Penetration of Organic Compounds Through Isolated Cuticular Membranes with Special Reference to C^{14} Urea [1, 2, 3]

Y. Yamada [4], S. H. Wittwer, and M. J. Bukovac

Department of Horticulture, Michigan State University, East Lansing, Michigan

Studies of solute penetration into living cells are becoming legion. Protective or barrier membranes must be traversed. Cell particulates, cells themselves, and external plant surfaces are covered with membranes of varying permeabilities. Not the least among the membranes are the noncellular cuticles that cover all aerial plant parts including leaves. Absorption by living leaf cells of any foliar applied chemical (mineral nutrients, growth regulators, pesticides, antibiotics) must be preceded by transcuticular penetration (14).

Some permeability, surface binding, and ion exchange characteristics have been reported for enzymically isolated cuticular membranes (16, 17). They include the stomatous green onion leaf cuticle and the astomatous tomato fruit cuticle. Penetration through stomatous and astomatous cuticles was much greater from the outer to the inner surface (influx) than from the inner to the outer surface (efflux), more pronounced for cations than anions, and a function of the extent of ion binding on the surface opposite the site of initial entry. Ion exchange capacities of cuticular membrane surfaces were greater for Ca^{++} than for SO_4^{--}, and for green onion leaf cuticles than for those isolated from ripe tomato fruit.

These observations suggested a study of the comparative rates of penetration and extent of cuticular surface binding of certain C^{14} labelled organic compounds with those already reported for inorganic ions. Special attention was given urea which is commonly applied as a nutrient spray to some agricultural crops to provide nitrogen. The results of a few studies are also included for the plant growth inhibitors maleic hydrazide and N,N-dimethylaminosuccinamic acid. Organic molecules, because of their size, functional groups, and charge pose more complex problems than the inorganic ions.

Materials and Methods

Isolation of Cuticular Membranes. Cuticles were enzymically separated as previously reported from the stomatous surfaces of green onion leaves and the astomatous surfaces of ripe tomato fruits (15, 16, 17). The addition of merthiolate as a disinfectant was found unnecessary and discontinued.

C^{14} Labelled Compounds. Urea solutions of 0.01, 0.1, 0.2, 1.0 and 10 mM were prepared with a specific activity of 15 μc per μmole. Solutions of 0.25 mM for maleic hydrazide and 0.75 mM for N,N-dimethylaminosuccinamic acid were formulated with specific activities of 4 μc per μmole and 2 μc per μmole, respectively. Lower concentrations of maleic hydrazide and N,N-dimethylaminosuccinamic acid were not prepared because of the relatively low specific activities available.

Binding on Cuticular Surfaces. The procedure was the same as previously reported for inorganic ions (17). Cuticular discs, 1.3 cm in diameter, were floated with either the inner or outer surface only in contact with 0.1 mM solutions of urea (15 μc/μmole, pH 7), maleic hydrazide (4 μc/μmole, pH 5.4), or N,N-dimethylaminosuccinamic acid (2 μc/μmole, pH 4.4). After 5 minutes contact, the adhering solutions on the discs were blotted with tissue paper. They were then washed by shaking in deionized water for 5 minutes. That retained on the cuticular surfaces after blotting and washing was considered the bound fraction and was expressed as mμmoles per cm^2.

Permeability Measurements. The same apparatus was used and procedures were followed as formerly described (15, 16). A large test tube containing the outer solution (30 ml) was suspended in a water bath maintained at 20°. Into this tube of deionized water was suspended a small tube with the cuticular membrane (diameter = 15 mm) affixed over the tube opening. The desired C^{14} labelled compound [inner solution (3 ml)] was added to the small tube. Rates of penetration through the membrane were determined by radioassay of aliquots taken periodically from the outer solution.

