected



the transpiration data for the increase in the water vapor concentration gradient caused by the temperature rise, he found that transpiration was constant over a wide light intensity range of  $10$  to  $50 \times 10^4$  erg  $\text{sec}^{-1} \text{cm}^{-2}$ . Thus,  $S$  did not change with light intensity above  $10 \times 10^4$  erg  $\text{sec}^{-1} \text{cm}^{-2}$ . Only at light intensities less than  $10 \times 10^4$  erg  $\text{sec}^{-1} \text{cm}^{-2}$  was transpiration a function of intensity. At those low intensities the stomatal apertures change with light intensity as indicated earlier.

Having decided that, after the stomata are open, the primary effect of light is to change  $[\text{CO}_2]_{\text{chl}}$ , let us ask: Why do plants become light saturated? Why do different species not respond alike to light? In answer to our first question, when  $P$  does not increase as light increases it means that  $[\text{CO}_2]_{\text{chl}}$  does not decrease. We ask why, and one of two explanations seems possible. Either  $[\text{CO}_2]_{\text{chl}}$  is zero, in which case it obviously couldn't decrease or, as another possibility,  $[\text{CO}_2]_{\text{chl}}$  is some other minimum concentration determined either by the capacity of the chemical system of the species or by affinity of the enzymes for  $\text{CO}_2$ .

This leads us to our second question, why species differ in their response to light. Our interest in this question is twofold. First, we would like to know why some species are light saturated when others are not and, second, we would like to know why the absolute rates of photosynthesis on a unit leaf area basis are so much higher with some species than with others. Again, Equation 2 can be helpful. We can see that  $[\text{CO}_2]_{\text{chl}}$  could have the same minimum value for all species and that differences in  $P$  between the species could be due to differences in  $L$ ,  $S$ , or  $M$ .

Differences in these quantities could result from differences in leaf thickness, differences in number and size of stomata, or differences in the rate of respiration (i.e. the ratio of respiratory to assimilating components in a leaf). If  $L$ ,  $S$ , and  $M$  were greater for one species than another, the disadvantage could be overcome by increasing  $[\text{CO}_2]_{\text{A}}$ . Thus, a hypothesis is presented which can be tested.

#### Effect of Carbon Dioxide

Hesketh (4) found that tobacco leaves in an atmosphere containing 800 ppm  $\text{CO}_2$  had light response curve almost identical with corn in an atmosphere containing 300 ppm  $\text{CO}_2$ . However, increasing  $[\text{CO}_2]_{\text{A}}$  did not increase  $P$  of philodendron leaves. Thus, differences in physical barriers in leaves do exist between species, and these differences explain, in part, why plants have different rates of photosynthesis. However, in the case of plants which do not respond to increasing  $[\text{CO}_2]_{\text{A}}$ , under conditions of light saturation, another explanation must be found. Such plants have limitations to the photosynthetic process which are

not physical and no manipulation of the environment can increase photosynthesis to the high rates which other species are capable of producing.

#### Effect of Temperature

The effect of varying both  $[\text{CO}_2]_{\text{A}}$  and the air temperature on  $P$ ,  $T$ , and stomatal opening of corn is shown in Figures 5, 6, and 7. For these experiments three 18-inch corn plants were put in the environment control chamber and a constant vapor pressure deficit was maintained between the leaves and the air as the temperature and  $[\text{CO}_2]$  were varied. The points are averages for two separate experiments. In each experiment six consecutive readings were taken for each environment change after a steady state had been reached.

$P$  increased as the temperature increased up to  $30^\circ$  at any given  $[\text{CO}_2]_{\text{A}}$ . This occurred despite the fact that respiration undoubtedly increased with increasing temperature. Much of the increase in  $P$  was due to a decrease in  $S$  indicated by the increasing stomatal width and increased  $T$  shown in Figures 6 and 7. However, the stomatal apertures were greatest at  $40^\circ$  while both  $T$  and  $P$  were less at  $40^\circ$ . Part of this seeming paradox may be due to errors in accurately measuring the stomatal width. Measurements of 100 stomata on a single leaf were taken at random for each setting and data for one experiment at  $40^\circ$

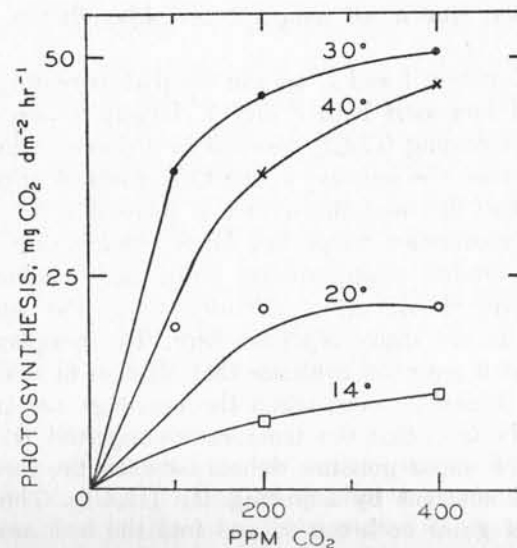


Fig. 5. The effect of temperature on photosynthesis of Maize at varying  $\text{CO}_2$  concentrations.