Paper Chromatographic Procedure. Single dimensional paper chromatographic procedures were used for identifying the penetrated solutes. At the termination of a typical experiment both the outer and inner solutions were concentrated 30-fold by freeze-drying. A phenol-water mixture 80: 20 (v/v) was used as the solvent in the separation of urea and its possible derivatives. After chromatographing on Whatman No. 1 filter paper and drying, color development was achieved with a spray solution of 1% di-

[1] Received July 20, 1964.

[2] Report No. COO-888-39 in cooperation with the Division of Biology and Medicine of the United States Atomic Energy Commission. Contract No. AT (11-1)-888.

[3] Journal Article 3422 of the Michigan Agricultural Experiment Station.

[4] Research Associate on leave from Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan.

Table I. *Retention after Blotting and Washing of Organic Compounds from a 0.1 mM Solution, by Outer and Inner Surfaces of Ripe Tomato Fruit Cuticular Membranes and by a Dialyzing Membrane*

Compound	Surface	Cuticular membrane		Dialyzing membrane
		Tomato	Onion	
		(× 10^{-2} mμmole/cm^2		
Urea	Outer	10	12	0.5
	Inner	8	13	0.5
Maleic hydrazide	Outer	18	...	2.0
	Inner	16	...	2.0
N, N-dimethylamino-succinamic acid	Outer	29	...	2.4
	Inner	27	...	2.4

methylaminobenzaldehyde dissolved in 1 N HCl (2). Solutions containing maleic hydrazide were chromatographed in a solvent containing isopropanol, concentrated ammonium hydroxide, and water (70: 10: 20 v/v). Maleic hydrazide was detected on the dried paper by spraying with a freshly prepared 1: 1 mixture of 1 % aqueous solutions of ferric chloride and potassium ferricyanide (1, 12).

Association of the C^{14} label with the urea or maleic hydrazide on the chromatograms that had penetrated through the cuticular membranes was confirmed by autoradiography and radiation scanning. No satisfactory paper chromatographic techniques were available for N,N-dimethylaminosuccinamic acid.

Results

Binding on Cuticular Surfaces. The data for binding of urea, maleic hydrazide, and N,N-dimethylaminosuccinamic acid on cuticular surfaces are summarized in table I. Retention of organic solutes per unit area against blotting and washing was essentially the same on the outer and inner surfaces. Binding was 10 to 20-fold greater on the surfaces of tomato fruit cuticle than on those of the dialyzing membrane. Urea binding on the onion leaf cuticle was slightly greater than on the tomato fruit cuticle.

Permeability. Data for the 3 organic compounds are summarized in figure 1. Flux, through the astomatous fruit cuticle during a 30-hour interval, was greater from outside to inside than from the inside to the outside. With a dialyzing membrane there was equal penetration of urea in both directions, but the rate exceeded by almost 10-fold that for the tomato cuticle. The rate of urea penetration through the ripe tomato fruit cuticle was approximately twice that of N,N-dimethylaminosuccinamic acid (B995) and almost 6 times that for maleic hydrazide (MH). Penetration rates with time became progressively less for MH and B995, while that for urea increased.

Somewhat different urea permeability results were obtained with the green onion leaf cuticle. Penetration during the first 10 hours was linear. This was followed by a decrease in rate between 10 and 25 hours and a final precipitous rise as the time approached 30 hours. This pattern resulted in the sigmoid curves of figure 2. Influx and efflux of urea through this stomatous cuticular membrane were approximately equal. The total amount which had penetrated at 30 hours was greater than that for the tomato fruit cuticle (fig 1C).

The amounts of urea which penetrated the ripe tomato fruit cuticle increased, but not proportionally, with the concentration of urea in the inner solution (fig 3). There was a linear relationship with time at the 0.01 and 0.1 mM levels, and a curvilinear response at concentrations of 1.0 and 10 mM.

Chromatographic Confirmation of Structures of Penetrated Molecules. Penetration of C^{14} labelled urea and maleic hydrazide as intact molecules was confirmed by establishing identical R_F values and chromogenic responses for the solute before and after cuticular penetration. Radiation scanning for the C^{14} label and its localization in the same spots provided the confirmatory evidence. Further observations showed that chromatograms of the outer solutions in the maleic hydrazide experiments consistently developed oil spots with no radioactivity or response to the color reagent.

Discussion

Role of Diffusion in Solute Penetration of Cuticular Membranes. There is some evidence that organic solutes in contact with cuticular membranes penetrate by diffusion (5). This may also be true of inorganic cations and anions. The equation

$$\log_{10}(a\text{-}x) = \left(\frac{-k_1}{2.303}\right) t + \log_{10} a$$

for a first order reaction (10) was applied to the results of these studies, wherein a is the initial concentration of solute in the inner solution and x is the concentration in the outer solution at time t. Therefore $(a\text{-}x)$ represents the solute remaining in the inner solution at time t. The specific rate constant is k_1 for a first order reaction. Values of k_1 were obtained from the following equation: $k_1 = 2.303$ (slope). These are shown in table II for the influx and efflux of MH and B995 through tomato fruit cuticles and for urea through a dialyzing membrane. Neither the influx or efflux of urea through a stomatous (from green onion leaf) or astomatous (from ripe tomato fruit) cuticular membranes followed first order kinet-

ics. Penetration of urea, however, through a dialyzing membrane corresponded to a first order reaction (table II). Thus, cuticular penetration of urea was other than simple diffusion. Perhaps a facilitated diffusion resulting from a change in the structure of the cuticular membrane itself may account for the increase in rate of urea penetration (fig 1C, 2). There is supporting evidence for facilitated diffusion in that urea greatly accelerates the penetration of cations and anions through cuticular membranes (15).

Surface Binding and Permeability Relationships. The order of permeabilities through cuticular membranes is urea > cations > anions (fig 4, table III). The more rapid penetration of cations than anions may be partially explained by the greater binding of cations on negatively charged cuticular surfaces opposite the site of initial entry (16, 17). This, however, does not explain the high permeability of urea. Penetration rates exceeded by 10 to 20-fold those for the cations Rb^+, Ca^{++} and the anions Cl^- and