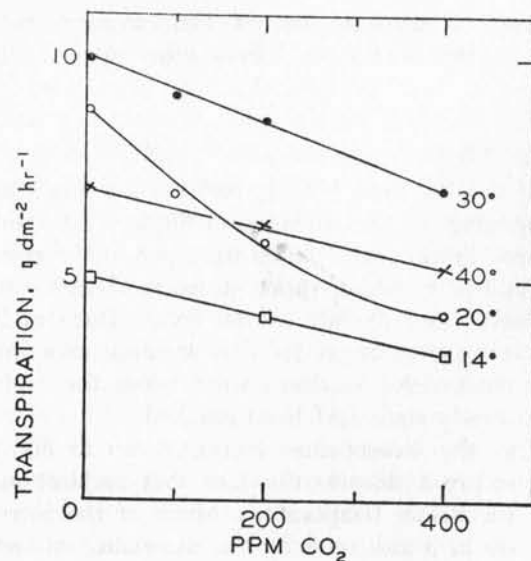


Fig. 6. The effect of temperature on transpiration of Maize at varying CO<sub>2</sub> concentrations.

showed abnormally large stomatal openings. However, it could also be due to increased non-stomatal resistance.

As  $[CO_2]_A$  increased,  $P$  increased, but  $T$  and stomatal aperture decreased. This was true at all temperatures although the changes were small at 14°.

In terms of Equation 1 and 2, we can see that increasing the temperature of the leaf increased both  $P$  and  $T$ , largely by decreasing  $S$ . Increasing  $S$  by increasing  $[CO_2]_A$  resulted in a decrease in  $T$ . However,  $P$  increased because the increase in the CO<sub>2</sub> gradient apparently had a greater effect than the inhibiting effect of increasing  $S$ .

During this conference there has been considerable discussion of diffusion shells around multiporate septa, and whether changes in stomatal apertures should affect diffusion when the apertures are 5 to 10 microns as are many reported here. The temperature data in Figures 4, 5, and 6 are good evidence that changes in stomatal aperture do affect both  $T$  and  $P$ , even when the openings are large. Again it should be emphasized that the temperature reported is the leaf temperature, and the vapor pressure deficit between the leaf and the air was maintained constant by adjusting the  $[H_2O]_A$ . Thus, it is clear that diffusion of gases both out of and into the leaf increased as the stomatal apertures increased. It seems likely that the increase was due to lowering the stomatal resistance to gas flow.

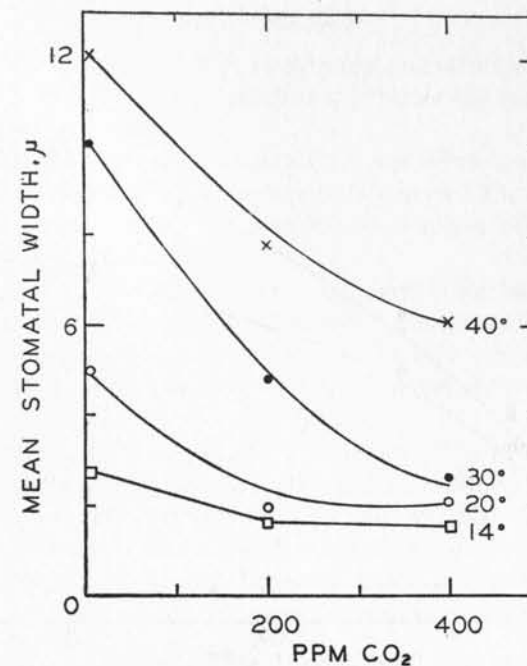


Fig. 7. The effect of temperature on mean stomatal width of Maize at varying CO<sub>2</sub> concentrations.

We have also discussed the relative magnitude of cuticular and stomatal diffusion. Undoubtedly the amount of diffusion through the cuticle varies with the species as the thickness of the cuticle varies. However, in corn, tobacco, tomato, sunflower, and pumpkin, cuticular diffusion of CO<sub>2</sub> is apparently very slow. This is indicated by measurements of photosynthesis when the stomata are closed by reason of incipient wilting, or the plants have been kept in the dark prior to the measurement. Under these conditions the rate of CO<sub>2</sub> absorption is very near zero. Therefore, unless the cuticle is more permeable to water than to CO<sub>2</sub>, the cuticular transpiration of these species must also be very much less than the stomatal transpiration.

#### Effect of Turbulence

Another factor of the environment which affects  $T$  and  $P$  is wind velocity. To determine the effect of air movement on the processes, a 30 cm diameter glass jar 50 cm tall was put in 10 cm of ice water and a light was mounted above it to provide energy for photosynthesis (10). The light was also a source of heat so a stable air situation was estab-

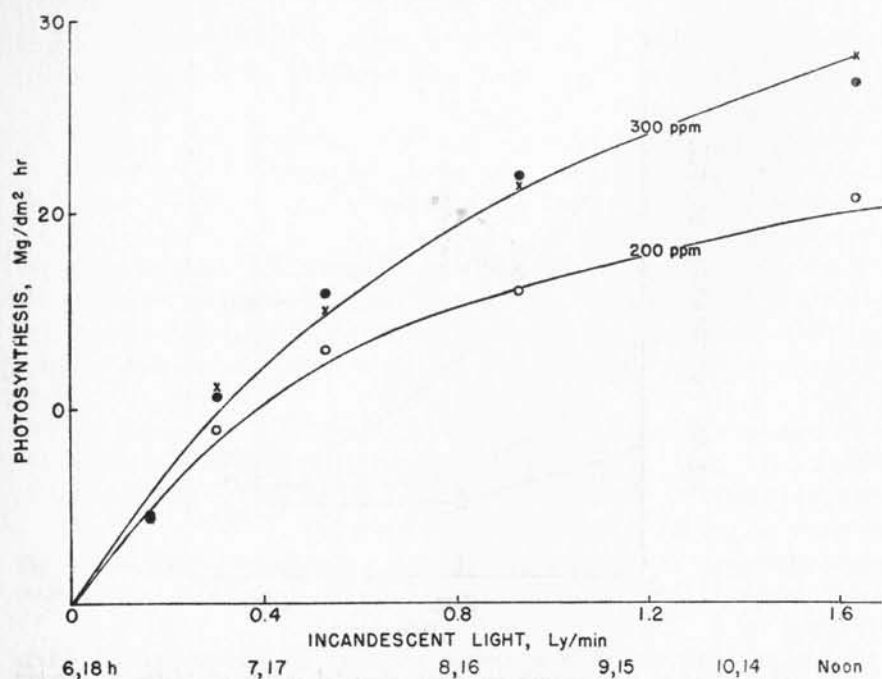


Fig. 8. The effect of turbulence on photosynthesis. See text for details.