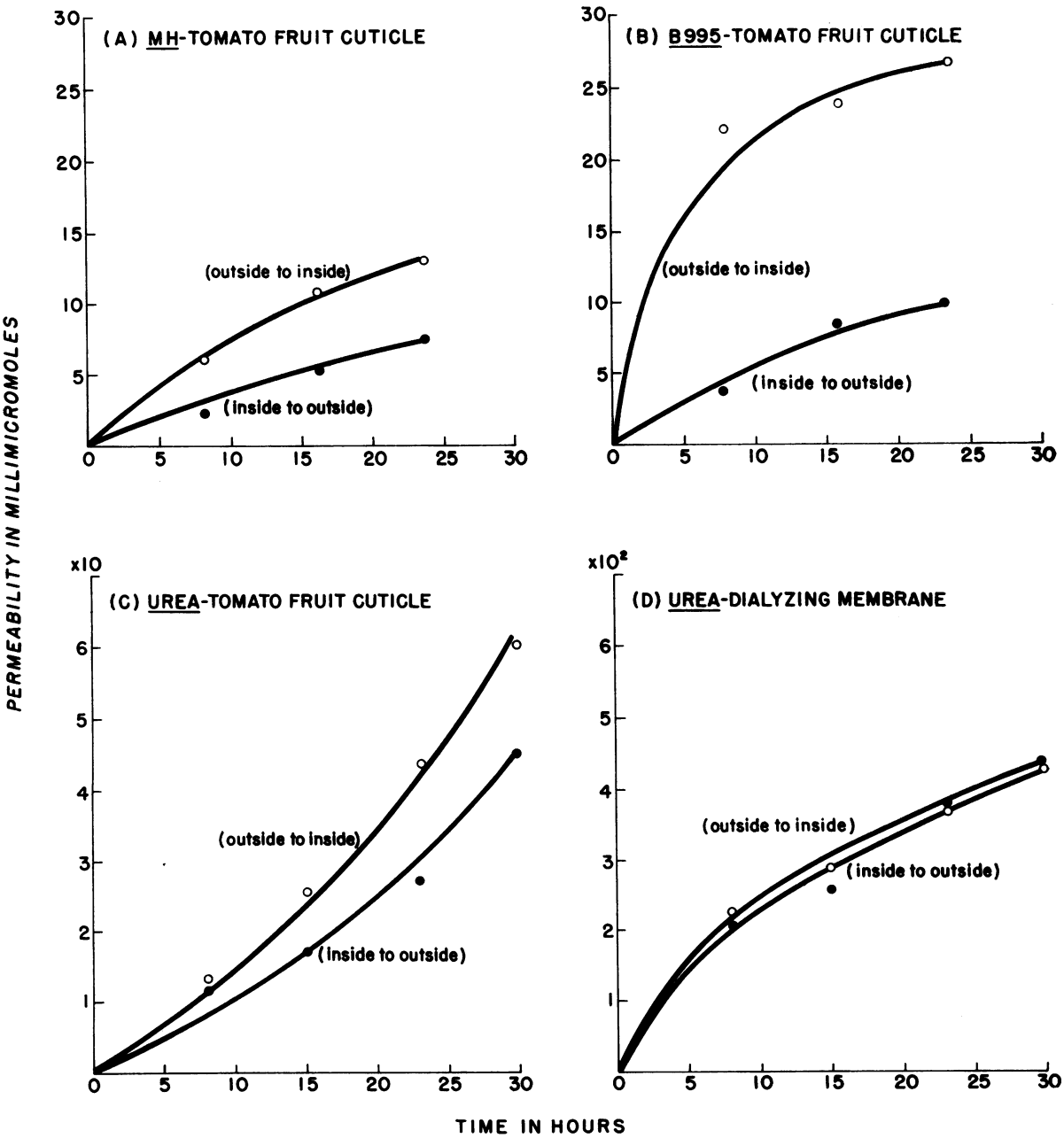

FIG. 1. Rates of unidirectional penetration of maleic hydrazide (MH), N,N-dimethylaminosuccinamic acid (B995), and urea through ripe tomato fruit cuticular membranes: A, MH (0.25 mM); B, B995 (0.75 mM); C, urea (0.1 mM); D, urea (0.2 mM) through a dialyzing membrane.

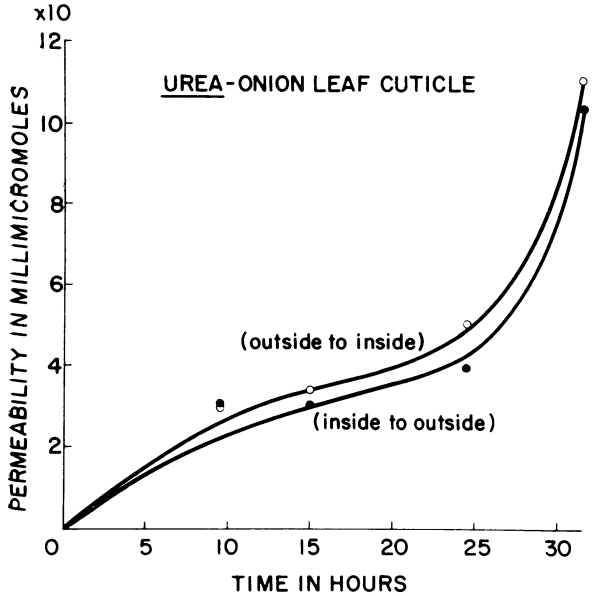

FIG. 2. Rates of unidirectional penetration of urea (0.1 mM) through onion leaf cuticles with stomatal pores.

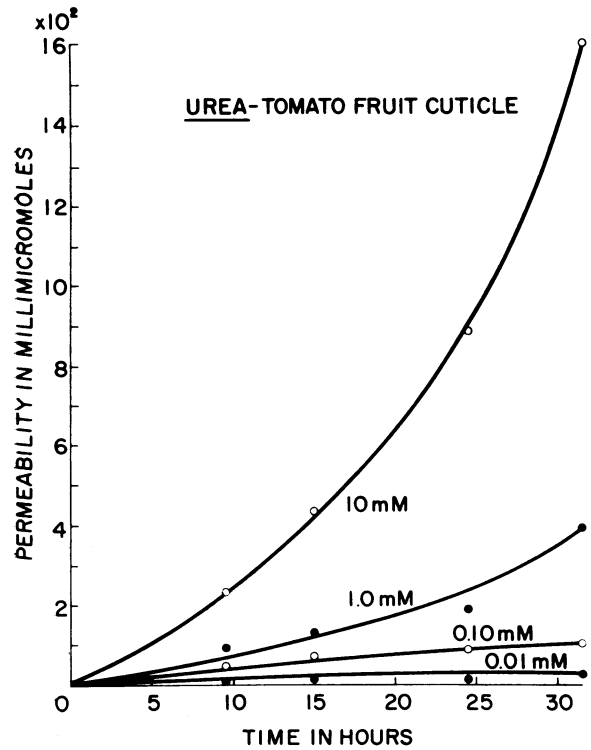

FIG. 3. The effect of concentration on urea penetration through tomato fruit cuticles. Urea concentration is 0.01; 0.1; 1.0 and 10 mM.

SO_4^{--} (fig 4, table III), yet binding on cuticular surfaces was equal to (outer) or much less (inner) than for Ca^{++} (table III).

Cuticular Permeability and Foliar Absorption. A remarkable parallel exists between the present data on comparative rates of penetration of urea, cations, and anions and the results of field trials wherein differences in the effectiveness of urea and nutrient salts have been evaluated when applied as nutrient foliar sprays (7). The nitrogen from urea is absorbed, translocated, and metabolized more rapidly than any other nutrient. Urea also enhances the rapidity of penetration of phosphate through cuticular membranes (15), and certain iron sprays applied with urea are more effective (14). Foliar absorption rates for cations exceed, in general, those for anions (14).

Factors Relating to the Permeability of Cuticular Membranes. Possible direct effects of penetrating compounds on the physical and chemical properties of enzymically isolated cuticular membranes are not precluded. Nonradioactive oil droplets appeared in the outer solutions in the present maleic hydrazide permeability experiments. According to Roelofsen and Houwink (11) and Crafts and Foy (3), cutin in cuticular membranes is a polymolecular network of unsaturated acids and esters, dicarboxylic acids, hydrocarboxylic acids and alcohols connected by ester, ether, and diether linkages. These bondages may be changed

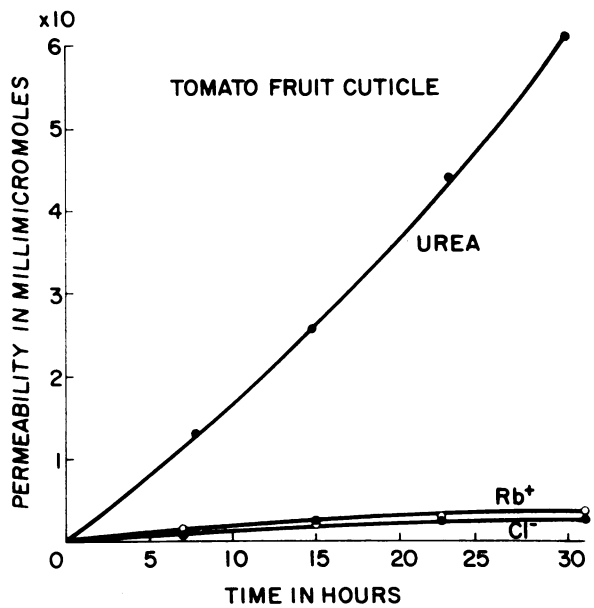

FIG. 4. Comparative rates of penetration of urea, Rb^+ and Cl^- through tomato fruit cuticles. All concentrations are 0.1 mM.