lished. Excised sugar cane leaves were put in the cylinder in this air which was as still as we could make it. The light response curve of the leaves was determined at 200 and 300 ppm carbon dioxide. Then a small fan in the cylinder was turned on which stirred the air vigorously. The light response at 200 ppm CO<sub>2</sub> was determined again in the moving air. The results of this experiment are shown in Fig. 7.

Stirring the air in the cylinder reduced  $L$  in Equation 1 and  $P$  increased. In Fig. 8 the open circles are for 200 ppm in still air and the X's are for 300 ppm in still air. The solid circles are for 200 ppm in stirred air. Thus, stirring the air was equivalent to increasing the CO<sub>2</sub> by 50 per cent in this system. From this, the effect of changing  $L$  in other systems can be predicted. In the open field the air will seldom be as calm as in this laboratory chamber, nor will CO<sub>2</sub> be as low as 200 ppm. At least half the daylight hours will have winds of 3 mi. hr<sup>-1</sup> according to Hellman (2). Thus,  $L$  will seldom be as large as for the lower curve in Figure 8. However, during still periods in bright light considerable increase in  $P$  and  $T$  would be expected by stirring the air and reducing  $L$ .

## SUMMARY

We have discussed the effect of environmental factors on gas exchange of leaves and have seen how environment controls the individual factors of the diffusion equation.

Study of diffusion requires a carefully controlled and reproducible environment and the simultaneous measurement of CO<sub>2</sub> absorption and water loss by leaves. A detailed description is given of our controlled environment growth chambers.

Light increases CO<sub>2</sub> absorption both by decreasing the concentration of CO<sub>2</sub> at the chloroplast and by decreasing the stomatal resistance to gas flow. The magnitude of the effect of the decrease in stomatal resistance can be measured by the increased water loss as the stomata open.

Increasing CO<sub>2</sub> in the atmosphere increases CO<sub>2</sub> absorption by increasing the gradient between the leaf and the atmosphere. However, increasing CO<sub>2</sub> in the atmosphere does not increase CO<sub>2</sub> absorption as much as predicted because the concentration of CO<sub>2</sub> at the chloroplast apparently increases and because stomatal resistance increases. Again, the magnitude of the increased stomatal resistance can be determined by the changed water loss.

Increasing the temperature increases both transpiration and CO<sub>2</sub> absorption, in part by decreasing stomatal resistance.

Stirring calm air is equivalent to adding carbon dioxide to the quiet atmosphere.

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### Discussion

DUGGER: I would like to comment on Figure 1. We are inclined to think of the stomatal cavity as the area where the air-water interface occurs. I believe several people have made measurements of the cell surface in contact with air and have found that this area in the palisade parenchyma is greater than in the spongy parenchyma. Also, in experiments with  $C^{14}O_2$ , autoradiograms show that practically all the activity is found in the palisade tissue; even though the entrance of  $CO_2$  has been through the stomata on the lower surface of the leaf. These palisade cells are in contact with a great deal more air than what is depicted with just a substomatal cavity. That would tend to lengthen  $L$ .

MOSS: Figure 1, of course, is diagrammatic. As I have used Equation 1 and 2,  $L$  represents the apparent pathlength between the atmosphere and the air-water interface inside the leaf wherever it is found.

ZELITCH: You are using a method which is quite different in principle from ours, namely, that by increasing the partial pressure of carbon dioxide you close stomata in the light and thus reduce transpiration rates. I wonder if you, and perhaps Dr. Gardner, would comment on how your results are influenced by boundary layers around leaves.

GARDNER: The influence of the boundary layer depends on the resistance it offers compared to the resistance of the stomata. In many experiments conducted in the laboratory, care is taken to provide a great deal of turbulence so the boundary layer is small and you are studying what you want to study, the stomatal effect. Outdoors in the field, where the wind velocity varies from 0 to 20 or 30 m.p.h., a sizeable boundary layer may develop at times. That is why micrometeorologists can frequently ignore the plant without even asking what kind of plant it is or whether it has stomata at all. They do not ask these questions because the boundary layer is limiting transpiration and all of these other questions become minor.

PALLAS: What would be the effect of trichomes on leaves?

GARDNER: They would tend to increase the size of the boundary layer.

MOSS: In reference to Dr. Zelitch's comment, I see no reason why varying the carbon dioxide concentration in our experiments would have any effect on the size of the boundary layer ( $L$  in equations 1 and 2). As Dr. Gardner has suggested, turbulence was high in most of our experiments. If turbulence were low, however, and the boundary large then we would expect a linear response of photosynthesis to increasing carbon dioxide concentrations in the atmosphere.

## The Role of Stomata in Plant Disease

By Saul Rich

### INTRODUCTION

Stomata are the most important infection courts for those foliage pathogens that cannot penetrate unbroken epidermis. For this reason alone, chemical regulation of stomata is of interest to plant pathologists. Our interest in stomata, however, is not limited only to their function as infection courts. Stomata are both entrances and exits, allowing pathogens to enter, and serving subsequently as exits, permitting the spread of infective propagules. Aside from serving as portals for pathogens, stomata may permit the entrance of foreign liquids, such as pesticides, and allow natural liquids to exude. As we shall see later, the movement of these liquids through stomata can play a prominent role in the cause and control of plant diseases.

The first section of this discussion will deal with the entrance of injurious agents through stomata. We shall consider ozone, bacteria, fungi, nematodes, and viruses. Next we shall discuss how organisms exit through stomata, and examine the importance of guttation. The section following will consider the effect of plant diseases on stomata. We shall conclude with the possible uses of chemical regulation of stomata in plant pathology.

### ENTRANCE THROUGH STOMATA

*Ozone.* Here in Connecticut, our interest in the role of stomata in plant disease stems from our interest in ozone damage to plants, particularly "weather fleck" on tobacco. Weather fleck is caused by damaging concentrations of ozone that must enter through stomata to cause injury (27). Weather fleck stipples the valuable shade grown tobacco leaves, and makes them worthless for wrapping cigars. In 1959, Connecticut tobacco growers lost over a million dollars because of weather fleck.

The ozone that flecks our Connecticut tobacco is an air pollutant resulting principally from the action of sunlight on exhaust gases from automobiles. Both nitrogen dioxide and waste hydrocarbons are thrown off by automobiles. Under ultraviolet light, nitrogen dioxide reacts with atmospheric oxygen to give nitrous oxide and ozone (23). This reaction is rapidly reversible, and ordinarily would not produce excessive ozone. Here the waste hydrocarbons enter the picture. They combine with

nitrous oxide, slowing the reverse reaction, and ozone accumulates. Fortunately for us, ozone is unstable. With sunset, the ozone-producing process stops, and the accumulated ozone degrades.

From the literature (27) and from our own experience with ozone fumigation of plants in the laboratory, we knew that closed stomata protect plants from ozone. Hence, Zelitch's report (51) raised the possibility that we could protect plants from ozone damage by chemical regulation of stomata. Whether this could be done would depend on the length of the fleck-producing periods compared with how long stomata could be kept closed by chemical regulators.

In Connecticut, natural accumulation of ozone begins in the morning and gradually increases to a peak in late afternoon or early evening. We find that atmospheric ozone must exceed 0.05 ppm to cause fleck. This threshold is rarely reached before 1000 or 1100 hours. Damaging levels of ozone usually disappear by 1900 to 2100 hours. To protect crops during a single high ozone period, a chemical regulator of stomata must have two properties. If applied as soon as the damaging threshold of ozone is exceeded, the regulator must close stomata within one hour; and it must keep stomata closed for the rest of the day. Because we may have fleck-producing periods on successive days, a regulator would be most practicable if one application could keep stomata closed for 48 hours.

It is obvious that chemical regulators of stomata are suitable. According to Zelitch and Waggoner, phenylmercuric acetate can close stomata and keep them closed for more than a week (53).

Our first evidence that phenylmercuric acetate can protect tobacco against weather fleck came in an unexpected way. Dr. D. Shimshi was using tobacco plants in the greenhouse to investigate the relation of stomatal aperture to photosynthesis and transpiration. He applied the inhibitor to upper and lower surfaces of separate attached leaves, spraying half leaves only. The opposite half of each leaf was left untreated as a control. Fortunately for us, there was a period of high ozone the following day. Two days later the plants showed typical fleck. But the fleck appeared only where the under surface of the leaf half had not been sprayed with phenylmercuric acetate. Half-leaves sprayed underneath with the compound were fleck-free even though the opposite half-leaf was badly flecked. Presumably the lower stomata were the important ones. Spraying the upper surface of the leaves did not protect from ozone. With the microscope, we verified that the effect of phenylmercuric acetate on stomatal closing was restricted to the surface sprayed (52). This lucky sequence of events demonstrated strikingly that chemical closure of stomata can protect tobacco against natural weather fleck. We need close only the lower stomata to prevent



fleck, even though the upper stomata remain open. Closing the upper stomata does not prevent fleck, so long as the lower stomata are open.

Subsequently, we verified these observations with tobacco plants in our fumigation chamber. The importance of the lower stomata, and the unimportance of the upper stomata to ozone damage was demonstrated on single leaves. Phenylmercuric acetate was sprayed on the lower surface of one quarter of a leaf and on the upper surface of another quarter of the same leaf. The other half was left untreated as a control. The leaf was then fumigated with 0.1 ppm ozone for 7 hours. The only portion of the leaf that remained undamaged was that quarter sprayed underneath with the inhibitor.

We know now that stomatal regulators which can close lower stomata and keep them closed can protect tobacco against ozone damage.

In tobacco, ozone preferentially damages the palisade parenchyma and its associated upper epidermis (41). Why, then, are the lower stomata so important in fleck? Ozone entering the lower stomata must pass the spongy mesophyll to attack the upper tissues of the leaf. The flecks should appear first on the lower surface, but they do not. The upper stomata of tobacco are only half as numerous as the lower stomata, but this should not make the difference. The problem remains unanswered.

Peroxyacetyl nitrate is another air pollutant that damages crops. Like ozone, this substance does not damage plants if stomata are closed (15). One would expect that phenylmercuric acetate would also prevent damage by peroxyacetyl nitrate.

**Bacteria.** We know now that bacteria regularly infect plants through stomata. At the turn of the century, however, the importance of stomata as infection courts was one of the major differences in a turbulent scientific argument between E. F. Smith and Albert Fischer.