Table II. *Specific Rate Constants (k_1) for First Order Reactions*

Solute	Membrane	Influx	Efflux
MH	Tomato cuticle	6.909×10^{-4}	3.984×10^{-4}
B995	Tomato cuticle	2.994×10^{-4}	1.612×10^{-4}
Urea	Dialyzing membrane	3.768×10^{-2}	3.768×10^{-2}

Table III. *Permeability and Surface Binding Characteristics of Tomato Fruit Cuticles and a Dialyzing Membrane*

	Substance penetration (% after 30 hr)		Binding (mμmole/cm²)	
	Influx	Efflux	Outer surface	Inner surface
Tomato fruit cuticle				
SO_4^{--}	1.9	0.3	0.01	0.02
Ca^{++}	2.1	0.7	0.9	13
Urea	20	15	0.10	0.08
Dialyzing membrane				
Ca^{++}	89	89	2.0	2.0
Urea	89	89	0.005	0.005

by such compounds as maleic hydrazide and urea. Certain cuticular constituents may even be extracted during the penetration process. Yamada (15) has reported that the fraction extracted with urea is soluble in ether. Urea might break hydrophobic bonds which could be involved in the linkage of urea extracted material to cuticular constituents. The time course sigmoid curves (fig 2) which characterize urea penetration through the green onion leaf cuticle, suggest that some factor other than free diffusion is involved.

Many nonpolar organic solutes are absorbed by plant foliage more rapidly than the highly polar inorganic salts (9). Thus, permeability of cuticular membranes is apparently not limited by pore size which is also true for a cellular animal membrane (13). Craig (4) has suggested that molecular size and structure of solutes can, within limits, be correlated with diffusion rates through calibrated dialyzing membranes. Our studies have consistently demonstrated that dialyzing membranes are far more permeable than cuticular membranes to both organic compounds and inorganic ions, and differences in penetration relating to orientation of the dialyzing membrane are nonexistent (table III). The nearest parallel to a dialyzing membrane thus far encountered has been with urea penetration through the stomatous onion leaf cuticle. The process is rapid and differences in unidirectional movement are almost nil.

Molecular size, charge, partition, volatility, solubility, and adsorbability (sorption) are important in solute penetration. Current studies suggest that partition and solubility may play an important role in cuticular penetration of urea and maleic hydrazide, while molecular size, charge, and adsorbability are relatively important for inorganic ions.

Important contributions on the movement of organic solutes through chemically isolated apple leaf cuticles have been made by Goodman and associates (6, 8). Penetration of large organic molecules is possible and independent of the presence of stomata. There is also the conclusion that stomatal openings in the membranes are not perforations but invaginations of the surface cuticle. Some results on urea penetration of chemically isolated apple leaf cuticles are not fully in agreement with those reported herein for enzymically separated green onion leaf and tomato fruit cuticles. Penetration rates were not appreciably greater than for an inorganic cation, and movement from the inner (mesophyl) surface to the outer (air) surface exceeded that from the outer to the inner surface. Penetration of the intact molecule through the cuticular membrane was not confirmed, and concentration of the solutes in the permeability experiments were not given.

Summary

Rates of penetration of C^{14} labelled urea, and in some instances maleic hydrazide and N,N-dimethylaminosuccinamic acid, through enzymically isolated astomatous tomato fruit and stomatous onion leaf cuticular membranes were determined. Results were related to previous permeability studies with inorganic ions and with binding on cuticular surfaces and dialyzing membranes.

Retention of urea was essentially the same on the outer and inner surfaces of both types of cuticles, and 10 to 20 times greater than on the surface of a dialyzing membrane.

Penetration of all 3 organic compounds through tomato fruit cuticles was greater from the outer to the inner surface than in the opposite direction. With the onion leaf cuticle, however, influx and efflux of urea was approximately equal.