Burrill reported in 1879 (5) that bacteria cause a plant disease, fire blight. About 1894, E. F. Smith began his own studies of bacterial plant diseases. Yet in 1899, Albert Fischer, an eminent German bacteriologist, declared that there could be no such diseases (18). As one of his arguments, he stated that it was extremely unlikely that bacteria would enter stomata. Even if they did enter, he said, the bacteria could not thrive in the substomatal cavity, which he thought to be devoid of nutrient. This was too much for Smith. He exploded in a series of articles using language that must have shocked the staid academic cloisters of the Old World (26).

Within the next 10 years, Smith brilliantly established the existence of bacterial diseases of plants. He left no doubt that bacteria can infect plants not only through nectaries and hydathodes, but also through

stomata (45). Stomata, then, are important infection courts for bacterial plant pathogens.

That stomata could play a role in resistance to bacteria was demonstrated by McLean in 1921 (37). He was studying the resistance of citrus leaves to bacterial canker. The leaves of both the Szinkum mandarin and the Florida seedling grapefruit can be readily infected with canker through wounds, such as pinpricks. In nature, however, the mandarin is canker-resistant while the grapefruit is susceptible.

McLean found that the stomata of the mandarin had a ridge at their entrance. Even at their widest, the external apertures of the mandarin stomata never exceed 1.5  $\mu$ . The grapefruit stomata, however, have no entrance ridges. The external apertures of the grapefruit stomata average 9.8  $\mu$  in width, and can open as wide as 11  $\mu$ . McLean proposed that this morphological difference in stomata accounted for the difference in natural susceptibility of these citrus species to bacterial canker.

Later, McLean and Lee (38) showed that the canker bacteria could be forced through the stomata of the Szinkum mandarin leaf by pressure, and that infection would result. They could infect the susceptible grapefruit leaf merely by dipping it into a suspension of canker bacteria.

The importance of natural openings to bacterial infection becomes obvious when we realize that bacteria cannot breach intact leaf surfaces.

**Fungi.** Unlike bacteria, fungi can penetrate the outer barriers of the intact leaf. This ability has made it most difficult to assess the importance of stomata in fungal diseases of plants.

Fungal germ tubes and swarm spores do enter stomata. De Bary (2) in 1861 described "potato mold" spores (*Phytophthora infestans*) germinating and entering through stomata. But does *P. infestans* infect only through stomata? If so, must the stomata be open for successful infection?

Although *P. infestans* has been one of our most intensively studied fungi, it was not until almost a century after de Bary that we had a definitive answer. In 1954, Pristou and Gallegly (42) showed clearly that germ tubes of *P. infestans* zoospores form appressoria. Infection pegs from these appressoria then penetrate either directly through the epidermis or between guard cells. The infection pegs penetrated the stomata whether they were open or closed.

Studies of the role of stomata in fungal diseases of plants have been most confusing. One of the few unequivocal examples of fungal penetration only through open stomata is infection of beets by *Cercospora*

*beticola*. Pool and McKay (40) showed that germ tubes of *C. beticola* infect only through stomata. Further, they showed that the germ tubes bypass closed stomata, entering only the open stomata.

The role of stomata in fungal diseases of plants perhaps has been most intensively studied in the rusts. All studies concur on one point: germ tubes of aeciospores and urediniospores infect only through stomata. Johnson (32) reported that germ tubes of rust urediniospores grow transversely across wheat leaves. Wheat leaf stomata are arranged in parallel, longitudinal rows, and stomata in adjacent rows are not usually opposite each other. Johnson pointed out this was advantageous for infection because a germ tube inevitably would hit a stoma as the tube continued across the leaf. But must stomata be open for infection? Do closed stomata prevent infection?

Although these questions have been explored with other diseases, the most comprehensive studies have been made with two wheat rusts: stem rust caused by *Puccinia graminis* Pers. f. sp. *tritici*, and leaf rust caused by *P. recondita* Rob. ex Desm. f. sp. *tritici*. We shall begin with Hart's work (24), though she notes that others before her had studied the relation of wheat stomata to rust.

Hart worked only with stem rust, and studied the stomatal behavior of wheat varieties that varied in rust resistance. She found that the stomata of susceptible varieties opened sooner and stayed open longer at maximum aperture than did stomata of resistant varieties. Hart felt that the critical infection period in the field usually lasts from dawn until the dew disappears at about 0730 hours. She noted that during this important period her susceptible varieties had wide open stomata for over an hour. The stomata of her intermediate varieties were wide open for less than an hour. The stomata of her resistant varieties were late-opening, and were never fully open until the heavy dew had gone and there was no free water for spore germination.

From her artificial infection studies, Hart could find no evidence that *P. graminis* can penetrate closed stomata of wheat.

The prevention of infection by stomatal behavior Hart termed "functional resistance." She expanded on this concept later (25).

Shortly thereafter Caldwell and Stone (6, 7) published conflicting evidence. They, however, studied leaf rust of wheat rather than stem rust. Their results left little doubt that *P. recondita* penetrated as readily through closed stomata as it did through open stomata. They also found that wheat guard cells close as soon as a leaf rust appressorium forms over the stoma. In every case infection occurred by a peg forced down between the guard cells from the appressorium.

The infection processes of the two rusts were re-examined by Yirgou

and Caldwell (50). They confirm again that closed stomata cannot exclude *P. recondita* from leaves of seedling wheat. They are convinced that the appressoria of *P. graminis* also close guard cells and that both fungi can penetrate closed stomata. The difference between the two species, they believe, is correlated with sensitivity to ambient carbon dioxide. They found that infection of wheat seedlings by *P. recondita* is not influenced by the carbon dioxide concentration. Infection by *P. graminis*, however, is enhanced by carbon dioxide-free air, and decreased by 5 per cent carbon dioxide. This effect, they are convinced, is independent of the influence of carbon dioxide on stomatal movement.

Recently, Romig (43) took another look at the possibility of functional resistance of wheat to leaf rust. With plants at anthesis, he found that the thick-walled guard cells of the peduncles and sheaths of many wheat varieties can exclude *P. recondita* when the stomata are closed. Thin-walled guard cells of the leaves, however, afford no protection. Curiously, when Romig decapitated the tillers, the thick-walled guard cells no longer excluded the fungus. The ability to exclude was regained when Romig placed 1 per cent auxin-lanolin on the cut stump.

Strains of a single fungal species may also differ in ability to penetrate stomata. Ullstrup (47) inoculated begonia leaves with four isolates of *Rhizoctonia solani*. He found that two of the isolates penetrate only through stomata. The other two isolates form a stroma from which the epidermis is punctured.

Environmental conditions may alter the infection route of fungi. Bald (1) studied the effect of free moisture on the penetration of gladioli by two different fungi: *Pleospora* sp., and *Botrytis gladiolorum*. He found that *Pleospora* penetrates gladioli stomata only when there is a little free moisture. When the gladiolus leaf is constantly covered with a film of free water, *Pleospora* spores germinate but the germ tubes do not penetrate. *B. gladiolorum* penetrates only through the cuticle when the gladiolus leaves are continuously wet. When the leaves are alternately wet and dry, this fungus penetrates both through the cuticle and through stomata.

Stomatal penetration by fungi may be important even when the part of the plant that is damaged has no stomata. An example is infection of tomato fruits by *Cladosporium fulvum*. This fungus infects through stomata, attacks the aerial parts of the tomato, and can invade the fruit to cause serious damage. Yet tomato fruits have no stomata or lenticels. Gardner (19) could not infect tomato fruits with the fungus, even when he wounded the fruit surface. He demonstrated conclusively that the mycelium invading the fruit originates from infections through the stomata of the sepals and torus.



*Other Leaf-damaging Agents.* Other leaf-damaging agents that can enter through stomata are viruses and nematodes.

It is well known that the most important infection route for tobacco mosaic virus is through wounds. Boyle and McKinney (4) wiped the virus on pepper leaves, then studied the relation of number of local lesions to trichomes and to stomata. They found the lesion number was related to trichomes, and not to stomata. They did not report whether the stomata were open at inoculation.

Duggar and Johnson (14) dropped inoculum of tobacco mosaic virus on tobacco and observed the relation of open stomata to infection. They concluded that the virus can infect intact leaves through stomata. This route is probably not of practical importance, but at least the virus can enter through stomata.

A very common problem is the killing of chrysanthemum leaves by the leaf nematode *Aphelenchoides ritzema-bosi*. The eggs of this nematode hatch in debris. The emerging larvae travel in a moisture film up the stem to the leaves. Here, the tiny larvae enter the leaves through open stomata (21). Once inside, the growing nematodes kill the leaves as they feed. Apparently, two other species, *A. besseyi* and *A. fragariae*, also penetrate leaves through stomata (48).

#### EXIT THROUGH STOMATA

*Pathogenic Organisms.* Smith (45) clearly demonstrated with *Xanthomonas pruni* that bacteria, which enter through stomata, can also exude through them. Once on the surface, the bacteria can be splashed to healthy leaves, to enter other stomata and infect anew.

Many fungi erect their sporophores out through stomata. This was observed by Berkeley in 1846 (3). About late blight of potatoes, he wrote: "The upper surface rarely if ever exhibits the mould, it being almost physically impossible for its delicate threads to penetrate the closely-packed cells which, being arranged side by side, leave scarcely any intercellular passages. The mould, in a few hours from its first piercing the apertures of the stomata, perfects its fruit, and in so doing completely exhausts its matrix, which in consequence withers."

Most of the plant pathogenic fungi which have imperfect forms with sporophores borne singly or in simple fascicles erect these sporophores through stomata. This includes the downy mildews, the powdery mildews, and many other groups of fungi important in plant pathology. As these sporophores are taller than 250  $\mu$ , the spores are borne beyond the leaf's still air barrier and within reach of the moving air. Here the wind can carry the spores to new victims.

Can sporophores penetrate closed stomata? It is well known that downy mildews can sporulate on leaves in the dark, but it may be that the sporophore initials must exit through open stomata first.

*Guttation.* We shall consider guttation through both hydathodes and stomata. When we speak of hydathodes we mean the so-called epithem-hydathodes. These have sub-pore cavities and guard cells. Even though these guard cells are supposedly unable to close (17), epithem-hydathodes are analogous to stomata.

Guttation produces a water bridge from the inside of the leaf through stomata and hydathodes to the outside of the leaf. Both Diachun *et al.* (10) and J. Johnson (31) demonstrated that a water bridge greatly facilitates the entrance of bacteria through open stomata. Bald (1) proposed that the alternate exudation and retraction of guttation fluid through stomata actually carries plant pathogens into the leaf.

Guttation fluid is not just pure water. It is a solution of soluble materials from the plant's interior. These solutes may play many roles. The first we shall discuss is a case of self-inflicted damage.

Maladies classed as "tipburns" are common on many plants. As the name implies, these diseases are characterized by searing and death of the leaves at their tips, and at edges adjacent to the tips. Tipburn has been described on potatoes, cabbage, and lettuce.