Permeability of urea through tomato fruit cuticles was twice that of N,N-dimethylaminosuccinamic acid and 6 times that for maleic hydrazide. The amounts of urea which penetrated the tomato fruit cuticle exceeded by 10 to 20 times the inorganic ions (Rb^+, Ca^{++}, Cl^-, SO_4^{--}), and increased, but not proportionally, with the concentration of urea in the test solution.

Penetration of C^{14} labelled urea and maleic hydrazide as intact molecules through cuticular membranes was confirmed by paper chromatographic procedures, radiation scanning, and autoradiography of chromatograms.

Acknowledgment

Appreciation is extended to Dr. A. E. Smith and Gracie M. Stone of the United States Rubber Company for supplying the C^{14} labelled maleic hydrazide and N,N-dimethylaminosuccinamic acid. The authors wish to acknowledge the helpful suggestions of Dr. David R. Dilley.

Literature Cited

1. ANDREAE, W. A. 1958. The estimation of maleic hydrazide by paper chromatography. Can. J. Biochem. Physiol. 36: 71–74.
2. BLOCK, R. J., E. L. DURRUM, AND G. ZWEIG. 1955. A Manual of Paper Chromatography and Paper Electrophoresis. Academic Press, New York. p 250–56.
3. CRAFTS, A. S. AND C. L. FOY. 1962. The chemical and physical nature of plant surfaces in relation to use of pesticide and to their residues. Res. Rev. 1: 112–39.
4. CRAIG, L. C. 1964. Differential dialysis. Science 144: 1093–99.
5. DARLINGTON, W. A. AND N. CIRCULIS. 1963. Permeability of apricot leaf cuticle. Plant Physiol. 38: 462–67.
6. GOODMAN, R. N. AND S. K. ADDY. 1962. Penetration of excised apple cuticular membranes by radioactive pesticide and other model compounds. Phytopathol. Z. 46: 1–10.
7. HINSVARK, O. N., S. H. WITTWER, AND H. B. TUKEY. 1953. The metabolism of foliar-applied urea. I. Relative rates of $C^{14}O_2$ production by certain vegetable plants treated with labelled urea. Plant Physiol. 28: 70–76.
8. KAMIMURA, S. AND R. N. GOODMAN. 1964. Penetration of excised apple cuticle membranes. II. Diffusion of radioactive model compounds and antibiotics and an analysis of membrane properties. Phytopathol. Z. In press.
9. MIDDLETON, L. J. 1959. The absorption of fertilizer by leaves. New Biology 30: 91–102. Penguin Books, Harmondsworth, Middlesex.
10. PRUTTON, C. F. AND S. H. MARON. 1957. Fundamental Principles of Physical Chemistry. The Macmillan Company, New York. p 612–22.
11. ROELOFSEN, P. A. AND A. L. HOUWINK. 1951. Cell wall structure of staminal hairs of *Tradescantia virginica* and its relation with growth. Protoplasma 40: 1–22.
12. SMITH, A. E. 1957. Chromatography of maleic hydrazide. MH Bulletin (#550), U.S. Rubber Company, Naugatuck, Connecticut.
13. TAYLOR, A. E., A. C. GUYTON, V. S. BISHOP, K. B. DELOZIER. 1964. Use of isotopes to establish a method for determining the permeability characteristics of the Pulmonary membrane. Proc. 2nd Oak Ridge Radioisotope Conf. Oak Ridge National Laboratory, Oak Ridge, Tennessee. p 52–53.
14. WITTWER, S. H. 1964. Foliar absorption of plant nutrients. Advan. Front. Plant Sci. 8: 161–82.
15. YAMADA, Y. 1962. Studies on foliar absorption of nutrients by using radioisotopes. Ph.D. Thesis. Kyoto University, Japan.
16. YAMADA, Y., S. H. WITTWER, AND M. J. BUKOVAC. 1964. Penetration of ions through isolated cuticles. Plant Physiol. 39: 28–32.
17. YAMADA, Y., M. J. BUKOVAC, AND S. H. WITTWER. 1964. Ion binding by surfaces of isolated cuticular membranes. Plant Physiol. 39: 978–82.