On potatoes, Lutman (36) related tipburn to hydathodes and stomata. He believed the damage was caused by excessive transpiration. Spraying potatoes with Bordeaux mixture appears to reduce tipburn. Lutman laid this effect to clogging of the hydathodes and stomata by the colloidal particles of Bordeaux. Lutman was able to close hydathode "guard cells" by solutions of appropriate osmotic concentration. He believed these cells were functional, but did not report whether they operated in the field as do the guard cells of stomata.

Lutman meant by "excessive transpiration" that the damage was caused by drying out of the cells surrounding hydathodes and certain stomata. Curtis (8) explained tipburn in another way. He showed that repeated evaporation of guttation water greatly concentrated the salts brought to the surface by the exudation. The salts eventually would become sufficiently concentrated at the surface to injure the leaf. He further considered the possibility that the salt deposits could be dissolved and sucked back into the tender interior of the leaf. Curtis also proposed that microorganisms could grow in the guttation fluid to produce other compounds toxic to the leaf.

Later, Munnecke, and Chandler (39) made use of Curtis' last proposal to explain the cause of a leaf spot of *Philodendron*. The spots are centered on the stomata. Guttation droplets from the stomata have

a high sugar content and support the growth of miscellaneous microorganisms. None of these microorganisms appear to be the specific cause of the spotting. Munnecke and Chandler concluded that the spots on *Philodendron* are caused by toxic materials from miscellaneous bacteria growing in the guttation exudate from stomata.

Guttation fluid may also change the performance of pesticides. Curtis (8) proposed that the fluid could dissolve pesticides, and these would be sucked back into the leaf. He pointed out that this could lead to phytotoxicity by ordinarily safe pesticides; or make a systemic toxicant out of a protective one. In his later paper (9), Curtis also demonstrated that guttation fluids enhanced the fungitoxicity of both yellow copper oxide and Bordeaux mixture.

The whole subject of guttation has been reviewed by Ivanoff (30).

#### EFFECT OF DISEASE ON STOMATA

Leaf damaging agents can affect stomata. Smog can close the stomata of tomatoes and oats, but has little effect on endive stomata (29, 33). Hull and Went thought this might explain differences in the sensitivity of these plants to smog, because stomata must be open for smog damage. Todd and Propst (46), however, could find little effect of smog components on stomata. This question needs more investigation.

We have found that the upper stomata may remain wide open in the fleck spots on ozone injured tobacco, even when the stomata are closed in the surrounding healthy tissue. The upper epidermis in the flecks appear to be killed, and the dead guard cells may be pulled open as the spots dry.

Gäumann (20) described the infection of pine needles by *Lophodermium pinastri*. Pine needle stomata are located at the bottom of sunken pits. The infecting mycelium of *L. pinastri* first grows down into the pit and fills it with a vesicle. Side branches grow from the vesicle to pierce the guard cells and "put them out of action." Then the fungus penetrates through the stoma to invade the interior of the pine needle.

Graf-Marin (22) found that barley plants infected with powdery mildew transpired more rapidly than healthy plants. He reported that the disease causes barley stomata to open sooner and wider than the stomata of healthy plants. He believed this was the main reason for the increase in transpiration of the mildewed barley.

Yarwood (49) confirmed that powdery mildews and other leaf pathogens increase transpiration. However, he could find no differences in stomatal behavior. He attributed enhanced transpiration of diseased plants to "increased permeability of the host cells."

When we think of the wilt diseases of plants we think of those

caused by the pathogens that infect the vascular bundles, for example, species of *Fusarium* and *Verticillium*. The wilting caused by these fungi is generally presumed to result from water shortage as the xylem vessels become dysfunctional. Dimond and Waggoner (12) found that this chronic water stress closes the stomata of healthy looking leaves of *Fusarium*-infected tomatoes. However, shortly after inoculation and before the stage of water stress, the infected plants transpire more than healthy plants (35). Linford (34) demonstrated that peas infected with *Fusarium* also transpire excessively. He proposed that the wilting of infected peas resulted from excessive water loss rather than diminished water supply.

The wilting toxins and their possible role in causing excessive transpiration was reviewed by Dimond (11). It is not certain whether stomatal behavior plays any role in the temporary increase in transpiration noted in the early stages of vascular wilt. However, the vascular wilts probably cause stomata to close and remain closed as chronic water stress begins.

Excessive transpiration may also result from virus infection. Heuberger and Norton (28) reported this for tomato mosaic. Selman (44) found the same was true for tomatoes inoculated with tobacco mosaic, spotted wilt, or yellow mosaic of tomatoes. With use of cobalt chloride paper, Selman discovered that the enhanced transpiration of infected plants was greatest from the upper epidermis. Because there are few upper stomata and many lower stomata on tomato leaves, Selman reasoned that the virus effect was mainly on cuticular transpiration.

Whether viruses affect stomatal behavior is not known, but we do know that viruses may invade guard cells. Esau (16) reported viral inclusions in the guard cells of tobacco infected with mosaic.

Disease may influence stomatal number as well as stomatal behavior. A striking example is the effect on black raspberry of systemic infection by the sporidial mycelium of the orange rust, *Gymnoconia peckiana*. As reported by Dodge (13), "The systemic stage of these orange rusts regularly so affects the host as to lead to the development of a large number of stomata on the upper side of the leaf where normally there would have been only a very few or none at all." He found that "As long as the epidermis is in a plastic condition the advance of the mycelium into new areas is accompanied by the development of additional stomata."

#### POSSIBILITIES

Where, then, can chemical regulation of stomata be of use in plant pathology? In most cases it would be advantageous to close stomata and keep them closed during a critical period. Fortunately, we now have



many compounds that will do this. The closing of stomata could protect plants against air pollutants, such as ozone and peroxyacetyl nitrate, and against bacteria and certain leaf nematodes. Closed stomata would not generally protect plants against infection by fungi, except in the case of *Cercospora* on beets.

Stomatal closing agents may serve as antisporegents if sporophores must exit through open stomata.

It may be possible to restrict guttation by closing stomata or hydathodes. This might be of value in the control of the tipburn diseases.

Finally, chemical regulation of stomata should prove most useful for investigating the role of stomata in plant disease.

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### Discussion

van OVERBEEK: I am interested in the decapitation effect described by Romig (43). Is there more infection of the decapitated plants because the stomata remain open?

RICH: The abstracts of Romig's work report that decapitation increased infection, but there is no mention of the effect of decapitation on stomata. Romig may have the information in his thesis, which, I understand, is to be published soon.

van OVERBEEK: We have studied the interaction of gibberellins and auxins. It is well known that decapitating a plant removes the auxin source, and then the lateral buds start to grow. So auxin inhibits the growth of lateral buds, but what starts their growth when auxin is removed? Thimann believes it is kinetin, but we have evidence that it is probably gibberellin. We have been able to force the lateral buds of bean plants by treating them with gibberellin. We could then stop the gibberellin effect by treating with auxin. We know that auxins close stomata, and we have a hint from this conference that gibberellins may open stomata. Decapitation, then, should allow the natural gibberellins to open stomata, permitting the fungus to enter. Auxins would have the opposite effect.

I am also interested in the increased transpiration of mildewed plants. Perhaps mildew fungi produce gibberellins. I remember one observation that may bear on this point. Many years ago, Dr. Yarwood at Berkeley showed me mildew, on grape I think, that caused an ab-

normal increase in growth. At that time we didn't know about gibberellins.

RICH: Powdery mildews apparently increase transpiration, but whether this is caused by an effect on stomata is questionable.

van OVERBEEK: You mentioned that systemic infection with orange rust caused upper stomata to appear on blackberry leaves developing after infection. We have observed that treating a smooth-leaved crabgrass with a kinin will cause the new leaves to be hairy. Another example of hormones changing the morphology of the leaf epidermis.

MEUSEL: At Southern Connecticut State College, we sprayed annual bluegrass with phenylmercuric acetate, then placed the sprayed plants in a saturated atmosphere. The treated leaves did not guttate, but as the grass grew, the new, untreated leaves showed guttation. Phenylmercuric acetate apparently prevented guttation, but I do not know if it did so by closing hydathodes.

WITTWER: We have noticed that treating certain crop plants with gibberellin increases their tendency to wilt. We are not sure this results from a disproportionate growth of tops in relation to roots, or to stomatal regulation.

I would like to ask one question about ozone injury. Is light necessary for flecking to appear?

RICH: I can only repeat what Dr. Dugger told us at this conference. Once a susceptible plant with open stomata has been fumigated with a damaging concentration of ozone, the symptoms will appear. Whether the fumigated plant is kept subsequently in light or dark apparently does not influence the symptoms.

WITTWER: Does leaf orientation influence flecking?

RICH: We have been able to obtain fleck on excised tobacco leaves hung vertically in our fumigation chambers. It appears, then, that leaf orientation is not too important.

WITTWER: Does stomatal entry of pathogenic fungi and bacteria always lead to infection?

RICH: No it does not. Host-specific pathogens often penetrate through the stomata and epidermis of many plant species that they are unable to infect.

DUGGER: Getting back to oxidant damage, Yarwood and Middleton (Yarwood, C. E., and J. T. Middleton. 1954. Smog injury and rust infection. *Plant Physiol.* 29: 393) noted that rust on beans prevented smog



injury. Carbohydrates are known to accumulate around rust pustules on beans (Wang, D. 1961. The nature of starch accumulation at the rust infection site in leaves of pinto bean plants. *Can. J. Bot.* 39: 1595). Perhaps this increase in carbohydrates protects the rusted bean leaves from smog.

RICH: This is possible. Other evidence also supports Dr. Dugger's report that both high and low concentrations of carbohydrates in leaves afford resistance to oxidant damage. For example, it is known that the light-colored areas of variegated leaves are resistant to ozone damage.

ZELITCH: I would like to describe some unpublished experiments that show that compounds able to close stomata may inhibit rust infection. Dr. Corden, when he was here at the Station, used bean rust to evaluate chemotherapeutants. In his preventive assay, he first floated bean leaves on the test solution and afterwards inoculated the leaves with rust spores. In his therapeutic assay, he inoculated the leaves first, and then treated them to see if the test material would destroy the infection. Dr. Corden found that our  $\alpha$ -hydroxysulfonates were effective in the preventive assay, but not in the therapeutic assay. He used concentrations that we now know will close stomata, and his assays were similar to our leaf disk assays. We did not know then that these compounds will close stomata. It appears that Dr. Corden may have inhibited rust infection of bean leaves by closing stomata.

Dr. Rich, you spoke of the effect of Bordeaux mixture on transpiration. I know Dr. Horsfall was interested in that at one time, and I wonder if he would care to comment?

HORSFALL: We found Bordeaux mixture had two effects on potato. Sprayed on the upper surface of the leaf, Bordeaux increased transpiration. Sprayed on the lower surface, it decreased transpiration. We assume that the effect on the upper surface of the potato leaf, where there are no stomata, is caused by saponification of the fatty acids of the cuticle. The lower surface effect, we thought then, would be caused by mechanical plugging of the stomata. It may be that instead of plugging, Bordeaux may prevent the opening of the stomata. The copper in Bordeaux may act like mercury in phenylmercuric acetate to regulate stomata.

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